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Biosynthetic Chlorination of the Piperazate Residue in Kutzneride Biosynthesis by KthP

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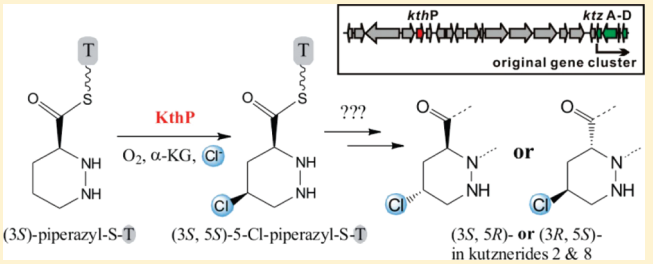
Supporting Information

ABSTRACT: Kutznerides 2 and 8 of the cyclic hexadepsipeptide family of antifungal natural products from the soil actinomycete *Kutzneria* sp. 744 contain two sets of chlorinated residues, a 6,7-dichlorohexahydropyrroloindole moiety derived from dichlorotryptophan and a 5-chloropiperazate moiety, as well as a methylcyclopropylglycine residue that may arise from isoleucine via a cryptic chlorination pathway. Previous studies identified KtzD, KtzQ, and KtzR as three halogenases in the kutzneride pathway but left no candidate for installing the C5 chlorine on piperazate. On the basis of analysis of the complete genome sequence of *Kutzneria*, we now identify a fourth halogenase in the pathway whose gene is separated from the defined kutzneride cluster by 12 open reading frames. KthP (kutzneride halogenase for piperazate) is a mononuclear nonheme iron halogenase that acts on the piperazyl ring tethered by a thioester linkage to the holo forms of thiolation domains. MS analysis of the protein-bound product confirmed chlorination of the piperazate framework from the (3*S*)- but not the (3*R*)-piperazyl-S-pantetheinyl thiolation proteins. After thioesterase-mediated release, nuclear magnetic resonance was used to assign the free imino acid as (3*S*,5*S*)-5-chloropiperazate, distinct from the 3*S*,5*R* stereoisomer reported in the mature kutznerides. These results demonstrate that a fourth halogenase, KthP, is active in the kutzneride biosynthetic pathway and suggest further processing of the (3*S*,5*S*)-5-chloropiperazate during subsequent incorporation into the kutzneride depsipeptide frameworks.



The family of antifungal cyclic hexadepsipeptides known as kutznerides 1−9 (Figure 1A), produced by the soil actinomycete *Kutzneria* sp. 744, is of nonribosomal origin.[1,2] Of the six building blocks, one is an unusual *β-tert*-butyl-α-hydroxy acid, while the other five are nonproteinogenic amino acids, including methylcyclopropylglycine and the 1,2-hydrazo-containing piperazate, whose biosyntheses remain obscure. As part of our prior efforts to study halogenases in biosynthetic pathways, we have focused on three of the four halogenations that occur on the Ktz scaffold: halogenations at C6 and C7 of the indole ring of a tryptophan-derived pyrroloindole residue[3] and a cryptic halogenation[4] that may underlie conversion of the proteinogenic amino acid Ile into methylcyclopropylglycine. Unexplored has been the biological chlorination reaction at the unactivated γ-CH_2 position (C5) of piperazate in kutznerides 2 and 8, where the chloropiperazate has been assigned the 3*S*,5*R* configuration (Figure 1B).[1] Piperazates are aza analogues of pipecolate, itself a higher homologue of proline, and are found in chair conformations of several NRPS and hybrid NRPS−PKS natural products.[5−7]

Our initial approach to identification of the *ktz* gene cluster involved the design and use of consensus deoxyoligonucleotide probes against both $FADH_2$-dependent halogenases and mononuclear nonheme iron halogenases.[8] We found two from the first category and one from the latter category clustered together in the midst of six NRPS modules, representing a convergent approach to identification of *ktz* biosynthetic genes.[8] We have subsequently shown that the two $FADH_2$-dependent enzymes KtzQ and KtzR, when heterologously expressed in and purified from *Escherichia coli* and *Pseudomonas putida*, respectively, act in tandem to make 6,7-dichlorotryptophan.[3] The mononuclear nonheme iron enzyme KtzD chlorinated the methyl side chain of isoleucine while that amino acid was tethered as an amino acyl thioester to the phosphopantetheinyl arm of the stand-alone thiolation protein KtzC, on the way to generation of cyclopropane-containing

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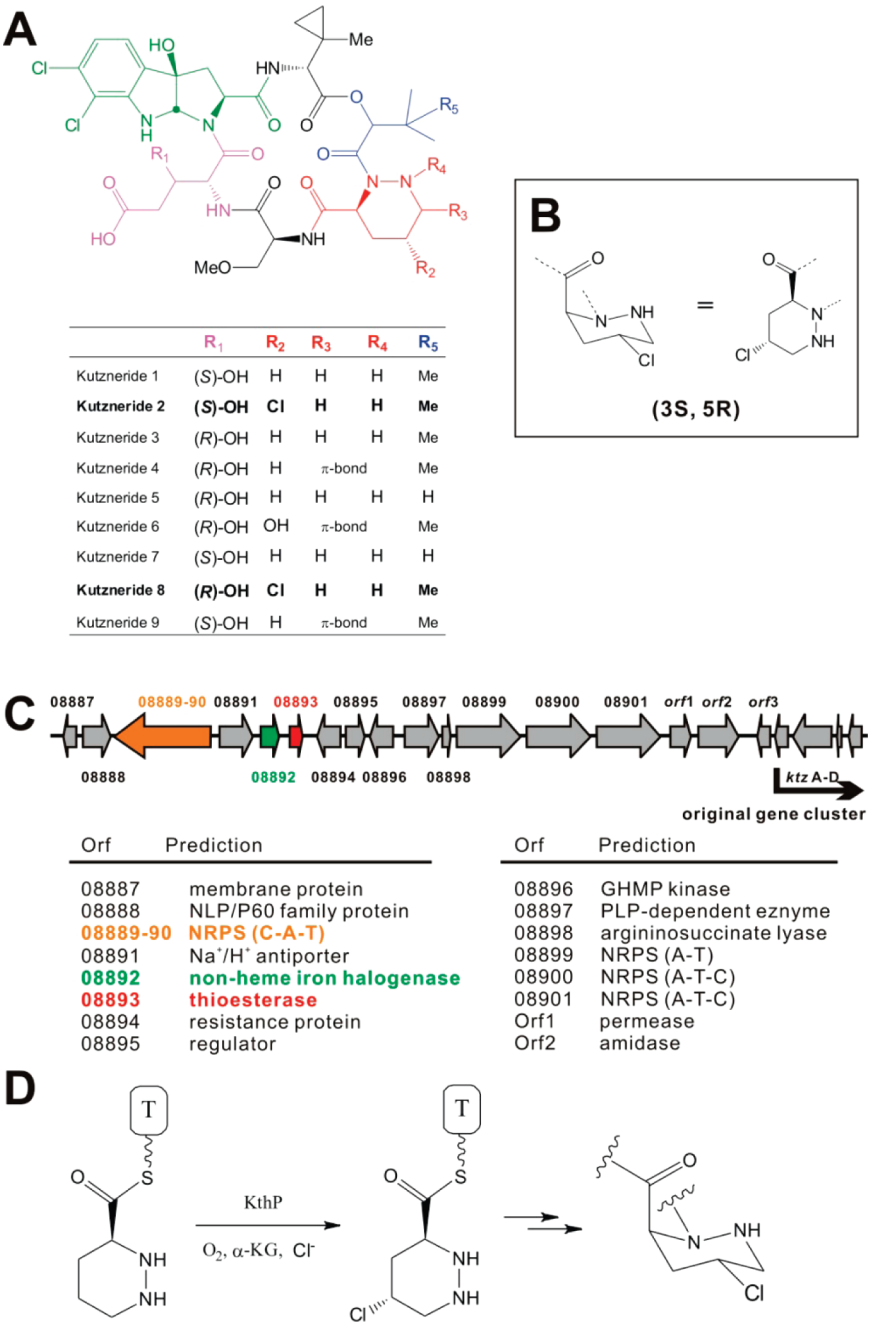

	R_1	R_2	R_3	R_4	R_5
Kutzneride 1	(*S*)-OH	H	H	H	Me
Kutzneride 2	**(*S*)-OH**	**Cl**	**H**	**H**	**Me**
Kutzneride 3	(*R*)-OH	H	H	H	Me
Kutzneride 4	(*R*)-OH	H	π-bond		Me
Kutzneride 5	(*R*)-OH	H	H	H	H
Kutzneride 6	(*R*)-OH	OH	π-bond		Me
Kutzneride 7	(*S*)-OH	H	H	H	H
Kutzneride 8	**(*R*)-OH**	**Cl**	**H**	**H**	**Me**
Kutzneride 9	(*S*)-OH	H	π-bond		Me

Orf	Prediction
08887	membrane protein
08888	NLP/P60 family protein
08889-90	NRPS (C-A-T)
08891	Na^+/H^+ antiporter
08892	non-heme iron halogenase
08893	thioesterase
08894	resistance protein
08895	regulator

Orf	Prediction
08896	GHMP kinase
08897	PLP-dependent eznyme
08898	argininosuccinate lyase
08899	NRPS (A-T)
08900	NRPS (A-T-C)
08901	NRPS (A-T-C)
Orf1	permease
Orf2	amidase

Figure 1. (A) Structures of kutznerides 1−9.[8] (B) Structure of the chloropiperazyl moiety in kutznerides 2 and 8. (C) Map and predicted functions of genes located upstream of the originally proposed gene cluster for kutzneride synthesis. Open reading frame 8892 is subsequently designated KthP in this work. (D) Proposed pathway for incorporation of chloropiperazate into kutznerides.

allo-coronamic acid, suggesting the presence of an additional isomerization step that remains to be identified.[4]

None of these three halogenases were active on piperazate scaffolds, indicating there might be even a fourth halogenase in the pathway that had not yet been identified. Subsequently, the genome of *Kutzneria* sp. 744 was sequenced as part of an actinomycete genome project at The Broad Institute.[a] Bioinformatic analysis indicates the presence of one and only one additional putative halogenase gene in the genome, a short distance upstream [by ∼12 open reading frames (orfs)] of the *ktz* gene cluster we had initially identified from a cosmid library by the halogenase gene probes. Specifically, orf 08892 (Figure 1C)[b] has the features predicted for a nonheme iron oxidation enzyme[9] in which the conserved HxD/E...H triad of iron ligands in hydroxylases is converted to an HxS...H triad. We have previously noted that the HxA...H triad is a signature ligand set for mononuclear iron halogenases in which the D/E ligand to iron is absent, allowing for first-shell coordination of chloride to iron as a reactant to be oxidized during the catalytic cycle.[10] orf 08889-90 comprises a predicted NRPS module and orf 08893 a thioesterase, suggesting this three-gene set could be involved in generating and then releasing the 5-chloropiperazate building block as a tethered intermediate on the NRPS module, similar to the logic of other nonheme mononuclear iron halogenases that work on

unactivated CH_2 and CH_3 centers of aminoacyl-S-thiolation domains (Figure 1D).[11]

In this study, we have validated the hypothesis that orf 8892 (renamed here as KthP for kutzneride halogenase of piperazate) is the missing fourth halogenase of the pathway by heterologous expression and purification from *E. coli*. Fe^{II} reconstitution allows assay of both free piperazate and piperazyl-S-pantetheinyl thioesters tethered to candidate protein thiolation (T) domains, as well as detection of chlorinated piperazyl product, both bound in a thioester linkage to the free-standing thiolation domain protein KtzC and as a free chloropiperazate derivative after hydrolytic release and appropriate derivatization.

MATERIALS AND METHODS

Cloning, Overexpression, and Purification of Kutzneria Proteins, TycF and Sfp. *KthP.* The *kthP* gene (assigned as KUTG_08892 by the Broad Institute) was amplified via polymerase chain reaction (PCR) from genomic DNA of *Kutzneria* sp. 744 using the forward primer 5′-AAT CAA TCA TAT GAC CAC CGC CGA CGA GTT C-3′ (*Nde*I restriction site underlined) and the reverse primer 5′-GAT CAA GCT TTC ACG GCC GGC GGG CCG C-3′ (*Hin*dIII restriction site underlined). Polymerase chain reactions were performed using Phusion High-Fidelity DNA Polymerase following the program and conditions provided by New England Biolabs. The amplified gene sequence was digested with *Nde*I and *Hin*dIII (NEB), ligated into a similarly digested pET28a vector using T4 DNA ligase, and transformed into *E. coli* TOP10 cells (Invitrogen). The identity of the resulting pET-28a (N-His_6) construct was confirmed by DNA sequencing.

The expression construct was transformed into *E. coli* BL21-(DE3) cells (Invitrogen), grown to saturation in LB medium supplemented with 30 μg/mL kanamycin at 37 °C, and diluted 1:100 into LB medium containing 30 μg/mL kanamycin. The culture (2 × 1 L) was incubated at 37 °C with shaking at 200 rpm, induced with 100 μg/mL IPTG at an OD_{600} of 0.5−0.7, and then incubated at 15 °C for 16 h. The cells were harvested by centrifugation (6000 rpm × 20 min), resuspended in 30 mL of buffer A [20 mM HEPES and 300 mM NaCl (pH 7.5)] with 5 mM imidazole, and then lysed via two passes through an Emulsiflex-C5 cell disruptor (Avestin). The lysate was clarified by centrifugation at 15000 rpm for 30 min, and the supernatant was incubated with ∼2.0 mL of Ni-NTA resin (Qiagen) for 1 h at 4 °C. The slurry was loaded into a column. The column was washed with buffer A containing 5 mM imidazole (1 × 20 mL) and 20 mM imidazole (2 × 20 mL), followed by buffer A containing 200 mM imidazole (3 × 10 mL) to elute the protein. Fractions containing the desired protein, as identified by denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis analysis (4 to 15% Tris-HCl gel, Bio-Rad) with Coomassie staining, were pooled and concentrated to ∼2 mL. It was dialyzed to equilibrium against 500 mL of buffer B [20 mM HEPES and 80 mM NaCl (pH 7.6)] containing 10 mM EDTA followed by two changes of buffer B to remove the EDTA.

The concentration was determined spectrophotometrically by assuming molar absorptivities (ε_{280}) of 61880 M^{-1} cm^{-1} as calculated by the method of Gill and von Hippel.[12]

KtzH T1. The first T domain of the *ktzH* gene was PCR amplified from genomic DNA of *Kutzneria* sp. 744 using the forward primer 5′-AAT CAA TCA TAT GTT CAC CGG CAC TGC CTA C-3′ (*Nde*I restriction site underlined) and the reverse primer 5′-GTC AGC AAG CTT CTC GGC CAC GGG C-3′ (*Hin*dIII restriction site underlined). The amplified gene was inserted into the pET-24b overexpression vector, and the steps that followed were the same as those described above.

KutG_08889-90 T. The T domain of the *kutG_08889-90* gene was PCR amplified from genomic DNA of *Kutzneria* sp. 744 using the forward primer 5′-GCA TTA TTC ATA TGG ACC GCG CGG CGC TG-3′ (*Nde*I restriction site underlined) and the reverse primer 5′-GCA ATA ATC TCG AGT CAT CAC GCG CCG ACG TC-3′ (*Xho*I restriction site underlined). The amplified gene was inserted into the pET-28a overexpression vector, and the steps that followed were the same as those described above.

TycF and Sfp. The cloning, overexpression, and purification of TycF and Sfp have been described previously.[13,14]

Reconstitution of KthP. Anaerobic reconstitution of KthP with Fe(II), α-KG, and DTT was conducted as described above for other nonheme Fe(II)-dependent halogenases.[15]

Chlorination Assay. The chlorination assay was conducted in two steps. First, the aminoacyl-CoA (see the Supporting Information for synthesis) was loaded onto the T domain by Sfp. It is initiated by addition of 10 μM Sfp to a 100 μL solution containing 25 mM Hepes, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM aminoacyl-CoA, and 200 μM T domain (pH 7.0). The mixture was incubated at ambient temperature for 1 h. Second, KthP was applied for chlorination. Anaerobically reconstituted KthP at various concentrations was added together with 2 mM α-ketoglutarate. If KthP in the as-isolated form was used, 0.5 mM $(NH_4)_2Fe(SO_4)_2$ was also added. The assay vial was periodically tapped from the bottom during the reaction to help with oxygen delivery.

MALDI. At desired times, 2 μL of chlorination assay mixture was added to 8 μL of a 0.5% TFA/H_2O mixture to stop the reaction. C4-ZipTip (Fisher) was prewet three times with 0.1% TFA in a MeCN/H_2O mixture (1:1, v/v) followed by three times with a 0.1% TFA/H_2O mixture. The assay sample was bound to the tip by pipetting it up and down six times. The tip was then washed five times with a 0.1% TFA/H_2O mixture. The protein was eluted by pipetting up and down with 4 μL of MALDI matrix solution, saturated sinapic acid in a MeCN/H_2O mixture (1:1, v/v). Finally, 0.8 μL of eluent was spotted onto a MALDI plate for analysis on a high-performance ABI 4700 MALDI-TOF/TOF mass spectrometer. Spectrometer conditions were as follows: acceleration voltage, 20000 V; grid voltage, 93%; grid wire, 0.2%; delay time, 250 ns.

HPLC and Liquid Chromatography with Mass Spectrometry on Fmoc Derivatives. TycF (10 μM) was added and the mixture incubated at ambient temperature for 1 h to release the product from the phosophopantetheine arm of the T domain. The solution was filtered through a Microcon YM-3 (Amicon) microcentrifuge filter; 50 μL of flow-through was collected and combined with 50 μL of MeCN, 50 μL of 0.2 M sodium borate (pH 8.0), and 20 μL of 10 mM Fmoc-Cl. After 5 min, 20 μL of 0.1 M 1-adamantanamine was added to stop the reaction. The mixture was subjected to HPLC using a solvent gradient of 20 to 100% B over 25 min (solvent A, 0.1% TFA/H_2O; solvent B, 0.1% TFA/MeCN) and monitored at 259 nm. The solution from the absorption peak was collected and injected onto the Agilent 6520 Q-TOF/LC-MS instrument for high-resolution mass spectrometric analysis.

Measurement of Turnover of KthP by HPLC. To 15 μL of the solution from the chlorination assay described above was added 85 μL of a 0.1% TFA/H_2O mixture to stop the reaction at a specified time, followed by HPLC using a solvent gradient from

20 to 40% B for 5 min and 40 to 65% B for 20 min (solvent A, 0.1% TFA/H_2O; solvent B, 0.1% TFA/MeCN). Typically, data from four or five different time points were collected to analyze as a set for one reaction. The chromatographic traces at 280 nm were simulated by three Gaussian distributions representing three species: the piperazyl-S-KtzC, the Cl-piperazyl-S-KtzC, and the S-pantetheinyl holo form. For each set of kinetic measurements, the mean and variance of each distribution are kept the same. The area of the Gaussian distribution curve is proportional to the concentration of the species it represents (assuming the molar absorptivity of KtzC is the same among three species).

NMR Spectroscopic Instrumentation and Analysis. The NMR spectra of the final deprotected *cis*- and *trans*-5-Cl-piperazic acid TFA salts (see below) were recorded using a Varian INOVA 600 MHz NMR spectrometer equipped with an HCN indirect-detection probe. Nongradient phase-cycled dqfCOSY spectra were recorded using the following parameters: 0.6 s acquisition time, 200−500 complex increments, and 16−128 scans per increment. NMR samples were prepared by dissolving the sample in ∼0.3−0.6 mL of methanol-d_4 (D, 99.95%). For analysis of KthP-derived 5-chloropiperazic acid, susceptibility-matched NMR tubes (Shigemi) were used. NMR spectra were processed using MNOVA (MestreLabs), using 4096 complex data points in F2 and 2048 complex data points in F1.

The 400 MHz ^{1}H and 100 MHz ^{13}C NMR spectra of all the synthetic intermediates prepared en route to (3*S*,5*R*)-N(1)-Fmoc-5-chloropiperazate (see the Supporting Information) were recorded on a Bruker Avance 400 NMR spectrometer.

Deprotection of Fmoc-5-chloropiperazate. A sample of ∼200 nmol of Fmoc-5-chloropiperazic acid (synthetic or KthP-derived) was dissolved in 100 μL of dry DMF; 50 μL of diisopropylamine was added, and the reaction vessel was flushed with argon and sealed. The reaction mixture was stirred in the dark for 3−5 h. Subsequently, DMF was removed in vacuo at room temperature. To form the trifluoroacetic acid (TFA) salt, a solution of 1 mL of methanol containing 50 μL of TFA was added to the residue and the resulting solution evaporated in vacuo.

High-Resolution Mass Spectrometry on Chloropiperazate. High-resolution mass spectrometry was performed on a LTQ Orbitrap Velos (Thermo Scientific) mass spectrometer in ESI^+ ionization mode with a mass range of m/z 120−400. For MS analysis, the sample was dissolved in ∼0.5 mL of 0.1% formic acid in water to give an ∼400 nmol/mL solution. (3*S*,5*S*)-5-Chloropiperazate: m/z calcd for $C_5H_{10}{}^{35}ClN_2O_2$ $[M + H]^+$ 165.0425, found 165.0426; m/z calcd for $C_5H_{10}{}^{37}ClN_2O_2$ $[M + H]^+$ 167.0396, found m/z 167.0395. The ratio of the two ions, $C_5H_{10}{}^{35}ClN_2O_2$ and $C_5H_{10}{}^{37}ClN_2O_2$, was ∼75:25, consistent with the presence of one Cl atom.

RESULTS

Cloning, Heterologous Expression, and Characterization of orf 8892 (KthP) from the Kutzneride Genome. The 963 bp *8892* (=*kthP*) gene was subcloned from *Kutzneria* sp. 744 genomic DNA and ligated into the pET-28a expression vector with an N-terminal His_6 tag. Induced overproduction of the protein in *E. coli* allowed subsequent elution from a nickel affinity column as a soluble 38 kDa protein in high yield (40 mg/L) and good purity (Figure S1 of the Supporting Information). The KthP protein could be added to assays separately from the anticipated Fe^{II} and α-ketoglutarate (α-KG) cofactors, or it could be reconstituted anaerobically with Fe^{II}, followed by anaerobic gel filtration to yield 0.89 equiv of bound Fe^{II}. The latter preparation of enzyme exhibited higher activity and was thus utilized unless otherwise specified.

Assays for KthP Halogenase Activity on Piperazyl-S-Thiolation Domains. To test for piperazate halogenation activity of pure KthP, we conducted initial studies with free L- or D-piperazate. No conversion to chlorinated products could be detected by LC−MS analysis, following conversion of the cyclic hydrazo acids to their N1-Fmoc derivatives to generate a readily detected chromophore (Figure S2 of the Supporting Information). The lack of activity on free piperazates was consistent with our prior studies on six mononuclear iron amino acyl halogenases, which demonstrated that they acted only on aminoacyl moieties tethered via a thioester linkage to the pantetheinyl arms of 8−10 kDa thiolation domains.[16]

To assay piperazyl-thioesterified T domains then required a source of likely thiolation domains and a way to install the piperazyl residues. For the choice of T domain platforms that might present the attached piperazyl group to KthP, we thought either KtzH-T1[8] or the adjacent orf 8889-90 NRPS module would be a candidate for piperazate activation as piperazyl-AMP and that these could then transfer to the holo form of the adjacent T domains in those NRPS modules. Unfortunately, despite several efforts to heterologously express the intact C1-A1-T1 (condensation-adenylation-thiolation) modules of KtzH and orf 8889-90 or the A-T didomain fragments or the T domains themselves, only the KtzH T1 and orf 8889-90 T domain construct gave a small amount of soluble protein in the apo form. As an alternative, we also purified the apo form of KtzC, a stand-alone thiolation domain that we have previously shown is overproduced in *E. coli* in soluble form and is recognized, when bearing an Ile-S-pantetheinyl prosthetic group, for chlorination by the mononuclear iron halogenase KtzD.[4]

With three candidate T domains in hand, we turned to the phosphopantetheinyl transferase (PPTase) technology we have developed and utilized in many contexts to convert the apo form of T domains to the aminoacylated holo forms.[14,17] The *Bacillus subtilis* PPTase Sfp is highly promiscuous in catalyzing the attack of a conserved Ser-OH side chain in apo T domains on the phosphopantetheinyl portion of CoASH and various acylated CoAs, creating a phosphodiester linkage to the Ser side chain.[14] This action converts the apo form of T domains to the holo form, with the phosphopantetheinyl arm installed at that Ser residue. With CoASH as the substrate, the Sfp PPTase generates the HS-pantetheinyl-P-thiolation domain. From aminoacyl/peptidyl-CoAs, the aminoacyl/peptidyl-S-pantetheinyl-P-forms of T domains are generated, thereby giving access to loaded T domains in the absence of a soluble and active adenylation domain (Figure 2A).

To that end, L- and D-piperazic acids (3*S* and 3*R*, respectively) were converted to the corresponding piperazyl-CoAs as detailed in the Supporting Information and utilized as substrates for Sfp-mediated post-translational modification of the three different T domains. KtzC gave the best yield for the final product and was thus utilized in the following experiments (Figure S2 of the Supporting Information). As shown in trace i of Figure 2B, MALDI MS analysis of the KtzC protein showed a mass of 10868.5 Da for the apo form of KtzC. When the Sfp-mediated post-translational modification used CoASH as a cosubstrate, the resulting holo HS-pantetheinyl form was detected (trace ii) with a mass of 11208.6 Da, showing the 342 Da (340 Da shift expected) mass shift from installation of the HS-pantetheinyl-P prosthetic group. When (3*S*)-piperazyl-CoA was used as a cosubstrate, the apo T

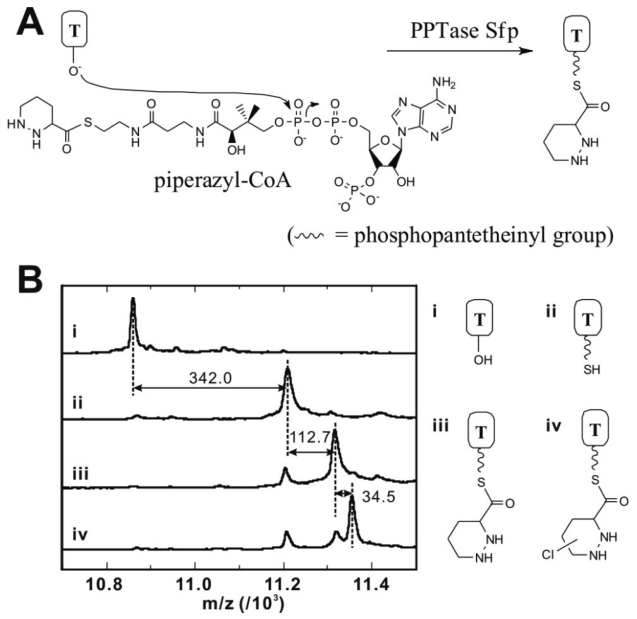

Figure 2. (A) Scheme for loading piperazyl-CoA onto the T domain by Sfp. (B) MALDI of (i) apo-KtzC, (ii) holo-KtzC, (iii) (3*S*)-piperazyl-S-KtzC, and (iv) (3*S*)-piperazyl-S-KtzC after KthP incubation. The mass differences between two species are given.

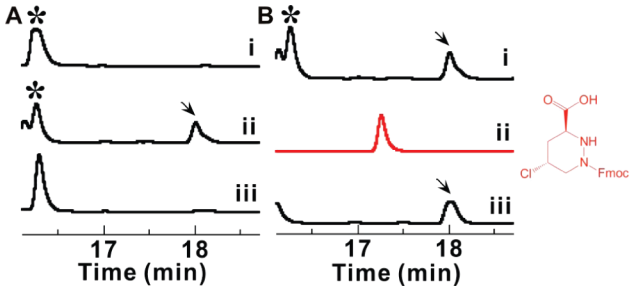

Figure 3. HPLC traces of cleaved reaction products as Fmoc derivatives. (A) Treatment of (3*S*)-piperazyl-S-KtzC (i) with KthP yields chlorinated product (ii), while no reaction for (3*R*)-piperazyl-S-KtzC was detected (iii). (B) Trace i is identical to trace ii in panel A as a comparison to the (3*S*,5*R*)-5-Cl-piperazate authentic standard (ii). The product peak collected from trace i was deprotected, analyzed by NMR, and rederivatized (iii). Asterisks denote Fmoc-piperazate (found m/z 353.1510 $[M + H]^+$, calcd for $C_{20}H_{21}N_2O_4$ m/z 353.1496). Black arrows denote the Cl-pip from enzymatic reactions (found m/z 387.1141 $[M + H]^+$, calcd for $C_{20}H_{20}{}^{35}ClN_2O_4$ m/z 387.1112).

domain was converted to a mass of 11320.8 Da (trace iii). The mass difference from the HS holo form was 112.2 Da, consistent with the expected 112 Da shift from the piperazyl group. Similarly, the (3*R*)-piperazyl-S-KtzC protein was generated from comparable incubations with (3*R*)-piperazyl-CoA (data not shown).

When pure KthP was added to such incubations in which the (3*S*)-piperazyl-S-KtzC species had been preformed, in the presence of O_2, NaCl, Fe^{II}, and α-KG as noted in Materials and Methods, the MALDI MS analysis indicated a new species at 11355.3 Da, corresponding to a mass shift of 34.5 Da, consistent with introduction of one chlorine (trace iv). In contrast, the (3*R*)-piperazyl-S-KtzC-tethered thioester was not processed by KthP, consistent with chiral recognition of the (3*S*)-piperazyl and not the (3*R*)-piperazyl ring. When the corresponding (3*S*)-N(1)-dehydropiperazate (see the Supporting Information) was converted to the CoA thioester and loaded onto apo T domains, again, no halogenation was detected.

Analysis of Chloropiperazate Products after Thioesterase-Mediated Release. To establish that the chlorination detected on the 11.3 kDa KtzC domain was introduced on the tethered piperazyl group (rather than on some amino acid side chain of the thiolation domain), we subjected the products to action of the thioesterase enzyme TycF from the tyrocidin synthetase cluster.[13] This enzyme hydrolytically removes aminoacyl and peptidyl moieties from the phosphopantetheinyl arms of T domains as part of the normal chain release mechanism of nonribosomal peptide synthetases and is a mild (pH 7, room temperature) and selective route for deacylation. The low-molecular mass material thereby released was subjected to Fmoc derivatization on N1 of the piperazates to introduce a chromophore and facilitate detection by LC−MS. An N1-Fmoc-piperazate standard served as reference for release of unhalogenated piperazyl-S-KtzC which gave the anticipated peak by LC−MS analysis (Figure 3A). Most notably, upon TycF release of the (3*S*)-piperazyl-KtzC thioester from the halogenase incubations, a new peak in LC was detected (Figure 3A, trace ii), and high-resolution mass spectrometry showed this to be an Fmoc-chloropiperazate (observed mass of m/z 387.1141, expected mass of m/z 387.1112). In-depth analysis of the LC−MS data failed to show evidence of any hydroxylated products, confirming that KthP is highly selective for chlorination over hydroxylation. These TycF/Fmoc derivatization assays could also be used to show the anticipated requirement for O_2 and for α-KG, previously established for this iron-dependent halogenase family (Figure S3 of the Supporting Information).[15,18,19] Consistent with the MALDI results, no chlorination product was detected from the substrate with the 3*R* configuration (trace iii).

Kinetic Analysis of (3*S*)-Piperazyl and (3*S*)-Pipecolyl-S-T Domain Halogenation. With halogenation of the piperazyl ring established by TycF-mediated release, Fmoc derivatization, and high-resolution LC−MS analysis, we turned back to the methodologies that we employed for the analysis of intact the chloropiperazyl-S-KtzC product (e.g., MALDI) to obtain initial information about kinetics. We chose this route rather than the Fmoc derivatization because of the large number of intervening steps in the Fmoc assays, including the requirement for a TycF-mediated incubation period for release of the Cl-piperazate. An HPLC protocol was developed to separate three different forms of KtzC for preliminary kinetic analysis: the piperazyl-S-KtzC (blue in Figure 4B), the Cl-piperazyl-S-KtzC (pink), and the HS-pantetheinyl holo form (green).[c] The analysis and quantification of each species are described in Materials and Methods. Figure 4B shows the dependence of the initial rates of tethered substrate chlorination on KthP halogenase concentrations between 5 and 20 μM (substrate piperazyl-S-KtzC at 170 μM). After a few minutes, the rate falls off, as is often the case for this superfamily of nonheme mononuclear iron oxygenative enzymes (oxygenases and halogenases) as they autoinactivate, presumably from derailment of high-valent oxoiron intermediates.[18,20] Deactivation of the enzyme over time is further supported by a second addition of freshly reconstituted KthP to the assay (Figure 4C). Figure 4D (blue trace) then shows the time course for chlorination of (3*S*)-piperazyl-S-KtzC with a linear rate for the first 2 min at a substrate:enzyme ratio of 17:1, allowing a crude estimate of the halogenation k_{cat} of 1.5 min^{-1} before inactivation during catalytic

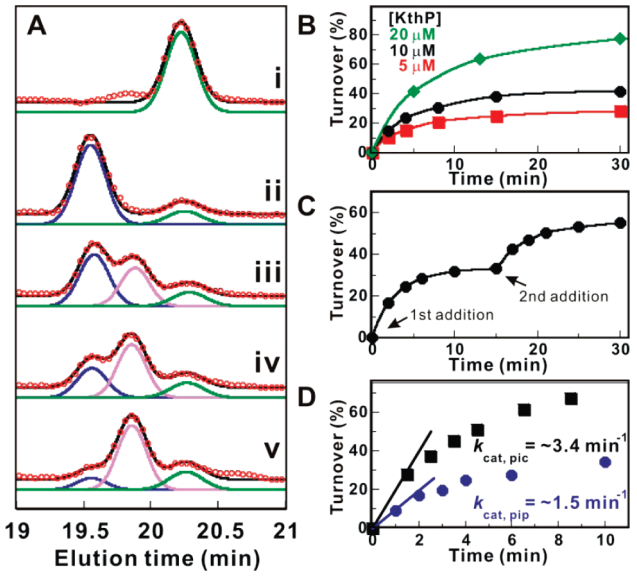

Figure 4. Kinetic analysis of KthP on (*S*)-piperazyl-S-KtzC and (*S*)-pipcolyl-S-KtzC. Substrate (170 μM) was applied in all assays. (A) HPLC traces and fittings for (i) holo KtzC, (ii) (*S*)-piperazyl-S-KtzC, and samples during kinetic measurement with 20 μM KthP that were quenched at 5 (iii), 13 (iv), and 60 min (v). Details for sample preparation and analysis are given in Materials and Methods. Red open circles show the experimental data. The black solid lines on top are the sums of simulations from three individual species: the HS-pantetheinyl holo form (green lines), piperazyl-S-KtzC (blue lines), and Cl-piperazyl-S-KtzC (pink lines). (B) Dependence of the initial rates of chlorination on KthP concentration at 5 (red), 10 (black), and 20 μM (green). (C) Turnover measurement with two additions of KthP (10 μM each). (D) k_{cat} measurement of KthP on (*S*)-piperazyl-S-KtzC (blue) and (*S*)-pipecolyl-S-KtzC (black).

turnover becomes significant. These kinds of analyses also further confirmed that the (3*R*)-piperazyl-S-KtzC was not detectably halogenated (data not shown).

Because piperazate is an aza homologue of the more common, but still nonproteinogenic, cyclic imino acid pipecolate, we undertook an evaluation of the permissivity of KthP. We synthesized the corresponding L- and D-pipecolyl-CoAs (in this case the 2*S* and 2*R* isomers, respectively; the 3*S* and 3*R* numbering in piperazate starts from the first hydrazo N) and used the Sfp-mediated post-translational route to yield the corresponding pipecolyl-S-KtzC protein thioesters as potential substrates. Again the 2*R* isomer was not halogenated, but the (2*S*)-pipecolyl-S-KtzC was active (Figure S4 of the Supporting Information). As shown in black in Figure 4D, a similar time course gives an estimate of 3.4 min^{-1} for chlorination of the (2*S*)-pipecolyl-S-KtzC, which is approximately double the rate with (3*S*)-piperazyl-KtzC. A 4-chloropipecolate residue is naturally observed in the mirabamide family of depsipeptides isolated from the sponges *Siliquariaspongia mirabilis* and *Stelletta clavosa*.[21,22] Our results here suggest this residue might arise from bacterial consortium members by a KthP-type halogenase. Subsequent synthesis of (2*S*)-prolyl-CoA and loading onto apo KtzC for halogenation assays indicated that the prolyl ring was not a chlorination substrate for Kth P (data not shown).

Determination of the Regiochemistry and Stereochemistry of Chlorination of the Piperazyl Framework by KthP as 3*S*,5*S*. To identify the site of chlorination on the piperazate ring by the KthP halogenase and to determine the stereochemistry of the product, we first compared the elution profiles of the Fmoc derivative of the enzymatic halogenated piperazate with that of an authentic sample of the Fmoc-(3*S*,5*R*)-5-chloropiperazate, synthesized by a variation of a previously described route (see the Supporting Information).[d] It was presumed that the (3*S*,5*R*)-5-chloropiperazate would be the enzymatic halogenation product, given the assignment of 3*S*,5*R* for the chlorinated kutzneride natural products.[1,2] However, LC−MS analysis shows that the Fmoc-chloropiperazate from the halogenase/thioesterase incubations (Figure 3B, trace i) elutes at a retention time different from that of the Fmoc 3*S*,5*R* standard (Figure 3B, trace ii). Both peaks had the anticipated identical mass (387.1141 ± 0.0002 Da) and so most likely reflected different regioisomers or diastereomers.

On the basis of a preliminary reanalysis of published NMR data,[1,2] we hypothesized that the two compounds had a diastereomeric relationship. Therefore, the enzymatically generated diastereomer must have the 3*S*,5*S* or 3*R*,5*R* configuration. We noted above that only the (3*S*)-piperazyl-S-KtzC was a substrate for chlorination by KthP, suggesting that the enzymatically generated chloropiperazate has a 3*S*,5*S* configuration. However, the 3*R*,5*R* configuration would be a formal possibility because aminoacyl thioesters may be subject to Cα deprotonation and epimerization. In this case of the chloropiperazyl thioester, epimerization at C3 could have led to formation of the 3*R*,5*R* product stereoisomer from an initially formed (3*S*,5*R*)-thioester. To evaluate whether the pure KthP halogenase had an unanticipated epimerase activity when halogenating (3*S*)-piperazyl-pantetheinyl-KtzC, the halogenation assays were conducted in deuterated buffer. Epimerization that involves deprotonation of C3 by an active-site base (B) would be expected to generate a BH^+ that equilibrates with solvent deuterons to BD^+, resulting in formation of a 3-deutero-5-chloropiperazyl-S-KtzC product. Hydrolysis by thioesterase action would yield deuterated derivatives of chloropiperazate and/or unreacted piperazate, which could be detected by MS. However, neither the starting piperazyl-S-KtzC nor the chloropiperazyl-S-KtzC product, after thioesterase-mediated release, showed any deuterium incorporation (data not shown), arguing against reversible formation of a C3 thioester enolate and epimerization at C3.

We then turned to ^{1}H NMR spectroscopic analysis of the thioesterase-released chloropiperazate to help prove the regiochemistry of chlorination and relative stereochemistry. We began our NMR spectroscopic studies with analysis of the synthetic sample of Fmoc-protected (3*S*,5*R*)-5-chloropiperazate prepared according to the route shown in the Supporting Information. As is often the case for Fmoc-containing compounds, ^{1}H NMR spectroscopic line shapes were extremely broad, precluding stereochemical analysis. In contrast, the TFA salt of (3*S*,5*R*)-5-chloropiperazic acid showed much better NMR spectroscopic properties and was thus chosen for analysis via double-quantum-filtered correlated spectroscopy (dqfCOSY). In small molecule structural analysis, dqfCOSY spectra exhibit a good signal-to-noise ratio down to nanomole amounts of analyte and allow determination of *J* coupling constants even for samples containing large amounts of impurities.[23] Analysis of the dqfCOSY spectrum of synthetic (3*S*,5*R*)-5-chloropiperazate (Figure S5 of the Supporting Information) yielded *J* coupling constants for the protons at C3−C6 that unambiguously confirmed the 3*S*,5*R* stereochemistry of the synthetic sample and demonstrated the feasibility of using dqfCOSY spectra for determining the relative

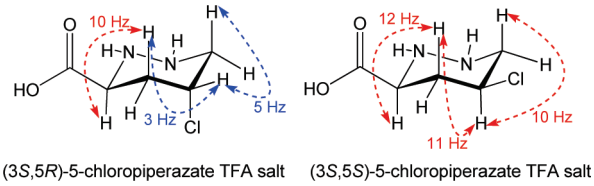

Figure 5. Structures of synthetic (3*S*,5*R*)-5-chloropiperazate and KthP-derived (3*S*,5*S*)-5-chloropiperazate TFA salt. Colored arrows highlight coupling constants diagnostic for relative stereochemistry (600 MHz, CD_3OD). Red arrows denote 1,2-diaxial coupling and blue arrows 1,2-axial−equatorial coupling.

stereochemistry of chlorinated piperazic acid derivatives (Table S1 of the Supporting Information).

For NMR spectroscopic analysis of KthP-derived 5-chloropiperazate, the Fmoc derivative purified by HPLC was deprotected and converted into the corresponding TFA salt. The dqfCOSY spectrum of the resulting sample (Figure S6 of the Supporting Information) revealed a large vicinal−diaxial coupling between H3 and the axial proton of the C4 methylene, H4a (Figure 5 and Table S2 of the Supporting Information), indicating that the carboxyl group had to be equatorial in KthP-derived chloropiperazate, as was found to be the case for synthetic (3*S*,5*R*)-chloropiperazate. However, in contrast to the synthetic standard, H5 in the KthP-derived chloropiperazate exhibited large couplings to both H4a (axial) and H6a (axial), indicating an axial orientation for H5 and thus an equatorial positioning of the C5-chlorine substituent (Figure 5). Given the equatorial positioning of both the carboxyl and the chlorine substituents, the enzymatic product had to be either 3*R*,5*R* or 3*S*,5*S*. Considering that the enzyme only uses the 3*S* isomer as a substrate, with no evidence of epimerization, we have concluded that, unexpectedly, the KthP-derived 5-chloropiperazate has the 3*S*,5*S* configuration. High-resolution mass spectrometry was used to further confirm the identity of KthP-derived 5-chloropiperazate after deprotection and NMR spectroscopic analysis (see Materials and Methods). The deprotected 5-chloropiperazate was also rederivatized with Fmoc and the product analyzed by HPLC, which confirmed that the stereochemistry of KthP-derived 5-chloropiperazate had remained unchanged throughout the entire process (Figure 3B, trace iii). The fact that the nascent KthP product is the (3*S*,5*S*)-chloropiperazate, rather than the 3*S*,5*R* diastereomer observed in mature kutznerides 2 and 8, suggests some subsequent steps must be involved for further processing of the chloropiperazate building block.

DISCUSSION

The piperazate ring (formally a hexahydropyridazine 3-carboxylate) is found in a number of *N*-acyl hexadepsipeptide natural products of mixed nonribosomal peptide/polyketide origin, including the immunosuppressant sanglifehrins,[24] the A83586C[25]/GE3[26]/kettapeptin[27]/polyoxypeptin[28] family of antitumor agents, and nonribosomal peptides such as the antibacterial monamycins[29] and the antifungal kutznerides.[1,2] The cyclic hydrazine framework of piperazate is an aza analogue of the more common nonproteinogenic amino acid pipecolate. X-ray structures of azinothricin,[30] A83586C,[25] piperazimycins,[31] and auranticins[32] all indicate that the piperazate rings are present as six-membered chair conformers that impose conformational constraints that rigidify the depsipeptide macrocyclic scaffolds.[5−7,25−34] In these natural product classes, D (3*R*)- and L (3*S*)-piperazate residues are known as well as, in some cases, 5-hydroxy-, 5-chloro-and N(1)-6-dehydro forms of piperazates.[5−7,29,31] In kutznerides 2 and 8, the 5-chloropiperazate ring has been assigned the 3*S*,5*R* configuration by Broberg et al. by NMR analysis[1,2] with the 5-chloro substituent equatorial as shown in Figure 1, based on the assumption that C3 has *S* stereochemistry.

The question of how and when the unactivated C5 methylene CH_2 group undergoes regio- and stereospecific chlorination in these kutzneride family members arose. From our past studies of the discovery and characterization of biosynthetic halogenases that act on comparably unactivated carbon sites in the side chains of amino acids,[15,18,20] we anticipated that a nonheme mononuclear iron^{II}-containing halogenase would be a likely candidate. Such enzymes use O_2, α-KG, and chloride ion as cosubstrates to generate a high-valent oxoiron species with Cl as a first-shell iron ligand.[10] These iron enzymes deliver a chlorine atom equivalent after removal of a hydrogen atom from an unactivated CH_3 or CH_2 group in an aminoacyl substrate. The mononuclear iron halogenases that we and others have discovered to date do not work on the free amino acids.[11] Rather, they catalyze reactions of aminoacyl thioesters tethered to the pantetheinyl prosthetic groups in thiolation domains of proteins affiliated with nonribosomal peptide synthetase assembly lines. Thus, we anticipated that the missing piperazate halogenase would be in this category of enzyme.

In fact, in our initial cloning effort to find the kutzneride biosynthetic gene cluster, we used both $FADH_2$-dependent halogenase gene probes and mononuclear iron halogenase gene probes and found a contiguous set of NRPS genes that also harbored two genes of the first halogenase gene category, *ktzQ* and *ktzR*,[3] and one of the second variety, *ktzD*.[4] However, none of them had activity on piperazate, suggesting that we might be missing relevant biosynthetic genes. In this work, we have built off the genome sequencing effort with *Kutzneria* sp. 744 conducted at the Broad Institute[a] on a series of actinomycetes of interest for their natural product biosynthetic capacity. Our starting focus was on bioinformatic prediction of any additional mononuclear iron halogenases in the *Kutzneria* genome, distinguishing the O_2- and α-KG-dependent halogenase subfamily from the more prevalent mononuclear iron oxygenase family. This approach predicts that the halogenases have only two histidine ligands instead of the canonical 2-His-1-carboxylate ligand set for Fe^{II} that characterizes the oxygenases.[10] In the half-dozen such halogenases studied so far,[11] the side chain carboxylate (Asp/Glu) ligand to iron is replaced with an Ala, leaving room for chloride ion now to become a first-shell ligand to the Fe^{II}.

There is only one additional orf that is a mononuclear iron halogenase candidate in the *Kutzneria* sp. 744 genome, and it is located ∼12 orfs upstream of the originally assigned core of the ktz cluster. Because several orfs without apparent relevance to kutzneride synthesis intervene (Figure 1), this was not recognized until the genomic sequence became available.[a] Intriguingly, orf 8892 (KthP) is a novel variant inasmuch as it has a Ser rather than an Ala side chain in place of the canonical Asp or Glu. In principle, one would like to make genetic deletions to observe the effects on kutzneride production, but no genetic system is yet available for *Kutzneria*.

As anticipated, there was no detectable chlorination of free L- or D-piperazate, so we turned to generation of piperazyl-S-T domain proteins as potential halogenation substrates. Despite several attempts to overproduce them, we could not obtain a soluble, active

piperazate-activating NRPS module from *E. coli* expression. Therefore, we turned to the strategy of enzymatic conversion of an apo form of a T domain protein, in this case the 10.8 kDa KtzC, to the aminoacylated holo form through the action of a broad-specificity phosphopantetheinyl transferase, Sfp from *B. subtilis*. Chlorination of piperazyl-S-KtzC by KthP was observed as an increase of 34 Da in MALDI analysis of the product still tethered via a thioester linkage to KtzC.

The piperazyl scaffold was capable of being evaluated in more detail by TycF-mediated mild release, followed by derivatization of the released piperazate at N1 with an Fmoc group to allow UV detection by HPLC analysis. The generation of the chloropiperazyl product depended on KthP and gave an accurate mass analysis, validating that the newly introduced chlorine substituent was indeed present. Parallel studies with synthetic D-piperazyl-CoA showed no chlorination, nor was any hydroxylation of the piperazyl-S-T domain detected, as might have been anticipated from the presence of the 5-OH-piperazyl residue in kutzneride 6 and a high-valent iron cofactor in KthP that should have both OH and Cl bound as ligands.[2]

The configuration of the unmodified piperazates in kutznerides 1 and 3 has been shown to be L (3*S*) on the basis of hydrolysis and hydrogenation to ornithine. The reasonable assumption that the chlorinated piperazates in kutznerides 2 and 8 would likewise be 3*S* was made. Assuming the carbonyl-bearing carbon was in the *S* configuration, the ROESY NMR data for kutznerides 2 and 8 indicated that the chlorine was equatorial and *trans*-disposed to the axial carboxy at C3, leading to a 3*S*,5*R* assignment.

Guided by the published structure, we synthesized an authentic sample of (3*S*,5*R*)-5-chloropiperazate. To our surprise, we did not see cochromatography of the N1-Fmoc derivative with the halogenated product released from KtzC by the thioesterase TycF, suggesting that other regioisomers or stereoisomers were the possible halogenase products. From NMR analysis, we were able to assign the chlorination regiochemistry and stereochemistry of the KthP product as either 3*S*,5*S* or 3*R*,5*R*. Given the halogenation of the (3*S*)-piperazyl thioester, and the lack of any detectable enzyme-mediated or chemical epimerization at C3, we have assigned the halogenation product as (3*S*,5*S*)-5-chloropiperazate.

It is not immediately clear how the KthP-derived (3*S*,5*S*)-5-chloropiperazate is biosynthetically converted to the 3*S*,5*R* diastereomer present in the assembled kutzneride scaffold. Once the (3*S*,5*S*)-chloropiperazate building block has been generated and then released as the free amino acid (orfs 8889-90 and 8892–93), we anticipate its ATP-dependent activation by the KtzH NRPS first module.[8] This module contains an epimerization (E) domain. On the basis of precedents from other E domain-containing modules,[35] it is probable that the activated Cl-piperazyl-S-pantetheinyl-KtzH intermediate would be epimerized at C3 and the resultant 3*R*,5*S* diastereomer (in a 3*S*,5*S* and 3*R*,5*S* mixture) condensed and elongated by the next condensation domain in the kutzneride assembly.[36] However, that would lead to a 3*R*,5*S* diastereomer, rather than the 3*S*,5*R* diastereomer assumed from the NMR analysis of kutznerides 2 and 8 in the original isolation paper.[e] Because we have, as yet, been unable to express KtzH domains and modules in active form in heterologous host expression systems, we have not yet been able to address this proposed next step in processing and elongation of (3*S*,5*S*)-5-chloropiperazate. Such studies must overcome the present hurdle of our inability to produce soluble, active piperazate loading and condensation modules in the kutzneride NRPS assembly line. There is the formal possibility that another piperazate halogenase exists in the *Kutzneria* genome and makes a 3*S*,5*R* diastereomer, but no such candidate is detected by genome bioinformatic analysis. Although we cannot exclude the possibility that tethering the piperazyl thioester to another T domain or in the context of a full NRPS module might affect the stereochemistry of chlorination at C5, this possibility seems unlikely.

We have also considered the possibility of an epimerization at C5 of (3*S*,5*S*)-5-chloropiperazate, which would lead to the 3*S*,5*R* configuration in line with the original assignment. That could proceed via a C6/N1 oxidation of the (3*S*,5*S*)-5-chloropiperazate to the cyclic hydrazone, an ensuing aza enolization event followed by stereospecific protonation at C5, and, finally, reduction of the new (3*S*,5*R*)-5-chlorodehydropiperazate, which shares the same moiety of dehydropiperazate as in kutznerides 4, 6, and 9. However, currently there are no good candidates for enzymes that might be involved in such a multistep conversion.

Further mechanistic and structural studies of KthP will be required to probe the stereo- and regioselectivity of chlorination at C5 in L-piperazyl residues (and C4 of pipecolyl residues). The same is true with regard to delineating the selectivity of transfer of $Cl^{\bullet}$ rather than $OH^{\bullet}$ to the C5 of bound substrate,[37] the degree of selectivity for cognate and noncognate T domains as presentation platforms, and the role of the active site Ser (rather than Ala) in creating space around Fe^{II} for the chloride ion to become a first-shell ligand.[10] Overproduction in *E. coli* gives high yields of soluble KthP, indicating this will be a promising candidate for characterization via structural biology.

Through their action, halogenases can convert simple amino acids into unique building blocks; in the model system presented by the kutzneride cluster, we see a broad display of Nature's halogenation strategies. The kutzneride biosynthetic pathway is now shown, remarkably, to have four biosynthetic halogenases interspersed with the NRPS modules and the enzymes for providing the dedicated nonproteinogenic amino acid building blocks. All four of the halogenases use O_2 as a cosubstrate, highlighting the oxidative nature of the four chlorinations. The enzymes are differentiated by the use of flavin cofactors (KtzQ and KtzR)[3] or nonheme iron cofactors (KtzD[4] and KthP) to tune the reactivity of the enzymes to match the electronic environments of their particular substrates. Halogenation can serve not only as an end in itself but also as an intermediate step in further diversification. In this case, chlorination by KthP tailors the piperazate scaffold with unique functionality, in addition to serving as a cue for subsequent stereochemical diversification of the residue. These results thus expand our knowledge of the important role halogenases play in the generation of the highly modified nonproteinogenic amino acids such as those found in the kutzneride antifungal scaffolds.

■ ASSOCIATED CONTENT

Supporting Information. Detailed procedure for the synthesis of various CoA substrates and the N(1)-Fmoc-(3*S*,5*R*)-5-Cl-piperazate, NMR spectroscopic data for synthetic (3*S*,5*R*)-5-chloropiperazate and KthP-derived (3*S*,5*S*)-5-chloropiperazate trifluoroacetic acid salt, overexpression and purification of KthP, HPLC traces of cleaved reaction products as Fmoc derivatives showing effects of different T domains and cofactors (O_2, Fe^{II}, and α-KG) on chlorination by KthP, and MALDI of (*S*)-pipecolyl-S-KtzC. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

[a]Actinomycetales group Database (http://www.broadinstitute.org/annotation/genome/streptomyces_group/MultiHome.html, accessed April 25, 2011).

[b]In the genomic sequence of *Kutzneria* sp. 744 reported by the Broad Institute (http://www.broadinstitute.org/), a stop codon was annotated between orf 8889 and orf 8890. We resequenced this region to confirm that the stop codon does not exist and orf 8889 and orf 8890 should be in one orf.

[c]KtzC in holo form could arise either form loading of CoA that is presented in synthesized piperazyl-CoA at a low level or by thioester hydrolysis at any point in the reaction, both of unreacted piperazyl and product Cl-piperazyl thioesters.

[d]For our first-generation synthesis of (3*S*,5*R*)-*N*1,*N*2-di-*tert*-Boc-5-chloropiperazic acid methyl ester, see ref 38. For our second-generation enantiospecific syntheses of (3*S*,5*R*)- and (3*R*,5*S*)-5-chloropiperazic acids from the individual enantiomers of diethyl tartrate, see ref 39.

[e]Comparing the reported NMR spectroscopic data of the largely identical kutznerides 1 and 2, we noted a surprisingly large difference in the chemical shift values of the α-protons of the *tert*-butylglycine residues adjacent to the piperazates. In addition, nuclear Overhauser effects between the protons of the *tert*-butylglycine residue and the α-proton of the piperazate in kutzneride 1 are absent in chlorinated kutzneride 2. These differences might be attributable to the C3 configuration of the 5-chloropiperazate of kutzneride 2 being epimeric to the piperazate unit of kutzneride 1.

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ABBREVIATIONS

KthP, kutzneride halogenase for piperazate; α-KG, α-ketoglutarate; Fmoc, fluorenylmethyloxycarbonyl; MALDI, matrix-assisted laser desorption ionization; C domain, condensation domain; A domain, adenylation domain; T domain, thiolation domain; HPLC, high-performance liquid chromatography.

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