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Insights into an Unusual Nonribosomal Peptide Synthetase Biosynthesis

IDENTIFICATION AND CHARACTERIZATION OF THE GE81112 BIOSYNTHETIC GENE CLUSTER[*][S]

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The GE81112 tetrapeptides (1–3) represent a structurally unique class of antibiotics, acting as specific inhibitors of prokaryotic protein synthesis. Here we report the cloning and sequencing of the GE81112 biosynthetic gene cluster from *Streptomyces* sp. L-49973 and the development of a genetic manipulation system for *Streptomyces* sp. L-49973. The biosynthetic gene cluster for the tetrapeptide antibiotic GE81112 (*getA-N*) was identified within a 61.7-kb region comprising 29 open reading frames (open reading frames), 14 of which were assigned to the biosynthetic gene cluster. Sequence analysis revealed the GE81112 cluster to consist of six nonribosomal peptide synthetase (NRPS) genes encoding incomplete di-domain NRPS modules and a single free standing NRPS domain as well as genes encoding other biosynthetic and modifying proteins. The involvement of the cloned gene cluster in GE81112 biosynthesis was confirmed by inactivating the NRPS gene *getE* resulting in a GE81112 production abolished mutant. In addition, we characterized the NRPS A-domains from the pathway by expression in *Escherichia coli* and *in vitro* enzymatic assays. The previously unknown stereochemistry of most chiral centers in GE81112 was established from a combined chemical and biosynthetic approach. Taken together, these findings have allowed us to propose a rational model for GE81112 biosynthesis. The results further open the door to developing new derivatives of these promising antibiotic compounds by genetic engineering.

The emergence of multi-drug resistant microbial pathogens is driving the search for novel antibiotics with new mechanisms of action and natural products continue to provide original scaffolds affecting essential bacterial targets (1, 2). As it is currently understood, most antibiotics act in three basic ways; 1) inhibition of DNA replication and repair, 2) inhibition of cell wall biosynthesis, and 3) inhibition of protein biosynthesis. Protein translation and cell wall biosynthesis in bacteria are currently the targets for the majority of antimicrobial natural products. The former is the target of the macrolides (*e.g.* erythromycin), the aminoglycosides (*e.g.* kanamycin), the streptogramins, the lincosamides, tetracycline, and chloramphenicol as well as of other classes of compounds that are not in clinical use. These compounds have been shown to affect protein biosynthesis at various steps (3, 4).

Some aspects of protein translation are rarely targeted, *e.g.* translation initiation and polypeptide chain termination. A microbial product screening program aimed at discovering novel inhibitors of bacterial protein synthesis revealed the new tetrapeptide GE81112 compounds (**1**-**3**) (Fig. 1) to selectively inhibit the formation of the prokaryotic 30 S initiation complex with an $IC_{50} = 0.9$ μM (5). To date, three GE81112 congeners, A (**1**), B (**2**), and B1 (**3**), have been described from a *Streptomyces* sp. (Fig. 1). Extensive NMR and MS studies revealed the tetrapeptides to comprise hydroxypipecolic and hydroxypentanoic acids, an (amino)histidine, and a hydroxychlorohistidine (5). A retro-biosynthetic analysis of the GE81112 core structure suggests a NRPS[2] origin with additional tailoring steps occurring at some point during assembly. NRPS multienzymes are composed of successive catalytic units or "domains" that are themselves organized into biosynthetic modules which catalyze the assembly reactions in a coordinated, often "co-linear" manner. Normally, each module in the assembly line performs one cycle of chain extension (condensation of one residue into the growing peptide chain). A typical, minimal NRPS module consists of an adenylation (A) domain, a peptidyl carrier protein (PCP) domain (also referred to as a thiolation (T) domain), and a condensation (C) domain (6). However, a growing number of gene clusters encode systems that deviate in their domain organization from the standard C-A-PCP architecture and comprise partial modules or isolated domains acting in *trans* to complement the functionality of the multimodular NRPSs (7–9).

To better understand GE81112 biosynthesis and to generate structural analogues, we sought to develop a strategy for the cloning and identification of the biosynthetic gene cluster. To this end, the cluster was identified on two overlapping cosmids. In addition, we expressed five A-domains from the corresponding NRPS genes and characterized them *in vitro*. These results together with the assignment of the configuration of most chiral centers have

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S4.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) FN821996.

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[2] The abbreviations used are: NRPS, nonribosomal peptide synthetase; A, adenylation; PCP, peptidyl carrier protein; T, thiolation; C, condensation; *O*-HPA, 5-hydroxy-2-aminopentanoic acid; Pip, pipecolic acid.

allowed us to delineate this unusual NRPS pathway and propose a biosynthetic model for the GE81112 antibiotics.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—Streptomyces sp. L-49973 was grown in INA5 (glycerol, 30 g liter^{-1}; soya extract, 15 g liter^{-1}; $CaCO_3$, 5 g liter^{-1}; NaCl, 2 g liter^{-1}) or T6 (glycerol, 45 g liter^{-1}; soya extract, 25 g liter^{-1}; $CaCO_3$, 2 g liter^{-1}) media in baffled flasks for production of GE81112. Pre-cultures were grown in V6 medium (glucose, 20 g liter^{-1}; meat extract, 5 g liter^{-1}; yeast extract, 5 g liter^{-1}; peptone 5 g liter^{-1}; caseine, 3 g liter^{-1}; NaCl, 1.5 g liter^{-1}, pH 7.5) and used to inoculate production culture (1:100). The cultures were maintained at 30 °C and 180 rpm on a rotary incubator and harvested after 6 days. *Escherichia coli* DH10B, *E. coli* ET12567/pUZ8002, and *E. coli* SURE were grown in liquid LB medium at 37 or 30 °C with the appropriate antibiotic selection. Antibiotic concentrations were as follows; apramycin (60 μg ml^{-1}), chloramphenicol (34 μg ml^{-1}), kanamycin (60 μg/ml^{-1}), and ampicillin (100 μg ml^{-1}) were used for selection in *E. coli*. Apramycin (60 μg ml^{-1}) was used for selection of *Streptomyces* sp. L-49973 recombinants. Nalidixic acid (25 μl ml^{-1}) was used to select against *E. coli* donor after conjugation.

Molecular Biology Methods—The pET28b (+) (Novagen) and pCR2.1 TOPO (Invitrogen) cloning vectors were from commercial sources; pKC1132 and pOJ436 were described previously (10). Restriction enzymes were purchased from MBI Fermentas. All PCRs were carried out using Taq (MBI Fermentas) or Phusion (Invitrogen) polymerase. DMSO was added to the reaction mixture to a final concentration of 5%. Conditions for amplification with a Peqlab ThermoCycler were as follows: denaturation, 15–30 s at 95/98 °C; annealing, 8–20 s at 50–62 °C; extension, 15–60 s at 72 °C (30 cycles); final extension at 72 °C for 10 min. Oligonucleotides were obtained from Sigma. Plasmid DNA was isolated using the GeneJET^{TM} Plasmid Miniprep kit (Fermentas). DNA fragments from agarose gel were isolated and purified using the NucleoSpin Extract II kit (Macherey-Nagel). All other DNA manipulations in *E. coli* (11) and *Streptomyces* (10) were carried out using standard protocols. For colony hybridization analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed according to the manufacturer's protocol (Roche Diagnostics).

Construction of a Streptomyces sp. L-49973 Genomic Cosmid Library—A *Streptomyces* sp. L-49973 genomic cosmid library was constructed in *E. coli* SURE. Genomic DNA was isolated with the salting out procedure (10). Then the DNA was partially digested with *Sau*3A, yielding fragments with an average size greater than 35 kb. The fragments were ligated into cosmid vector pOJ436 (12) digested with BamHI and PvuII and *in vitro* packaged with the Gigapack III Gold packaging extract kit according to the manufacturer's handbook (Stratagene). 2304 colonies were transferred into six 384-well microtiter plates using a Qbot robot (Genetix) and grown overnight in 2YT medium. For storage at −80 °C, 50 μl of freezing solution (0.076% $MgSO_4 \cdot 7\ H_2O$, 0.45% sodium citrate·2 H_2O, 0.9% NH_4SO_4, 44% glycerol, 4.7% K_2HPO_4, 1.8% KH_2PO_4) was added to each well. Robotically produced high density colony arrays (Hybond N+, Amersham Biosciences) were utilized for the screening of the cosmid clones, as described previously (13).

Screening of a Streptomyces sp. L-49973 Genomic Cosmid Library—Cyclodeaminase and NRPS fragment sequences were initially used as probes under low stringency conditions. To create the cyclodeaminase probe, we used specific primers to amplify two known cyclodeaminase genes, *tubZ* (14) (primers Tubz_up and TubZ_down, 570-bp product)m from the tubulysin cluster, and *rapL* (15), from the rapamycin cluster (primers RapL_up and RapL_down, 534-bp PCR product) (see the primers in supplemental Table S1). As an alternative approach, we designed an additional probe to identify NRPS genes. For this, we used degenerate NRPS primers; NRPS-A1-up and NRPS-H1-dn (supplemental Table S1). These primers amplify A-domains between the structural regions A3 and A6 (16), where the amino acid binding pocket is located, giving a 760-bp PCR fragment. The amplicons were labeled with digoxigenin and used to probe the cosmid library at low stringency (40 °C). Several cosmids that hybridized with both probes were subjected to PCR analysis for the amplification of the cyclodeaminase and NRPS A-domains. In the first screening we identified a cosmid encoding a putative cyclodeaminase but not the expected GE81112 biosynthetic enzymes. A segment of this cyclodeaminase was amplified using the primers Cyclo_probe_for and Cyclo_probe_rev (supplemental Table S1) and used under high stringency conditions (42 °C) for further screening of the library. Cosmids hybridizing with this probe were analyzed by PCR using primers to amplify the cyclodeaminase and NRPS A-domains again. The resulting PCR products were gel-purified and subcloned into pCR2.1 TOPO vector and sequenced. Several NRPS sequences were found from different cosmids, and the eight critical residues responsible for substrate recognition could be determined enabling an *in silico* prediction of the substrate specificity of each cloned A-domain fragment. Cosmids harboring A-domains that were predicted to activate pipecolic acid were digested with BamHI, and the restriction pattern was compared with identify similar cosmids. End sequencing of the cosmids was carried out using primers T4 and T7 (supplemental Table S1). One cosmid (BI11) was identified containing a large part of the GE81112 biosynthetic gene cluster. To find a cosmid that overlapped with the 3′ (T7) end of the cluster, a 1-kb fragment was amplified from the T7 end of cosmid BI11 (primers BI11-T7end-for and BI11-T7end-rev, supplemental Table S1) to serve as a probe. The cosmid library was then screened with the new probe, with hybridization at 42 °C. Among the identified cosmids, BA23 showed the smallest extent of overlap with BI11 based on PCR analysis and restriction digest. Cosmids BI11 and BA23 were shotgun-sequenced on both strands as described previously (17).

Data Analysis—The annotation analysis of the sequence data was performed through FramePlot analysis (FramePlot 4.Obeta) (23) and data base comparison with the basic alignment search tool (BLAST) on the server of the National Center for Biotechnology Information. For alignment analysis of the sequence data, ClustalW on the server of EMBL-EBI was used. Specificity of the A-domains was determined by using the NRPS predictor Bioinformatics Toolbox from University of Tübingen and polyketide synthase/NRPS analysis web site.

Conjugation and Generation of Mutant Strains of Streptomyces sp. L-49973—DNA manipulation was carried out with *E. coli* DH10B as the host strain. To generate knock-out mutants by means of insert-directed homologous recombination, a 572-bp internal fragment of gene *getE* was amplified with primers pipA_for and pipA_rev (supplemental Table S1). The fragments were cloned into pCR2.1TOPO, and the constructs were digested with EcoRI. The fragments were ligated into knock-out vector pKC1132 (10) digested with EcoRI. The final pKC1132-derived plasmids were introduced into *Streptomyces* sp. L-49973 by intergeneric conjugation with the methylation-deficient donor strain *E. coli* ET12567 containing the conjugative vector pUZ8002 (10). Mutants were analyzed by PCR using appropriate control primers. One primer was designed to bind to the integrated vector backbone (lacZ1, lacZ2), whereas the second primer was designed to target the genome sequence either up- or downstream of the integration site (A1_for, A1_rev) (supplemental Table S1). PCR of the mutants yielded distinct amplicons, whereas no products were detected from the wild type.

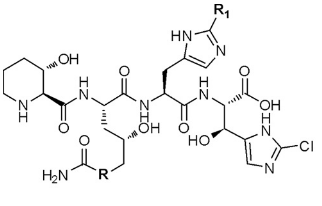
Analysis of GE81112 Production in Streptomyces sp. L-49973—*Streptomyces* strains (wild types and mutants) were cultured in 500-ml baffled shake flasks containing 100 ml of GE81112 production medium (INA5 or T6) at 30 °C and 180 rpm. Recombinant strains were amended with apramycin (60 μg ml^{-1}). A square of agar from a sporulating SM agar plate was used for inoculation. After 6 days of cultivation, cells were harvested by centrifugation at 12,000 rpm for 5 min. The culture supernatant was extracted two times with ethyl acetate, evaporated, and redissolved in 500 μl of methanol. LC-coupled FT-Orbitrap-MS analysis was carried out with an Accella UPLC system (Thermo Electron Corp.) operating in positive ionization mode at a scan range of *m/z* 100–2000. A Hypersil Gold column (2.1 × 50 mm; Thermo Fisher Scientific) was used for separation with a solvent system consisting of H_2O (A) and acetonitrile (B), each containing 0.1% formic acid. A gradient of 5–95% B was applied over 10 min. Measurements were carried out in single ion mode. GE81112 compounds were identified by comparison to the retention times and the MS data of authentic standards (GE congener A: $[M+H]^+ = 644.21858$; GE congener B: $[M+H]^+ = 659.22953$; GE congener B1: $[M+H]^+ = 658.24587$) in the positive ionization mode.

Construction of A-domain Overexpression Constructs—The genes encoding for the five A-domains (GetEA$_1$, GetGA$_2$, GetGA$_3$, GetJA$_4$, and GetMA$_5$) of GE81112 were PCR-amplified from cosmids BI11 and BA23. The forward and reverse primers for amplification of A-domain fragments *getEA*$_1$, *getGA*$_2$, *getGA*$_3$, and *getMA*$_5$ introduced NdeI and BamHI restriction sites (supplemental Table S1). A-domain fragment *getJA4* could not be amplified from the cosmids, probably due to the high GC content. Therefore, the A-domain sequence was synthesized, and the GC content was optimized for use in *E. coli* (ATG::biosynthetics GmbH). The fragment was obtained in pBluescript SK+ (pBSK) vector flanked by restriction sites NdeI and EcoRI (supplemental Fig. S1). All PCR products were cloned into the digested pET28b (+) vector using the corresponding NdeI/BamHI restriction sites. Fragment *getJA*$_4$ was obtained after restriction of pBSK/GetJA4 with NdeI and EcoRI and cloned into pET28b (+). Final plasmids were sequenced and transformed into *E. coli Rosetta* BL21 (DE3) pLysS/RARE for protein expression.

Expression and Purification of the A-domains—Purified A-domain pET28b (+) plasmids were transformed into *E. coli Rosetta* BL21 (DE3) pLysS/RARE competent cells for protein production and purification. Fresh transformants harboring the constructs were grown in LB-medium (1-liter batches started with 0.1% inocula from a 10-ml culture grown for 5 h at 37 °C) supplemented with kanamycin (50 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). All cells were grown at 37 °C to an OD$_{600}$ of ~0.8. The cells were then induced with 1 M isopropyl-β-D-thiogalactopyranoside to an end concentration of 0.2 mM and then grown at 16 °C overnight. The cells were harvested by centrifugation (6000 rpm, 10 min, 4 °C) and resuspended in buffer A (20 mM Tris-HCl, pH 7.8, 200 mM NaCl, and 10% (v/v) glycerol). The cells were then lysed (2 passes at 700 p.s.i., French press, SLM Aminco), and the cell debris was removed by centrifugation (21,000 rpm, 10 min, 4 °C). Prepacked HisTrap^{TM} HP columns were used for preparative purification of histidine-tagged recombinant proteins by immobilized metal ion affinity chromatography on the Äkta prime^{TM} plus system (GE Healthcare). 15-ml protein lysates were filtered through a sterile filter and loaded onto the 1-ml HisTrap column. Purification was performed as recommended in the GE Healthcare manual (HisTrap HP, Instructions 71-5027-68 AF). The desired protein was eluted from the column in a stepwise imidazole gradient with buffer B (20 mM Tris-HCl, pH 7.8, 200 mM NaCl, and 10% (v/v) glycerol and 60, 100, 200, 300, and 500 mM imidazole, 10-ml fractions). Fractions containing the pure target protein, as determined by SDS-PAGE, were combined and concentrated to ~200 μl by using Amicon Ultra PL-10 centricons. Then 800 μl of storage buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10% (v/v) glycerol) was added to the concentrated protein before flash-freezing in liquid nitrogen and storage at −80 °C. Protein concentrations were determined using the Bradford assay (Bio-Rad). 1–3 mg/ml purified protein were obtained for each protein per liter of culture.

Determination of Substrate Specificity by ATP-[^{32}P]PP$_i$ Exchange Assay—To determine substrate specificity, ATP-[^{32}P]PP$_i$ reactions (100 μl) containing Tris-HCl (pH 7.5, 75 mM), $MgCl_2$ (10 mM), dATP (5 mM), amino acid (5 mM), and protein (2 μg) were performed at 30 °C. ^{32}P-Labeled tetrasodium pyrophosphate was obtained from PerkinElmer Life Sciences (NEN #NEX019). The reactions were started by the addition of [^{32}P]PP$_i$ (0.1 μCi final amount) for up to 30 min before quenching with charcoal suspensions (500 μl, 1.6% (w/v) activated charcoal, 0.1 M $Na_4P_2O_7$, and 0.35 M perchloric acid in H_2O). The charcoal was pelleted by centrifugation before being washed twice with the wash solution (500 μl, 0.1 M $Na_4P_2O_7$, and 0.35 M perchloric acid in H_2O), resuspended in H_2O (500 μl), and counted by liquid scintillation (Beckman LS6500). The experiments were carried out in triplicate for each substrate concentration with a negative control (no amino acid).

Chemical Analyses—GE81112 was purified and hydrolyzed as described by Brandi *et al.* (5). Dehalogenation of GE81112 was carried out under H_2 at atmospheric pressure and room temperature in 10% acetic acid, with 10% palladium/carbon as



GE congener A (**1**) **R** = O; **R₁** = H

GE congener B (**2**) **R** = O; **R₁** = NH_2

GE congener B1 (**3**) **R** = NH; **R₁** = NH_2

GE81112

FIGURE 1. **Chemical structures of GE81112 tetrapeptides.** The GE81112 antibiotics are three closely related NRPS tetrapeptides A (**1**), B (**2**), and B1 (**3**). The absolute stereoconfiguration of each amino acid residue has now been determined by chiral GC/MS analysis of the acid-hydrolyzed natural product. These data indicate that the GE81112 factors **1**-**3** are composed of (modified) L-amino acids.

catalyst. Catalytic hydrogenation of hydroxypicolinic acid was performed under H_2 atmosphere (50 p.s.i.) at room temperature in aqueous NH_4OH with 10% palladium/carbon as a catalyst. Dihydroxylation of allylglycine was performed following published procedures (18). A sample of (2*R*,3*R*)-3-hydroxypipecolic acid was kindly provided from Prof. Jieping Zhu (CNRS, Gif-sur-Yvette, France). Chiral GC-MS analyses were performed on the methyl esters, and trifluoroacetyl derivatives were analyzed using a Finnigan TSQ700 triple stage quadrupole mass spectrometer interfaced with a Varian 3400 gas chromatographer (5). NMR and $[\alpha]_D$ literature data for 3-hydroxyhistidine were obtained from (19). Homonuclear ^1H and heteronuclear ^{13}C,^1H NMR experiments were recorded at 400 MHz on a Bruker Advance spectrometer in D_2O or in D_2O acidified with trifluoroacetic acid (TFA).

RESULTS

Isolation of the GE81112 Biosynthetic Gene Cluster—To capture the GE81112 biosynthetic gene cluster (*get*, for GE81112 tetrapeptide) a cosmid library containing 2304 clones was generated from the genomic DNA of the GE81112 producer strain (*Streptomyces* sp. L-49973). Hybridization probes were designed by applying a retrobiosynthetic strategy that allowed us to predict some probable genetic elements of the *get* cluster from analysis of the metabolite structures (Fig. 1). On this basis we designed a set of probes using degenerate primers based on A-domains of streptomycete origin using the CODEHOP software (20); priming was targeted against the core A3 and A6 motifs. Furthermore, from the structure of **1** we predicted that the starter unit likely involves pipecolic acid, which is known to be formed from lysine via the action of a lysine cyclodeaminase (21). As cyclodeaminase genes are relatively rare in bacterial genomes, this gene was used to design a second probe to identify the *get* cluster (22). We designed specific probes based on a cyclodeaminase sequence that was identified in previous experiments in the same strain.[3] Colony hybridization of the *Streptomyces* sp. L-49973 cosmid library with the cyclodeaminase probe led to the identification of 7 cosmid hits. The cosmids were then determined to contain the targeted cyclodeaminase sequence by PCR. As we expected NRPSs to be encoded by the identified cosmids, we used the degenerate NRPS primers to amplify and sequence A-domain segments from these cosmids. Sequence analysis and prediction of the A-domain substrate specificity revealed one cosmid (BI11) containing an A-domain with predicted substrate specificity for proline/pipecolic acid, as expected for the GE81112 starter unit (Table 1). Subsequent, complete sequencing of the cosmid revealed several genes expected for GE81112 biosynthesis. As the entire gene cluster was not present on the cosmid, we identified overlapping cosmids. In total, seven cosmids were identified, and after verification by PCR and restriction analysis, a single one (BA23) was selected for further analysis.

Sequence Analysis and Organization of the get Biosynthetic Gene Cluster—The two overlapping cosmids, BI11 and BA23, were sequenced. The obtained sequence was analyzed for the presence of putative open reading frames (*orfs*) with FramePlot 4.Obeta (23), and preliminary functional assignments of individual *orfs* were made by comparison of the deduced gene products with proteins of known function in the BLAST data base (Table 2). Annotation of the two cosmids (BI11 and BA23) revealed 29 *orfs*, of which 14, designated *getA-N*, are postulated to be involved in the GE81112 biosynthetic pathway (Fig. 2*A*). The first gene predicted to be involved is *getA*, as the proteins encoded by the *orfs* upstream of *getA* show no homology to proteins involved in biosynthetic pathways. *getA* (852 bp) encodes a type II thioesterase. Type II thioesterases are present in many NRPS and polyketide synthase systems, where they perform crucial proofreading functions by hydrolyzing aberrant substrates from the respective carrier protein domains (24). *getA* is found within an operon that also harbors the genes *getB-E*. *getB* and *getC* encode proteins having homology to known ABC transporter systems (25). The next gene, *getD*, encodes the cyclodeaminase that was targeted in our library-probing strategy. It shows 52% identity to TubZ, the cyclodeaminase from *Angiococcus disciformis* (14). The first gene encoding a NRPS protein (a freestanding A-domain) is *getE*, which likely starts with a GTG and is preceded by a putative ribosome binding site (GGAG) 7 bp upstream of the start codon. The next gene, *getF*, is oriented in the opposite direction and encodes a protein with homology to a putative L-(2*S*)-proline 3-hydroxylase. The following gene, *getG* (7212 bp), encodes another NRPS and is the likely starting point of a new operon which includes *getH* and *getI*. *getH* (1614 bp) encodes a di-domain NRPS (PCP-C) enzyme and again starts with a GTG, whereas *getI* exhibits homology to an oxygenase. The last segment of the cluster contains 5 genes (*getJ-getN*) and starts with another change in the transcription direction. It begins with *getJ* (2460 bp), which encodes a NRPS di-domain (A-PCP) enzyme. The next two genes, *getK* and *getL*, encode a GTPase protein and a halogenase, respectively. The NRPS-di-domain (A-PCP) encoding gene *getM* (1848 bp) is assumed to start with a TTG, with a ribosome binding site (AGGG) located 6 bp upstream. The last gene *getN* encodes a protein with homology to a type I thioesterase. The involvement of *orfs* 1–5 and 6–15 in GE81112 biosynthesis is unlikely but cannot yet be excluded. Further experiments will be carried out in the future to determine the exact boundaries of the gene cluster.

Analysis of NRPS Domains—For the seven *orfs* with homology to NRPS genes (Fig. 3*A*), the constituent domains were assigned using the polyketide synthase/NRPS predictor (PKS Analysis Web site) and confirmed by manual inspection with

[3] T. M. Binz, S. I. Maffioli, M. Sosio, S. Donadio, and R. Müller, unpublished data.

TABLE 1
Prediction of substrate specificity of GE81112 A-domains based on the specificity-conferring codes of A-domains
Orn, ornithine.

A-domain	Position of the amino acid within the A-domain								Predicted amino acid	Identity
	235	236	239	278	299	301	322	330		
										%
GetEA$_1$	Asp	Val	Gln	Tyr	Ile	Ala	Gln	Val	Pro/Pip	70
GetGA$_2$	Asp	Ala	Tyr	Asn	Leu	Gly	Leu	Ile	Orn/Gln/Asp	70
GetGA$_3$	Asp	Ala	Val	Gly	Val	Gly	Glu	Val	Tyr/Trp	70
GetJA$_4$	Asp	Ser	Ala	Ser	Thr	Ala	Glu	Val	His	70
GetMA$_5$	Asp	Ser	Ala	Leu	Thr	Ala	Glu	Val	His	70

TABLE 2
Predicted function of non-PKS/NRPS proteins present up- and downstream of the GE81112 biosynthetic gene clusters

Protein	No. of amino acids	Proposed function of the homologous protein	Origin	Identity/ similarity	Accession no.
				%	
Proteins encoded upstream of the GE81112 biosynthetic gene cluster					
Orf1	335	Partitioning-binding protein	*Nocardia farcinica* IFM 10152	89/95	CBL93707.1
Orf2	502	Hypothetical protein	*S. scabiei* 87.22	77/84	CBL93708.1
Orf3	476	Hypothetical protein 5443	*S. scabiei* 87.22	58/69	CBL93709.1
Orf4	1203	FtsK/SpoIIIE family protein	*S. scabiei* 87.22	78/86	CBL93710.1
Orf5	1238	Serine/threonine protein kinase	*Streptomyces avermitilis* MA-4680	53/67	CBL93711.1
NRPS portion of the biosynthetic gene cluster					
GetA	284	Thioesterase	*Streptomyces hygroscopicus* ATCC 53653	64/75	CBL93712.1
GetB	564	ABC multidrug transporter	*Streptosporangium roseum* DSM 43021	52/67	CBL93713.1
GetC	596	ABC multidrug transporter	*S. roseum* DSM 43021	58/74	CBL93714.1
GetD	402	Cyclodeaminase TubZ protein	*Angiococcus disciformis*	52/65	CBL93715.1
GetE	518	Syringopeptin synthetase	*Pseudomonas syringae*	40/54	CBL93716.1
GetF	364	L-Proline 3-hydroxylase	*Ralstonia solanacearum* GMI1000	33/52	CBL93717.1
GetG	2404	Peptide synthetase protein	*R. solanacearum* GMI1000	31/46	CBL93718.1
GetH	538	Peptide synthetase protein	*S. hygroscopicus* ATCC 53653	40/49	CBL93719.1
GetI	346	Oxygenase	*Streptomyces rochei*	48/66	CBL93720.1
GetJ	820	Bacitracin synthetase 3	*Bacillus licheniformis*	37/52	CBL93721.1
GetK	435	GTP-binding protein	*S. scabiei* 87.22	58/72	CBL93722.1
GetL	585	Halogenase	*Microcystis aeruginosa*	40/57	
GetM	616	Bacitracin synthetase 3	*B. licheniformis*	39/56	CBL93723.1
GetN	288	Thioesterase	*Myxococcus xanthus*	43/58	CBL93724.1
Proteins downstream of the GE81112 biosynthetic gene cluster					
Orf6	315	3-Oxoacid-CoA transferase subunit B	*Streptomyces ghanaensis* ATCC 14672	84/90	CBL93725.1
Orf7	260	Protocatechuate 3,4-dioxygenase α-subunit	*S. avermitilis* MA-4680	70/81	CBL93726.1
Orf8	196	Protocatechuate 3,4-dioxygenase, β-subunit	*Thermomonospora curvata* DSM 43183	76/83	CBL93727.1
Orf9	488	3-Carboxymuconate cycloisomerase	*Streptomyces sviceus* ATCC 29083	71/78	CBL93728.1
Orf10	260	Deoxyribose-phosphate aldolase	*S. scabiei* 87.22	73/79	CBL93729.1
Orf11	1194	Arthrofactin synthetase/syringopeptin synthetase	*Bradyrhizobium sp.* BTAi1	43/54	CBL93730.1
Orf12	818	Putative NRPS	*Streptomyces viridochromogenes* DSM 40736	35/47	CBL93731.1
Orf13	285	Translation-associated GTPase	*Streptomyces albus* J1074	88/92	CBL93732.1
Orf14	424	Hypothetical protein	*Streptomyces ambofaciens*	44/60	CBL93733.1
Orf15	486	Integrin-like protein	*S. viridochromogenes* DSM 40736	44/59	CBL93734.1

BLAST. To incorporate four amino acids into GE81112, the synthetases were expected to contain a loading module followed by three condensation modules with the standard C-A-PCP arrangement. Furthermore, the simplest model predicts that the four amino acid precursors would be incorporated in a co-linear manner: pipecolic acid-ornithine/glutamine/glutamic acid-histidine-histidine. However, the *get* NRPS genes exhibit a non-co-linear arrangement that did not fit our expected model (Fig. 3*A*). Although the domain complement necessary for the biosynthesis of a tetrapeptide could be identified, one extra A-PCP di-domain was present. Moreover, the NRPS modules show a highly split arrangement, as they occurred as freestanding domains (GetE) or di-domain units (GetH, GetJ, GetM) (Fig. 3*A*). Thus, from the domain assignment alone, the overall order of subunits could not be discerned. To determine the substrate specificity of the A-domains, the specificity-conferring code and eight conserved motifs were identified for each A-domain, and bioinformatic analysis was employed to predict their substrate specificities (Table 1 and supplemental Fig. S2) (26, 27). The first A-domain (GetEA$_1$), encoded by *getE* as a discrete protein, was a candidate for starter unit selection as its predicted specificity was for the incorporation of proline/pipecolic acid (Table 1). The second A-domain (GetGA$_2$) showed homology to ornithine/glutamine/asparagine-incorporating A-domains. The third A-domain (GetGA$_3$) was predicted to be specific for the incorporation of tyrosine/tryptophan and the two latter domains GetJA$_4$ and GetMA$_5$ for histidine (Table 1). These results correlated well with the prediction that an ornithine and two histidines are incorporated into the GE81112 metabolites. However, it remained unclear why two A-PCP di-domains (GetM and GetJ) are encoded by the cluster, as only one is required to give a full complement of four active modules. To check if any of the domains were inactive, the C- and PCP-domains were analyzed as well. The *get* cluster encodes three C-domains that aligned well with the C-domains from the rapamycin-, the gramicidin-, and the calcium-dependent antibiotic biosynthetic clusters (28–30). The seven conserved regions were identified in all of

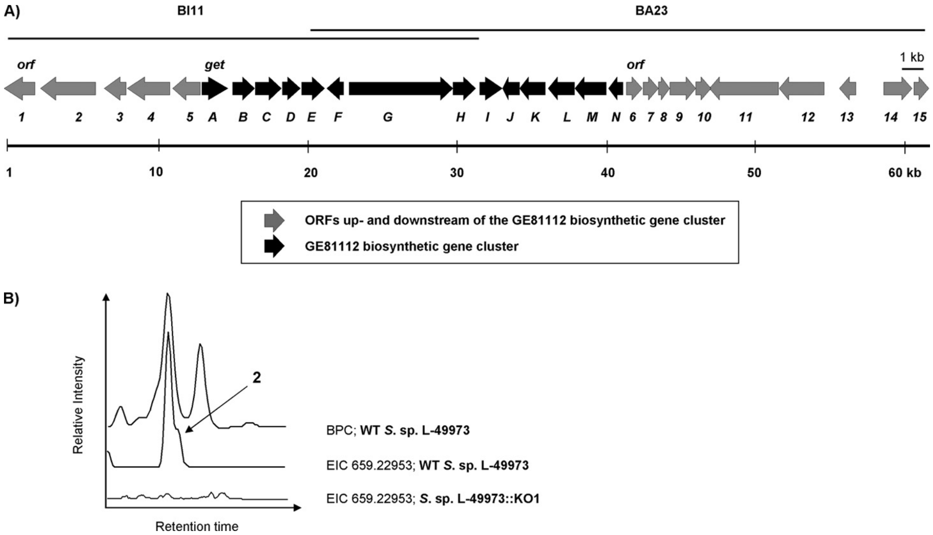

A)
BI11
BA23
orf
get
orf
1 kb
1
2
3
4
5
A
B
C
D
E
F
G
H
I
J
K
L
M
N
6
7
8
9
10
11
12
13
14
15
1
10
20
30
40
50
60 kb
ORFs up- and downstream of the GE81112 biosynthetic gene cluster
GE81112 biosynthetic gene cluster
B)
Relative Intensity
2
BPC; WT S. sp. L-49973
EIC 659.22953; WT S. sp. L-49973
EIC 659.22953; S. sp. L-49973::KO1
Retention time

FIGURE 2. **Organization of the GE81112 biosynthetic gene cluster in *Streptomyces* sp. L-49973.** *A*, a schematic representation of the GE81112 biosynthetic locus and flanking ORFs in *Streptomyces* sp. L-49973 is represented on two overlapping cosmids. Proposed functions for individual ORFs are summarized in Table 2. *B*, shown is LTQ high resolution Orbitrap MS analysis of extracts of *Streptomyces* sp. L-49973 wild type and *Streptomyces* sp. L-49973::KO1 mutant, showing a base peak chromatogram (*BPC*) of *Streptomyces* sp. L-49973 wild type extract (m/z = 100–2000) and extracted ion chromatograms (*EIC*) of GE81112 compound B (**2**) with a molecular ion of the mass m/z = 659.22953 $[M+H]^+$ from *Streptomyces* sp. L-49973 wild type and *Streptomyces* sp. L-49973::KO1 mutant extracts. GE81112 production is abolished in the mutant.

the C-domains and the same analysis was carried out for the PCP-domains, revealing the signature sequence and active serine residue, in each case (supplemental Fig. S3).

These results demonstrate that, in principle, all of the NRPS domains are active. Furthermore we annotated two discrete thioesterases (encoded by *getA* and *getN*), both containing the conserved motif *GXSXG*, present in functional enzymes (16). BLAST analysis (Table 2) revealed that the protein GetA is more related to type II thioesterases, whereas GetN is more related to type I thioesterases. This finding was surprising, as GetN is a discrete protein (like a type II thioesterase) not integrated into an NRPS-like type I thioesterase typically found in bacterial systems.

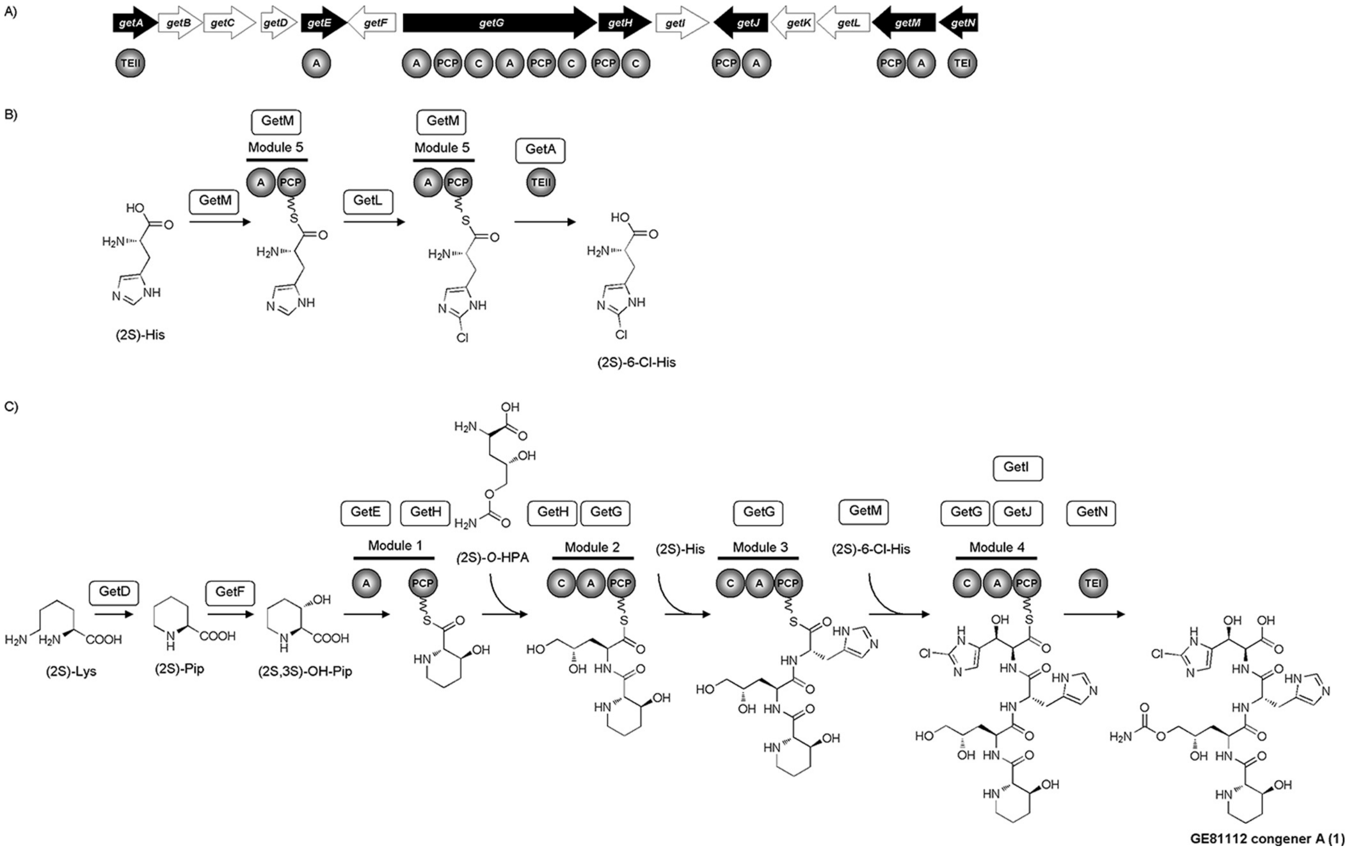


FIGURE 3. **A linear model of GE81112 biosynthesis.** *A*, genetic and modular organization of the GE81112 biosynthetic gene cluster is shown. *Black arrows* indicate NRPS genes, and *white arrows* non-NRPS genes. *TEI*, type I thioesterase. *B*, proposed biosynthesis of the fourth amino acid precursor is shown. Free (2*S*)-histidine is activated by the A-domain and loaded to the PCP of GetM. The PCP-bound histidine is chlorinated at position 6 by the halogenase GetL and hydroxylated at the *β*-position by GetI, although one of these reactions may not occur on the GetM-bound amino acid. It is not clear if the halogenation or hydroxylation occurs first, and it could be either way. The type II thioesterase GetA hydrolyzes the modified histidine to give the free amino acid. *C*, shown is the proposed biosynthesis of GE81112 congener A. Lysine is converted to pipecolic acid by the cyclodeaminase GetD followed by hydroxylation catalyzed by GetF. (2*S*,3*S*)-Hydroxypipecolic acid is then activated by the A-domain GetE and loaded to the PCP of GetH. *O*-HPA, histidine, and 3-hydroxy-6-chlorohistidine are activated and loaded by modules 2, 3, and 4 in the next steps, and the final tetrapeptide is released by the type I thioesterase (*TEI*) GetN.

Confirmation of the Role of the get Cluster by Gene Inactivation—To verify the identity of the proposed GE81112 biosynthetic gene cluster, we inactivated *getE,* which encodes a free-standing A-domain. For this, a knock-out construct was designed by amplifying an internal fragment, which was then cloned into the knock-out vector pKC1132 (10). Initial attempts to transform *Streptomyces* sp. L-49973 with the knock-out plasmid were unsuccessful, necessitating the development of an adapted transformation method. Only by using a larger ratio of *E. coli* cells (5×10^{10}) to 5×10^7 recipient cells were we able to obtain several exconjugants containing the knock-out vector, indicating that the number of donor cells is crucial for the conjugation efficiency with this strain (31). The resultant mutants were verified by PCR against the apramycin resistance gene as well as an internal region from the genomic DNA. The mutants were then cultivated in production medium, and the extracts were analyzed for the presence of the GE81112 compounds by high resolution MS. This analysis clearly showed that GE81112 production was abolished in the mutants, confirming the role of the cloned gene cluster (Fig. 2*B*).

Biochemical Analysis of Adenylation Domains—To obtain experimental evidence for A-domain substrate specificity, we expressed the five *get* A-domains as N-terminal His_6-tagged proteins. DNA fragments coding for the adenylation domains of *getE* (1 domain, $GetEA_1$, size 60.39 kDa), *getG* (2 domains, $GetGA_2$ and $GetGA_3$, sizes 61.50 and 63.13 kDa), *getJ* (1 domain, $GetJA_4$, size 60.0 kDa), and *getM* (1 domain, $GetMA_5$, size 63.40 kDa) were amplified from cosmids BI11 or BA23 and cloned into pET28b (+) vectors. The constructs were confirmed by sequencing and transformed into *E. coli Rosetta* BL21 (DE3) pLysS/RARE. Cultivation was carried out at 16 °C. Expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at $A_{600} = 0.8$–1. All proteins could be obtained in the soluble fraction and were used for the ATP-PP_i exchange assay after purification.

The substrate specificity of the five purified adenylation domains was evaluated using the established ATP-PP_i exchange assay (32, 33). Briefly, each protein was incubated with a panel of different amino acids, including the anticipated substrate of each A-domain. As a control, each protein was incubated in the absence of added amino acid. The results as shown in Fig. 4, *A–E*, indicate that $GetEA_1$ activated L-(2*S*)-Pip (100%), D-(2*R*)-Pip (82.9%). and L-(2*S*)-Pro (80.3%). The background controls were between 0.85 and 3.79%, confirming that the measured activity reflected the true substrate preference of the A-domain. $GetGA_2$ activated L-(2*S*)-ornithine (100%) as well as L-(2*S*)-Gln (62.7%) preferentially, whereas L-(2*S*)-Glu and L-(2*S*)-Asp were activated to a minor extent. $GetGA_3$, $GetJA_4$, and $GetMA_5$ all activated L-(2*S*)-His (100%) as well as L-(2*S*)-Lys to a minor extent (between 41 and 53%). So a clear preference for L-(2*S*)-His could be verified for all the three proteins. Taken together, these results establish that the five proteins exhibit the enzymatic activity of adenylation domains and show preference for substrates consistent with the GE81112 structure.

Stereoconfiguration of Chiral Centers—Before this work, the stereochemistry of the chiral centers in GE81112 was not

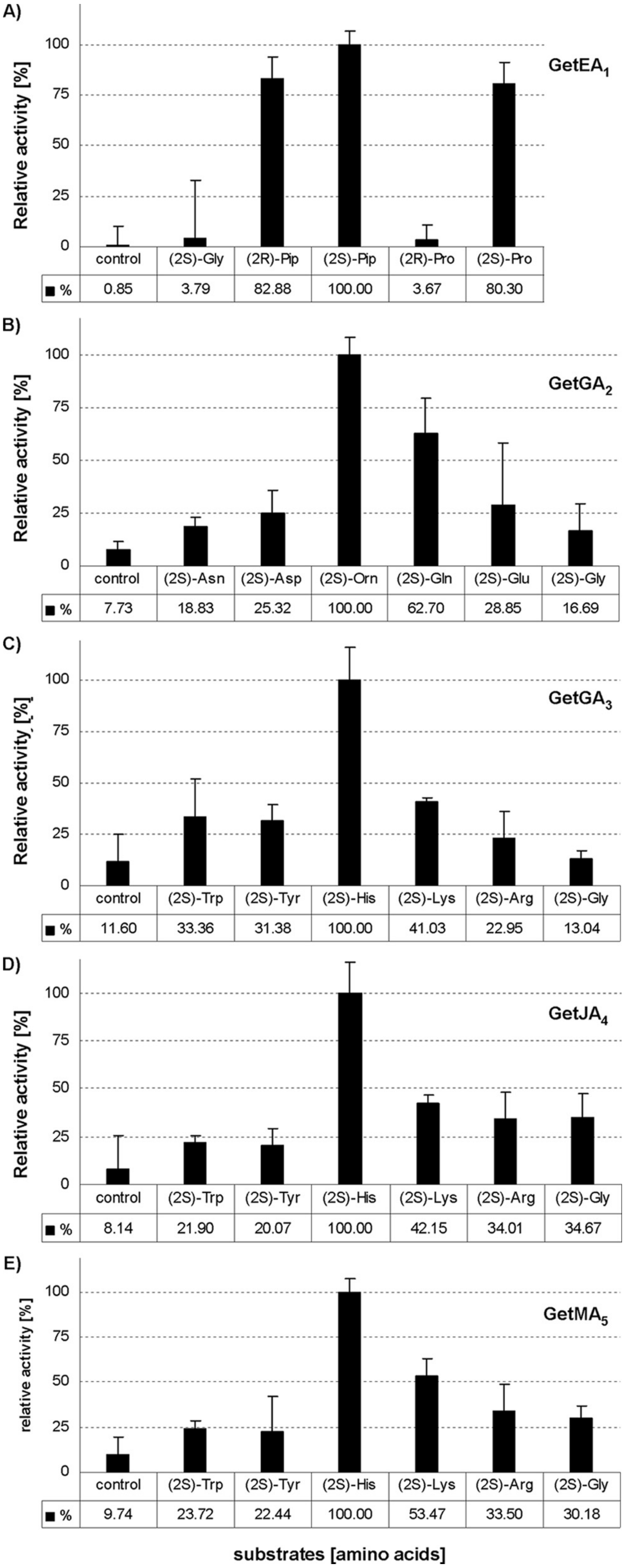


FIGURE 4. **Relative substrate specificities of internal adenylation domains from the GE81112 biosynthetic gene cluster.** Internal adenylation domains $GeEA_1$ (*A*), $GeGA_2$ (*B*), $GeGA_3$ (*C*), $GeJA_4$ (*D*), and $GeMA_5$ (*E*) were investigated in terms of activity in the ATP-PP_i exchange reaction with different amino acids and a control without amino acid. The highest activities were set at 100%. The background was below 10%. The specificities of the different domains coincide with the primary structures of the GE81112s.

TABLE 3

^{1}H and ^{13}C NMR chemical shift assignments of GE8112 congener A in D_2O at 298 K

The assignments were made by analysis of COSY, TOCSY (two-dimensional total correlation spectroscopy), HMQC (heteronuclear multiple quantum coherence), and HMBC (heteronuclear multiple bond coherence) spectra. Numbering is according to previous work (5).

Residue[a]	Group	δ s^{1}H	δ ^{13}C
AA1			
2	CH	4.09	60.3
3	CH	4.51	64.6
4	CH_2	1.82, 1.99	28.2
5	CH_2	1.75, 1.99	15.7
6	CH_2	3.02, 3.45	43.6
AA2			
2	CH	4.56	50.6
3	CH_2	1.84	33.7
4	CH	3.85	65.7
5	CH_2	3.92, 4.06	68.1
AA3			
2	CH	4.67	52.2
3	CH_2	2.98, 3.27	27
AA4			
2	CH	4.50	59.3
3	CH	5.20 (J = 3 Hz)	67.8
5′	CH	6.97	118

[a] Numbers in this column represent positions.

known. The chemical analyses described here have confirmed the indication from the ATP-PP_i exchange assays that all amino acids have the (2*S*)-configuration and provided preliminary evidence about the other stereocenters.

NMR analyses of GE81112 dissolved in DMSO-d_6 (5) do not provide useful information about the relative stereochemistry due to generally broad signals. Improved resolution and shape of the signals were achieved using D_2O (as such and acidified with TFA), allowing new assignments (Table 3). Under these conditions, the signal belonging to the β-hydrogen of chlorohistidine (5.20 ppm) shows a vicinal coupling constant J of 3 Hz, suggesting a *threo* relative stereochemistry according to literature data available for 3-hydroxy-His (19), whereas the opposite stereochemistry is expected to have a J of 6 Hz (34). Analogous indications about the 3-hydroxypipecolic acid residue were not achieved due to partially overlapping signals not allowing a satisfactory J resolution. Further experiments were, thus, necessary to determine the relative and absolute stereochemistry of GE81112.

The first three amino acids were investigated by chiral GC-MS of acid-hydrolyzed congener A (data not shown). The first amino acid did not match (2*R*,3*R*)-3-hydroxypipecolic acid or any of the (2*S*,3*R*) and (2*R*,3*S*) *cis*-diastereomers, easily obtained by catalytic hydrogenation of 3-hydroxy-picolinic acid. We, thus, assume the first amino acid has the (2*S*,3*S*) stereochemistry. For the second amino acid, protected L-allylglycine was dihydroxylated to (2*S*)-2-amino-4,5-dihydroxy-pentanoic acid, yielding a mixture of the two diastereomers in the γ position. The same procedure, performed on racemic allylglycine, provided standards of the remaining two (2*R*)- diastereomers. Based on the chiral analysis of the second amino acid, the configuration of the α-carbon is assumed to be (*S*), and based on the published facial diastereoselection of dihydroxylation (18), the γ-stereocenter should be (*S*).

The third amino acid matched (2*S*)-His by comparison with commercial (*S*) and (*R*) standards. For the fourth amino acid, GE81112 was hydrogenated to its dechloro analog, affording 3-hydroxyhistidine upon acid hydrolysis. Comparison of NMR and $[\alpha]_D$ data with literature values were consistent with the (2*S*,3*S*) configuration for the fourth amino acid (data not shown), in agreement with the *threo* relative stereochemistry suggested by the NMR experiments in D_2O.

These results together with the lack of epimerization domains (or dual condensation/epimerization domains), thus, strongly suggest that GE81112 is a tetrapeptide made of L-amino acids (2*S* configuration) only and suggest the stereochemistry of the other centers as shown for congener A in Fig. 1.

DISCUSSION

Many antibiotics target the prokaryotic translational apparatus, but few selectively inhibit initiation. Protein translation in prokaryotes is initiated by the binding of fMet-tRNA to the ribosomal P-site. Recently, the GE81112 tetrapeptides were shown to specifically inhibit this fMet-tRNA binding by blocking the P-site and, thus, represent a unique class of inhibitors with a new mode of action (35). Identification and biochemical characterization of the GE81112 biosynthetic gene cluster now provide insights into the biosynthesis of this unique family of secondary metabolites and sets the stage for the generation of new derivatives by genetic engineering. The identity of the cloned gene cluster was confirmed by inactivation of *getE*, which completely abolished GE81112 production. Although many of the enzymes encoded by the *get* cluster are consistent with GE81112 biosynthesis, the NRPSs are present with a highly split, non-co linear module arrangement (Fig. 3*A*). Unusual features include two freestanding A-PCP di-domains (encoded by *getJ* and *getM*) and a stand-alone A-domain (encoded by gene *getE*). Stand-alone A-domains have been identified in other nonlinear NRPS pathways, *e.g.* myxochelin (36) and yersiniabactin (37), whereas free-standing A-PCP di-domains are found in the zorbamycin and syringomycin gene clusters (38, 39). According to bioinformatic analysis, all NRPS domains are predicted to be functional (supplemental Fig. S2 and S3). Furthermore, because the *get* cluster encodes five distinct A and PCP domains instead of the four predicted for a tetrapeptide, the order of subunits (and corresponding modules) could not be predicted from sequence alone. The established specificity for the five A-domains and analysis of the functions predicted from the other enzymes encoded by the cluster allow us to draw a first model for GE81112 formation (Fig. 3*C*).

Accordingly, the biosynthesis of GE81112 starts with the formation of (2*S*)-pipecolic acid from (2*S*)-lysine via the action of the putative cyclodeaminase GetD (Fig. 3*C*). Pipecolic acid is directly activated by the A-domain $GetEA_1$ and then loaded onto the adjacent PCP domain. Consistently, an ATP-PP_i exchange assay confirmed that $GetEA_1$ preferentially recognizes (2*S*)-pipecolic acid (Fig. 4*A*). However, the pipecolic acid moiety in GE81112 is hydroxylated at the β-position. There is precedent for β-hydroxylation to occur at three different stages; on the free amino acid (40), whereas the amino acid is tethered to the PCP (41), or on the mature peptide after thioesterase hydrolysis from the NRPS (42, 43). As GetEA1 could not be directly assayed with hydroxypipecolate due to the commercial unavailability of this compound, we suggest that this domain might also recognize hydroxypipecolic acid. In any case, the presence of the 3-hydroxyl group on the pipecolate moiety is essential for activity, as GE81112 derivatives lacking this group are 3 orders of magnitude less active in the translation assay than the parent compounds.[4]

It was not obvious from which amino acid the second building block is derived. For GE81112 congeners A (**1**) and B (**2**), the second amino acid residue is 5-hydroxy-2-aminopentanoic acid (*O*-HPA), which is hydroxylated at position 4 and *O*-carbamoylated at position 5, whereas congener B1 (**3**) contains a hydroxylated and carbamoylated ornithine residue (Fig. 1). We hypothesize that the A-domain GetGA$_2$ is responsible for the incorporation of the second amino acid in GE81112. A recent example in the biosynthesis of the nucleoside antibiotic polyoxin shows that *O*-carbamoyl-polyhydroxypentanoic acid is generated from free (2*S*)-glutamate by stepwise reduction, *O*-carbamoylation, and hydroxylations (44). The complete building block is then attached to the nucleoside. Similarly, GE81112 congeners A and B could derive from activation of the *O*-HPA substrate (or of a precursor) by the A-domain GetGA$_2$ and loading on the corresponding PCP (Fig. 3*C*). However, we could not identify candidate genes in the *get* cluster for *O*-HPA biosynthesis, so they may be encoded elsewhere in the genome (45, 46). According to our hypothesis, the A-domain GetGA$_2$ must show a broad substrate acceptance, activating *O*-HPA- and ornithine-based amino acids to generate the different GE81112 congeners. As *O*-HPA or any derivatives were not commercially available, we tested the likely precursors of *O*-HPA, (2*S*)-glutamic acid and (2*S*)-glutamine in addition to (2*S*)-ornithine. Activation of all the three amino acids (ornithine, Glu, and Gln) was observed (Fig. 4*B*) with a preference for (2*S*)-ornithine. The reduced degree of activation of glutamic acid and glutamine may indicate that modified versions of glutamic acid and glutamine are preferred, consistent with the hypothesis that *O*-HPA is formed before loading to the PCP. It should be noted that the major congeners of the GE81112 complex produced by *Streptomyces* S. sp. L-49973 are A and B,[5] indicating that *in vivo* *O*-HPA or a precursor is the preferred substrate for the NRPS.

The third amino acid incorporated into the GE81112 peptide is either histidine (**1**) or aminohistidine (**2** and **3**). *In silico* analysis of GetGA$_3$ predicts specificity for tyrosine or tryptophan rather than histidine. This prediction may indicate a general preference of the A-domain for aromatic amino acid residues, which might account for activation of both histidine and aminohistidine. However, it is not clear how the aminohistidine moiety is generated: "amination" might occur at the PCP-bound histidine after release of the peptide from the NRPS, or alternatively, aminohistidine could derive from the cyclization of arginine by nucleophilic attack of the γ-carbon. According to the ATP-PP$_i$ exchange assay, histidine is the preferred substrate, and no activation was observed with arginine, tryptophan, or tyrosine (Fig. 4*C*). These data suggest that aminohistidine is not generated from PCP-bound arginine, tryptophan, or tyrosine, but we could not establish whether GetGA$_3$ also recognizes aminohistidine, as this amino acid is not commercially available. Thus, the nature and timing of histidine amination remains unclear.

A 3-hydroxy-6-chlorohistidine is the last amino acid to be incorporated in GE81112. Halogenation is a common modification found in bioactive natural products (47–49), and the timing of halogenation reactions has been shown to vary. For example, in rebeccamycin, pre-assembly line chlorination of tryptophan occurs (50), whereas a PCP-bound threonine is chlorinated for syringomycin (51). The putative halogenase GetL is the likely candidate for histidine chlorination in GE81112. GetI shows homology to non-heme iron-dependent oxygenases/hydroxylases and is, therefore, assumed to hydroxylate histidine at the β-position. Figs. 3, *B* and *C*, illustrate the proposed mechanism for the chlorination/hydroxylation of histidine and its incorporation into GE81112. As there is evidence from sequence analysis and *in vitro* experiments (supplemental Fig. S2 and Fig. 4*E*) that the A-PCP domains of the seemingly superfluous GetM protein are active, this di-domain may play a role in the biosynthesis of the fourth amino acid precursor. Indeed, there are a number of examples in which specialized A-PCP di-domains are essential for generating NRPS precursors (52–56). Here, we propose that the A-domain GetMA$_5$ activates (2*S*)-histidine, consistent with the results of the ATP-PP$_i$ exchange assay, and tethers it to the PCP of GetM. Hydroxylation and/or halogenation reactions then occur on the PCP-bound amino acid as catalyzed by GetI and GetL, respectively (Fig. 3*B*). The thioesterase GetA would then release the modified amino acid, as described for BarC from the barbamide biosynthetic pathway (53). In the subsequent step, the free, modified histidine is activated by the A-domain GetJA$_4$ and loaded onto the PCP of module 4 (Fig. 3*C*). This is supported by the ATP-PP$_i$ exchange assay showing that GetJA$_4$ has specificity for (2*S*)-histidine (Fig. 4*D*). Again, the relative timing of chlorination and hydroxylation is unclear, as it is conceivable that one of these reactions may occur on the GetJ-tethered amino acid or peptide. In Fig. 3*C*, GetA is predicted to function as a type II thioesterase to release the modified amino acid from GetM. Alternatively, GetA could act as an aminoacyltransferase, shuttling the PCP-bound modified histidine from GetM to GetJ. There are several examples of such PCP-to-PCP shuttling reactions (38, 55), and the aminoacyltransferases identified to date have been assigned into two groups (38); one group, comprising SyrC, CmaE, and ZmbVIId, contains a G*X*C*X*G motif at the active site, and these enzymes are predicted to act as acyltransferases, with the active-site cysteine shown to be directly involved in aminoacyl transfer (52); the second group includes the acyltransferases BarC (53) and CouN7 (54), with an active site serine in the G*X*S*X*G motif, and are predicted to function as ordinary thioesterases. GetA and GetN both contain active site serines, which suggest that they both function as normal thioesterases and not as aminoacyltransferases (supplemental Fig. S4). GetN is expected to catalyze the release of the peptide from the assembly line (Fig. 3*C*).

The combination of genetic, biochemical, and chemical analyses demonstrate that GE81112 is composed of L-amino acids only. This result is consistent with the observation that it is a

[4] S. I. Maffioli, unpublished results.

[5] M. Sosio and S. I. Maffioli, unpublished results.

substrate of the oligopeptide permease in some bacterial species.[6] Many unusual features were found in the biosynthesis of this all-L-ribosome binding tetrapeptide. The NRPS modular architecture includes A-domains that incorporate unusual amino acids, such as hydroxypipecolic acid, hydroxypentanoic acid, and hydroxylchlorohistidine. Although many questions about its biosynthesis remain, the availability of the GE81112 cluster as well as tools for genetic manipulation now provide a platform for attempts to decipher these issues and to generate new GE81112 derivatives by genetic engineering.

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REFERENCES

1. Hopwood, D., Levy, S., Wenzel, R. P., Georgopapadakou, N., Baltz, R. H., Bhavnani, S., and Cox, E. (2007) *Nat. Rev. Drug Discov.* **6,** 8–12
2. Baker, D. D., Chu, M., Oza, U., and Rajgarhia, V. (2007) *Nat. Prod. Rep.* **24,** 1225–1244
3. Walsh, C. (2003) *Antibiotics: Actions, Origins, Resistance*, American Society for Microbiology, Washington, D. C.
4. Kohanski, M. A., Dwyer, D. J., and Collins, J. J. (2010) *Nat. Rev. Microbiol.* **8,** 423–435
5. Brandi, L., Lazzarini, A., Cavaletti, L., Abbondi, M., Corti, E., Ciciliato, I., Gastaldo, L., Marazzi, A., Feroggio, M., Fabbretti, A., Maio, A., Colombo, L., Donadio, S., Marinelli, F., Losi, D., Gualerzi, C. O., and Selva, E. (2006) *Biochemistry* **45,** 3692–3702
6. Finking, R., and Marahiel, M. A. (2004) *Annu. Rev. Microbiol.* **58,** 453–488
7. Hutchinson, C. R. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 3010–3012
8. Wenzel, S. C., and Müller, R. (2005) *Curr. Opin. Chem. Biol.* **9,** 447–458
9. Wenzel, S. C., and Müller, R. (2007) *Nat. Prod. Rep.* **24,** 1211–1224
10. Kieser, T., Bibb, M., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics* pp. 562 and 566, The John Innes Foundation, Norwich, England
11. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Dürr, C., Schnell, H. J., Luzhetskyy, A., Murillo, R., Weber, M., Welzel, K., Vente, A., and Bechthold, A. (2006) *Chem. Biol.* **13,** 365–377
13. Rachid, S., Krug, D., Kunze, B., Kochems, I., Scharfe, M., Zabriskie, T. M., Blöcker, H., and Müller, R. (2006) *Chem. Biol.* **13,** 667–681
14. Sandmann, A., Sasse, F., and Müller, R. (2004) *Chem. Biol.* **11,** 1071–1079
15. Khaw, L. E., Böhm, G. A., Metcalfe, S., Staunton, J., and Leadlay, P. F. (1998) *J. Bacteriol.* **180,** 809–814
16. Marahiel, M. A., Stachelhaus, T., and Mootz, H. D. (1997) *Chem. Rev.* **97,** 2651–2674
17. Silakowski, B., Schairer, H. U., Ehret, H., Kunze, B., Weinig, S., Nordsiek, G., Brandt, P., Blöcker, H., Höfle, G., Beyer, S., and Müller, R. (1999) *J. Biol. Chem.* **274,** 37391–37399
18. Girard, A., Greck, C., and Genêt, J. P. (1998) *Tetrahedron Lett.* **39,** 4259–4260
19. Dong, L., and Miller, M. J. (2002) *J. Org. Chem.* **67,** 4759–4770
20. Rose, T. M., Schultz, E. R., Henikoff, J. G., Pietrokovski, S., McCallum, C. M., and Henikoff, S. (1998) *Nucleic Acids Res.* **26,** 1628–1635
21. Gatto, G. J., Jr., Boyne, M. T., 2nd, Kelleher, N. L., and Walsh, C. T. (2006) *J. Am. Chem. Soc.* **128,** 3838–3847
22. He, M. (2006) *J. Ind. Microbiol. Biotechnol.* **33,** 401–407
23. Ishikawa, J., and Hotta, K. (1999) *FEMS Microbiol. Lett.* **174,** 251–253
24. Weissman, K. J., and Müller, R. (2008) *Chembiochem* **9,** 826–848
25. Méndez, C., and Salas, J. A. (1998) *FEMS Microbiol. Lett.* **158,** 1–8
26. Stachelhaus, T., Mootz, H. D., and Marahiel, M. A. (1999) *Chem. Biol.* **6,** 493–505
27. Challis, G. L., Ravel, J., and Townsend, C. A. (2000) *Chem. Biol.* **7,** 211–224
28. Aparicio, J. F., Molnár, I., Schwecke, T., König, A., Haydock, S. F., Khaw, L. E., Staunton, J., and Leadlay, P. F. (1996) *Gene* **169,** 9–16
29. Turgay, K., Krause, M., and Marahiel, M. A. (1992) *Mol. Microbiol.* **6,** 529–546
30. Hojati, Z., Milne, C., Harvey, B., Gordon, L., Borg, M., Flett, F., Wilkinson, B., Sidebottom, P. J., Rudd, B. A., Hayes, M. A., Smith, C. P., and Micklefield, J. (2002) *Chem. Biol.* **9,** 1175–1187
31. Kopp, M., Irschik, H., Gross, F., Perlova, O., Sandmann, A., Gerth, K., and Müller, R. (2004) *J. Biotechnol.* **107,** 29–40
32. Mootz, H. D., and Marahiel, M. A. (1997) *J. Bacteriol.* **179,** 6843–6850
33. Thomas, M. G., Burkart, M. D., and Walsh, C. T. (2002) *Chem. Biol.* **9,** 171–184
34. Hecht, S. M., Rupprecht, K. M., and Jacobs, P. M. (1979) *J. Am. Chem. Soc.* **101,** 3982–3983
35. Brandi, L., Fabbretti, A., La Teana, A., Abbondi, M., Losi, D., Donadio, S., and Gualerzi, C. O. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 39–44
36. Gaitatzis, N., Kunze, B., and Müller, R. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 11136–11141
37. Miller, D. A., Luo, L., Hillson, N., Keating, T. A., and Walsh, C. T. (2002) *Chem. Biol.* **9,** 333–344
38. Galm, U., Wendt-Pienkowski, E., Wang, L., George, N. P., Oh, T. J., Yi, F., Tao, M., Coughlin, J. M., and Shen, B. (2009) *Mol. Biosyst.* **5,** 77–90
39. Vaillancourt, F. H., Vosburg, D. A., and Walsh, C. T. (2006) *Chembiochem* **7,** 748–752
40. Yin, X., and Zabriskie, T. M. (2004) *Chembiochem* **5,** 1274–1277
41. Chen, H., Thomas, M. G., O'Connor, S. E., Hubbard, B. K., Burkart, M. D., and Walsh, C. T. (2001) *Biochemistry* **40,** 11651–11659
42. Buntin, K., Rachid, S., Scharfe, M., Blöcker, H., Weissman, K. J., and Müller, R. (2008) *Angew. Chem. Int. Ed. Engl.* **47,** 4595–4599
43. Samel, S. A., Marahiel, M. A., and Essen, L. O. (2008) *Mol. Biosyst.* **4,** 387–393
44. Chen, W., Huang, T., He, X., Meng, Q., You, D., Bai, L., Li, J., Wu, M., Li, R., Xie, Z., Zhou, H., Zhou, X., Tan, H., and Deng, Z. (2009) *J. Biol. Chem.* **284,** 10627–10638
45. Steffensky, M., Mühlenweg, A., Wang, Z. X., Li, S. M., and Heide, L. (2000) *Antimicrob. Agents Chemother.* **44,** 1214–1222
46. Yu, T. W., Bai, L., Clade, D., Hoffmann, D., Toelzer, S., Trinh, K. Q., Xu, J., Moss, S. J., Leistner, E., and Floss, H. G. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 7968–7973
47. Neumann, C. S., Fujimori, D. G., and Walsh, C. T. (2008) *Chem. Biol.* **15,** 99–109
48. Eustáquio, A. S., Gust, B., Luft, T., Li, S. M., Chater, K. F., and Heide, L. (2003) *Chem. Biol.* **10,** 279–288
49. Yeh, E., Blasiak, L. C., Koglin, A., Drennan, C. L., and Walsh, C. T. (2007) *Biochemistry* **46,** 1284–1292
50. Yeh, E., Garneau, S., and Walsh, C. T. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 3960–3965
51. Vaillancourt, F. H., Yin, J., and Walsh, C. T. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 10111–10116
52. Strieter, E. R., Vaillancourt, F. H., and Walsh, C. T. (2007) *Biochemistry* **46,** 7549–7557
53. Chang, Z., Flatt, P., Gerwick, W. H., Nguyen, V. A., Willis, C. L., and Sherman, D. H. (2002) *Gene* **296,** 235–247
54. Garneau-Tsodikova, S., Stapon, A., Kahne, D., and Walsh, C. T. (2006) *Biochemistry* **45,** 8568–8578
55. Singh, G. M., Vaillancourt, F. H., Yin, J., and Walsh, C. T. (2007) *Chem. Biol.* **14,** 31–40
56. Pfeifer, V., Nicholson, G. J., Ries, J., Recktenwald, J., Schefer, A. B., Shawky, R. M., Schröder, J., Wohlleben, W., and Pelzer, S. (2001) *J. Biol. Chem.* **276,** 38370–38377

[6] A. Maio and S. Donadio, unpublished results.