Discovery of unusual dimeric piperazyl cyclopeptides encoded by a Lentzea flaviverrucosa DSM 44664 biosynthetic supercluster

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Rare actinomycetes represent an underexploited source of new bioactive compounds. Here, we report the use of a targeted metabologenomic approach to identify piperazyl compounds in the rare actinomycete Lentzea flaviverrucosa DSM 44664. These efforts to identify molecules that incorporate piperazate building blocks resulted in the discovery and structural elucidation of two dimeric biaryl-cyclohexapeptides, petrichorins A and B. Petrichorin B is a symmetric homodimer similar to the known compound chloptosin, but petrichorin A is unique among known piperazyl cyclopeptides because it is an asymmetric heterodimer. Due to the structural complexity of petrichorin A, solving its structure required a combination of several standard chemical methods plus in silico modeling, strain mutagenesis, and solving the structure of its biosynthetic intermediate petrichorin C for confident assignment. Furthermore, we found that the piperazyl cyclopeptides comprising each half of the petrichorin A heterodimer are made via two distinct nonribosomal peptide synthetase (NRPS) assembly lines, and the responsible NRPS enzymes are encoded within a contiguous biosynthetic supercluster on the L. flaviverrucosa chromosome. Requiring promiscuous cytochrome p450 crosslinking events for asymmetric and symmetric biaryl production, petrichorins A and B exhibited potent in vitro activity against A2780 human ovarian cancer, HT1080 fibrosarcoma, PC3 human prostate cancer, and Jurkat human T lymphocyte cell lines with IC50 values at low nM levels. Cyclic piperazyl peptides and their crosslinked derivatives are interesting drug leads, and our findings highlight the potential for heterodimeric bicyclic peptides such as petrichorin A for inclusion in future pharmaceutical design and discovery programs.

Significance

Actinomycetes produce many clinically useful drugs, especially antibiotics and anticancer agents. Rare actinomycetes are known to produce bioactive molecules but they remain underexplored compared to more common Streptomycetes spp. Natural molecules having piperazate building blocks are often bioactive, and genome analyses previously indicated the rare actinomycete Lentzea flaviverrucosa DSM 44664 may encode for the production of such molecules. To discover these from complex fermentation mixtures, we devised and employed a targeted metabolomic approach that revealed petrichorin A, an unusual heterodimeric biaryl-cyclohexapeptide. Its structure was determined by using multi-dimensional nuclear magnetic resonance, theoretical calculations, and strain mutagenesis, and its biosynthesis implicated an atypical cytochrome p450 heterodimerization event. Petrichorin A demonstrated potent cytotoxicity, highlighting heterodimeric-biaryls as interesting features for future drug design.
Bioinformatic efforts typically scan microbial genomes for BGCs encoding molecules having known pharmacophores, privileged scaffolds, or other desirable chemical motifs. Microbial producers are selected for genome-sequencing based on equally complex criteria, including their relatedness to traditional producers of approved drugs, isolation from unique environmental niches, their relative rarity in culture collections, and other factors (12).

Piperazate (piz) is a nonproteinogenic amino acid and proline mimic that imparts conformational constraint and other desirable properties to natural and synthetic molecules that incorporate it (13). Piz fits the classical definition of a privileged scaffold, a molecular substructure associated with compounds that can target diverse biology (14). Thus, piperazyl compounds are desirable for therapeutic discovery. In addition to being counted among several known drugs and important structural leads (e.g., cilazapril, the matlystatins, and sanglifehrins) (15), piperazyl molecules have additional research interest owing to their atypical associated enzymology (16–18) and proposed roles in microbial symbiosis and chemical ecology (19–21).

There are over 100 documented natural products with integral piz moieties, most of which are produced by actinobacterial members of the Streptomyces genus (22). The recent elucidation of piz biosynthesis via the unusual hemoprotein KtzT and related members of the PzbB-protein family has enabled straightforward recognition of piperazyl-molecule BGCs in microbial genomes (17, 18). Our prior work on actinobacterial piperazate metabolism revealed a pzbB-linked BGC in Lentzea flaviverrucosa DSM 44664, and the enzyme it encodes was competent for piperazate production in heterologous hosts (18). L. flaviverrucosa is a member of the Pseudonocardia family and is a member of the so-called “rare actinomycetes” (23). Rare actinomycetes, including Lentzea, Actinoplanes, Nonomuraea, and Salinispora plus several other genera, are phylogenetically diverse and biotechnologically interesting filamentous actinobacteria (23). These organisms are recognized for their potential to produce biotechnologically important molecules, but remain significantly underexplored compared to their more commonly encountered Streptomyces relatives (24). Understanding and accessing the biosynthetic potential of rare actinomycetes is a priority for continued natural drug discovery, but work exploring Lentzea for biotechnology remains relatively sparse. Known Lentzea products include rebeccamycin (25), lassomycin (26), and lentzeosides (27), and members of the genus have been used for the bioconversion of the cyclosporin A-derivative FR901459 into several new congeners (28). Published examples of Lentzea genetic systems to support biotechnological development are also limited, with Lentzea, sp. strain ATCC 31319 mutations for the study of thiolactomycin production being the lone example (29).

Motivated by our earlier findings indicating L. flaviverrucosa should produce a yet-undiscovered piperazyl compound (18), here we report the targeted discovery of the biosynthetically atypical piperazyl compounds petrichorins A and B (Fig. 1) from this strain. Identifying these molecules in growth extracts was enabled by combination pzbB gene disruptions, L-orn isotopic-labeling, and piperazyl-targeting tandem mass spectrometry (MS/MS) fragmentation analysis. After fully resolving their structures, both petrichorins were found to share several structural features with a growing family of cyclic peptides and depsipeptides (including chloptinsin [30], himastatin [31], and members of the alboflavinus [32, 33] and kutzneride [34] complexes) (Fig. 1, Top) that incorporate both piperazyl and hydroxyhexahydropyrrolo[2,3-b]indole-2-carboxamide (HPIC) substructural elements. Of the two petrichorins, the minor product petrichorin B was more structurally simplistic, consisting of a bicyclic-cyclopeptide homodimer whose structure is highly similar to chloptinsin. By comparison, the major product petrichorin A significantly differed from all previously known dimeric cyclohexapeptides in that it is a heterodimer comprised of one half of petrichorin B crosslinked to a different cyclohexapeptide, named petrichorin C (Fig. 1, Bottom). The biosynthesis of the petrichorins was pursued via additional genetic experiments in the native host, revealing that the production of the highly atypical asymmetric diimer petrichorin A requires an unusual cytochrome p450 crosslinking of two different cyclopeptides generated by distinct NRPS assembly lines. Finally, the bioactivities of petrichorins A, B, and C were tested against human HT1080 fibrosarcoma, PC3 prostate cancer, A2780 ovarian cancer, and Jurkat T lymphocyte cell lines. The petrichorin A heterodimer showed significant inhibition along with the B homodimer, and both of these were superior to the C monomer. Accordingly, we posit that heterodimeric biaryl-cyclohexapeptides and their depsipeptide analogs should be specifically considered for future therapeutic-lead synthesis.

Results and Discussion

Piperazate-Targeted Metabologenomics for Petrichorin Discovery.

Piperazate biosynthesis requires the formation of an unusual N–N bond, which is formed by heme b-dependent enzymes related to KtzT. We previously identified and heterologously expressed a kztT ortholog from L. flaviverrucosa (ltzT) that produces piperazate in recombinant Streptomyces lividans and Streptomyces flavoulosus (18). This suggested that L. flaviverrucosa likely has the native capacity to produce piperazyl compounds but none had been discovered in this strain or in any other Lentzea spp. Examining the ltzT gene neighborhood revealed a potential biosynthetic gene cluster (BGC, ~67 kb, ltzA–ltzV) encoding multiple predicted tryptophan halogenases, five NRPS enzymes having 11 total adenylation domains, a single transcriptional regulator, transporters, and several oxidative tailoring genes (Fig. 2A, SI Appendix, Table S1).

To discover piperazyl molecules in L. flaviverrucosa, we used a multipronged approach based on the genetics of piz monomer production and piperazate-targeted metabolomics (Fig. 2 B–D). Piz biosynthesis from L-ornithine (L-orn) requires an N-hydroxylase and an N-hydroxy-L-orn cyclase, and homologs of both were found in the liz locus (Fig. 2A, lizA–lizV and lizT, respectively). We tested L. flaviverrucosa for a metabolic response to exogenous L-piz and d7-L-orn to identify potential piperazyl compounds. After feeding the strain with these amino acids, organic extracts of spent growth media were analyzed via liquid chromatography/mass spectrometry (LC/MS), revealing a single strong peak that responded positively to both compounds (Fig. 2 B and C). Specifically, exogenous L-Piz approximately doubled the peak area of one L. flaviverrucosa metabolite (0.5–1 mM supplementation), and the same piz-responsive signal also incorporated deuterated-L-orn, consistent with a piperazyl metabolite.

We then utilized an MS/MS fragmentation scheme for more sensitive detection of piperazyl compound signals within complex microbial extracts. This method was established using matlystatin production in Amycolatopsis atramentaria (35, 36), where MS/MS was used to identify daughter ions containing the piperazyl-feature of matlystatin B via d7-L-Orn incorporation (SI Appendix, Fig. S1). By comparing labeled vs. unlabeled extracts, we identified a piperazyl-derived m/z 85.1 fragment, which we surmised could be used for the sensitive detection of other piperazyl compounds. After applying this method to
L. flaviverrucosa, we rediscovered the piz-responsive signal from Fig. 2B, plus an additional minor product missed by our initial efforts (Fig. 2D).

To confirm these molecules are indeed piz-dependent and encoded by the \textit{ltz} locus, we established a genetic system in \textit{L. flaviverrucosa} to create unmarked gene deletions. This was achieved using a standard \textit{rpsL}-counterselection approach (see Materials and Methods), and all mutants described herein were created in the same \textit{rpsL} background (S12 K88M, JV691). After deleting piz-essential \textit{ltzT}, the resulting mutant was deficient for both peaks identified in Fig. 2D. Both signals were rescued by ectopically expressing \textit{ltzT} under the control of the strong constitutive promoter \textit{PermE}*. Other \textit{ltzT} orthologs cloned from piperazyl BGCs in \textit{Kutzneria} sp strain 744 (\textit{ltzT}, kutznerides) and \textit{Streptomyces himastatinicus} (\textit{himtC}, himastatin) similarly restored function (Fig. 2D). These data clearly linked the production of the Fig. 2 piperazyl signals to the \textit{ltz} locus.

**Structure of the Petrichorins.** Both molecules detected in Fig. 2D were purified to homogeneity for structural elucidation (see Materials and Methods and SI Appendix). Both were isolated as white powders, and HR-ESI-MS revealed each had distinct masses and mass formulae (the major product, petrichorin A, \(C_{67}H_{92}Cl_2N_{18}O_{18}\) \(m/z\) 1507.6288, calculated for [M + H]+ 1507.6287; the minor product petrichorin B, \(C_{70}H_{98}Cl_2N_{18}O_{20}\) quasi-molecular ion peak at \(m/z\) 1603.6484 [M + Na]+, calculated 1603.6474).

NMR characterization of petrichorin A was challenging due to extensive signal overlap. Pursuing the final structure of this molecule thus required a combination of standard 1D and 2D
NMR and other chemical methods (SI Appendix and Table S2), plus in silico modeling and strain mutagenesis for confident structural assignment (latter discussed below). In brief, comprehensive 2D-NMR analysis (COSY, TOCSY, HSQC, and HMBC) (SI Appendix, Figs. S2–S8) suggested the presence of one alanine (ala), one N-methyl-alanine (Meala), one isoleucine (ile), one O-methyl-serine (OMe-ser), two threonines (thr-1 and thr-2), four piperazic acids (one being hydroxylated at γ-position), plus two chlorinated tryptophan (trp) derivatives (later identified as HPIC). Amino acid hydrolysis followed by Marfey’s analysis (37) indicated the absolute configuration of the component amino acids (SI Appendix, Figs. S9–S16), and HMBC and NOESY were used to determine amino acid sequences for both the asymmetric left and right rings of petrichorin A (SI Appendix, Fig. S28). Additionally, Mosher reactions coupled with 2D NMR (38) were used to determine the absolute configuration of secondary alcohols (SI Appendix, Figs. S17–S27). Finally, the absolute configurations of the asymmetrically chlorinated and cross-linked HPIC residues of heterodimeric petrichorin A required quantum calculations of NMR shifts, an established strategy to resolve difficult natural product structures (39). To do this with minimized computational cost, we theoretically modeled four simplified bicyclic HPIC derivatives (SI Appendix, Figs. S29, isomers 1a–d, and S30) and correlated calculated shifts with collected experimental values for the analogous region of petrichorin A. Following systematic conformational sampling at the MMFF level, followed by fast NMR calculations at B3LYP/6–31G** the J-DP4 calculations suggested (SI Appendix, Fig. S29, isomer 1a) as the most likely substructure (99.97% confidence) (40). This result further verified by DP4+ (>99.99% confidence) after refining the NMR calculations at the PCM/mPW1PW91/6–31+G**/B3LYP/6–31G* level of theory (see SI Appendix, Tables S5 and S6 and accompanying diagrams) (41). From these summed experiments, petrichorin A was revealed to be a unique asymmetric bicyclic hexapeptide, comprised of two distinct piperazyl cyclopeptides tethered by a biaryl linkage as shown in Fig. 1 (see SI Appendix for further data and detail).

Solving the molecular structure of petrichorin B was relatively straightforward compared to petrichorin A, owing to its symmetric, homodimeric structure. Following a similar battery of 1D and 2D NMR, hydrolytic component analysis, derivatization and theoretical NMR calculations used for petrichorin A (SI Appendix, Tables S3, S7, S8, Figs. S28, S33–S46) petrichorin B was found to consist of a symmetric homodimer of the same cyclopeptide that constitutes the left ring of petrichorin A (Fig. 1). While heterodimeric petrichorin A was unique in the chemical literature, petrichorin B was found to be highly similar to chloptosin (30). The only significant differences between them were that petrichorin B substitutes allo-isoleucine for chloptosin’s valine, and petrichorin B has two γ-hydroxyl-piz moieties while all chloptosin piz residues are nonhydroxylated.

**NRPS Assembly of the Petrichorins.** The clear structural parallels that exist between petrichorin B and chloptosin suggested they likely arise from highly similar pathways, but the chloptosin BGC remains unreported in the literature, making it unavailable for comparison. While the liz locus remains architecturally distinct in online sequence databases, published BGCs for the biosyntheses of the piperazyl cyclopeptides himastatin and kutzerinidene from Streptomyces himastatinicus ATCC 53653 and Kutzeria sp. strain 744, respectively (SI Appendix, Fig. S47), share partial parallels with the liz locus with regard to gene content and organization. However, the liz locus critically lacks genes for the polyketide enzymology needed for depsipeptide linkages and their associated biosynthetic intermediates, which are integral to the kutzerinides and himastatins.

The different peptide sequences making up each ring of the petrichorin A heterodimer (left ring: D-αllo-ile, L-Piz, D-(4S-OH)-piz, L-O-methyl-ser, D-thr, L-6-Cl-HPIC; right ring: D-ala, L-piz, D-thr, L-N-methyl-ala, D-piz, L-7-Cl-HPIC) indicated each half of the molecule must be synthesized via distinct NRPS enzymology. Assigning which liz-associated NRPS genes are responsible for petrichorin A and B biosyntheses initially involved bioinformatic analysis of the liz cluster’s NRPS enzymes to predict the order and chirality of possible peptide products.
The actual structures of the petrichorins wherever merization domains within both NRPS assembly lines agreed with molecular colinearity analyses. Finally, the presence of several epitetically found within LtzE and LtzV. Their placement within each sequence (42), and single-instances of these domains were respec-

within NRPS proteins are readily discernable from each other by

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HmtF shares the same X-A-T domain order as LtzA). Module 6 is missing a necessary adenylation domain, and likely to function in himastatin (31) production as well (HmtL ide pathway requires one (47), and homology suggests one is examples are now documented (46). Furthermore, the kutner-

ide pathway requires one (47), and homology suggests one is likely to function in himastatin (31) production as well (HmtL module 6 is missing a necessary adenylation domain, and HmtF shares the same X-A-T domain order as LtzA).

To determine which NRPS assembly line(s) function in petrichorin A and B production, ΔltzU and ΔltzD mutations were created and the accumulation of piperazyl peptides were monitored by LC/MS/MS (Fig. 4). Deleting ltzU abrogated heterodimeric petrichorin A production, but not the homodimer petrichorin B, which agrees with precursor production model in Fig. 3. In contrast, deleting ltzD lost all dimeric cyclohexapeptide production. Instead, the strain accumulated a molecule with a mass consistent with a monomeric cyclopeptide. Once purified to homogeneity and structurally characterized (essentially as done for petrichorins A and B) (SI Appendix, Table S4 and Figs. S28, S48–S55), this biosynthetic intermediate (petrichorin C) was confirmed as being equivalent to one-half of the petrichorin A heterodimer (Fig. 1, legend). In addition, the simplified NMR spectra obtained for petrichorin C were used to further strengthen the initial NMR interpretations of the corresponding subregion of petrichorin A. We found it curious that we were unable to detect the theoretically possible dimer of petrichorin C in the ltzD mutant, while the homodimer petrichorin B was readily produced in the ltzU strain. The reason why the latter homodimer is readily produced while the other one apparently is not remains unknown.

Finally, the above analyses suggested that most of the biosynthetic building blocks required for NRPS-elaborated precursor peptide formation are either supplied via central metabolism through standard proteinogenic amino acid pools, or are produced by enzymes encoded within the cluster itself (i.e., pipera-

zate, ltzF, and ltzT). The single exception to this was L-allo-ile, likely activated by LtzD module 1. L-allo-ile is known to be produced from L-ile in a pyridoxal phosphate-dependent, two-

enzyme pathway that requires enzymes homologous to MfnO/ DsdD and MfnH/DsaE (48). Mising from the ltz locus, searching the L. flaviverrucosa genome with MfnO and MfnH from the marformycin (49) cluster identified homologs (WP_090070987.1 and WP_090064152.1, respectively) encoded outside of the petrichorin supercluster that could potentially be involved in allo-ile production.

(SI Appendix, Table S9). These data led us to posit that an NRPS assembly line consisting of LtzDE most likely directs the precursor of the left ring of petrichorin A (Figs. 1 and 3). Due to obvious structural parallels, this also implicates LtzDE in the production of homodimeric petrichorin B (Fig. 1). Concordantly, the assem-

ly line comprised of LtzUV showed substantial collinearity with the expected peptide precursor of the right ring of petrichorin A. O-methyltransferase and N-methyltransferase domains found within NRPS proteins are readily discernable from each other by sequence (42), and single-instances of these domains were respec-

tively found within LtzE and LtzV. Their placement within each NRPS assembly line dovetailed seamlessly with the rest of our molecular colinearity analyses. Finally, the presence of several epimerization domains within both NRPS assembly lines agreed with the actual structures of the petrichorins wherever D-configured amino acids were encountered (Fig. 3).
Cytochrome P450 Involvement in Petrichorin (Hetero)Dimer Formation and Tailoring. Multiple cytochrome P450 enzymes encoded within the chloptosin, himastatin, kutzneride, and alboflavusin biosynthetic loci are known to be involved in the maturation of tryptophan into HPIC (KtzM, HmtT, and AfnD), piperazyl peptide oxidation (HmtN and AfnA), and the formation of homodimeric (chloro)biaryl crosslinkages between HPIC residues (HmtS and ClpS) (30–32, 47, 50). While these transformations have been investigated elsewhere, we probed the functions of their homologs encoded within the \( \Delta \)ltz-locus (LtzR, LtzS, and LtzH, respectively; Fig. 5) for multiple reasons. These include a general difficulty in predicting biosynthetic P450 functions from sequence similarity alone (51), and LtzR, LtzS and LtzH are all members of the same P450 family (CYP113, using CYPED) (52). Furthermore, Lentzea affords an interesting opportunity to study these P450s within the context of a seldom-studied rare actinomycete host. Finally, none of the other pathways yield heterodimeric products, necessitating further investigation.

While limited numbers of P450s sourced from characterized piperazyl-HPIC cyclopeptide BGCs are available for comparison, we employed a maximum-likelihood inferred phylogeny (Fig. 5A) of the proteins above and their \( \Delta \)ltz-locus encoded homologs to assess if an evolutionary approach might better guide functional prediction. We found P450s involved in HPIC formation (blue highlights), HPIC-biaryl crosslinking (red highlights) formed monophyletic groups, and LtzR and LtzS, respectively, claded within those groups. However, LtzH formed a separate group with HmtN, a piperazyl-peptide hydroxylating P450 from the himastatin (31) pathway and these were polyphyletic with AfnA (a P450 thought to have a similar function as HmtN) (32). Together, this suggested that biaryl linking and HPIC forming AfnA (a P450 thought to have a similar function as HmtN) (32). The himastatin (31) pathway and these were polyphyletic with AfnA (a P450 thought to have a similar function as HmtN) (32). Thus, this suggested that biaryl linking and HPIC forming AfnA (a P450 thought to have a similar function as HmtN) (32). The himastatin (31) pathway and these were polyphyletic with AfnA (a P450 thought to have a similar function as HmtN) (32). From this, we inferred that LtzR is likely involved in petrichorin HPIC formation, LtzS is required for cyclopeptide crosslinking, and LtzH is involved in piperazyl hydroxylation (by virtue of its similarity to HmtN and the presence of D-4(OH)-Piz subunits in both petrichorins). After constructing unmarked deletion mutants in letzR, letzS, and letzH, we found that each strain accumulated several biosynthetic intermediates having retention times and masses distinct from petrichorin A and B. In concert with the phylogenetic predictions and previous himastatin biosynthesis and engineered alboflavusin dimerization analyses, we predicted that a \( \Delta \)ltzR strain should be unable to produce crosslinked intermediates based on the inability of the strain to produce HPIC (where intact HPIC is apparently necessary for downstream P450 crosslinking) (32). Our high-resolution MS and LC/MS/MS of the piperazyl intermediates accumulated in this mutant found molecules having mass formulae corresponding to expected precursors of the left and right sides of petrichorin A (ZR1 and ZR2, Fig. 5B and E) which feature chloro-tryptophan residues in place of chloro-HPIC, in agreement with our predictions.

We found that deleting letzS led to the production of petrichorin C, which was initially discovered in this work after accumulating in our \( \Delta \)ltzD NRPS mutant (Fig. 4). In contrast with the letzD mutant (unable to synthesize the left cyclopeptide chain of petrichorin A and the entirety of petrichorin B), mutating letzS led to concomitant accumulation of an additional molecule (ZS2) having a mass formula equivalent to the predicted left cyclohexapeptide of the petrichorin A dimer (Fig. 5C and F). Accordingly, ZS2 was then also posited to be structurally equivalent to the cyclopeptide monomer that is dimerized to create petrichorin B. Finally, deleting letzH led to the production of biosynthetic intermediates with masses that correspond to petrichorin A missing a lone piz-hydroxyl group, or petrichorin B lacking both piz-hydroxyl decorations (Fig. 5D and G; ZH1 and ZH2 respectively, where ZH2 is potentially identical to chloptosin C) (53). Importantly, all of these P450s were genetically complemented to restore petrichorin A and B, indicating the accumulated intermediates were not influenced by transcriptional polarity (SI Appendix, Fig. S56). Taken together, these data offer strong evidence for letzD-locus P450 function that is congruent with our phylogenetic analysis.

Analysis of Petrichorin Regulation. Many actinomycete BGCs encode for cluster-situated regulators (CSRs), proteins that function to coordinate transcriptional regulation of the genes in their cluster neighborhood (55). The BGCs encoding for himastatin and the kutznerides both encode CSRs (31, 47), but appear to employ dissimilar regulatory strategies based on a lack of contextually conserved regulatory proteins between...
Fig. 5. Phylogenetic and metabolic analyses to assign ltx-locus cytochrome P450 gene functions. The cytochrome P450s associated with BGCs encoding molecules like those found in Fig. 1 share significant homology and are thus challenging to functionally assign via sequence identity alone (32). However, (A) a maximum-likelihood phylogeny bins P450s involved in HPIC maturation (blue branches) and biaryl-crosslinking (red branches) into monophyletic groups, indicating potential orthology. In contrast, putative piperazyl-hydroxylating P450s (HmtN, LtzH and AfnA) failed to show similar monophyly. CypX (AGS49593.1) (54), an exemplar CYP113C-family P450, was used as an outgroup; the tree was constructed with 500 bootstraps and branches <90% confidence were collapsed with actual values noted at nodes. (B–D) LC/MS/MS analysis of biosynthetic intermediates accumulated in ltxR, ltxS, and ltxH mutants, respectively, plus (E–G) homology-inferred and HRMS-supported structures of key molecules arising in each mutant. For each proposed intermediate, the shaded circles indicate the target of each P450, with colors matching those used to highlight the tree in (A). Hydroxylation targets are indicated in dark charcoal (G). Several mutants accumulated intermediates having masses commensurate with hydride [2H] or hydroxyl [OH] additions or losses (±) to the illustrated structures; these are denoted within chromatogram peak callouts for clarity. Peak in (C) indicated with * has a mass equivalent with ZS2; indicating a likely geometric isomer thereof.
Table 1. Petrichorin IC\textsubscript{50} values (nM) against select human cancer cell lines with paclitaxel control

| Cell lines | Petrichorin A | Petrichorin B | Petrichorin C | Paclitaxel |
|------------|--------------|--------------|--------------|-----------|
| A2780      | 28.12 ± 2.329| 35.32 ± 4.289| 169.14 ± 2.860| 8.127 ± 2.156 |
| HT1080     | 35.51 ± 4.194| 36.36 ± 3.423| 72.41 ± 3.021| 75.0 ± 2.511 |
| PC3        | 34.49 ± 2.262| 68.43 ± 3.296| 273.46 ± 3.385| 136.0 ± 1.494 |
| Jurkat     | 20.25 ± 2.624| 72.47 ± 1.551| 101.9 ± 3.014| 8.694 ± 2.699 |

them. Xie et al. (56) found that himastain biosynthetic regulation is complex, suggesting two canonical transcriptional regulators encoded within the compound’s BGC (HmtA and HmtD) are chiefly involved. These proteins respectively belong to the MerR and ParB regulatory families, which contrasts with the single SARP-family transcriptional regulator (KtzK) encoded within the core of the kutzeride BGC. Comparing the lzt locus against these clusters revealed a single SARP-encoding gene (ltzP), suggesting closer regulatory parallels with the kutzeride locus. While kutzeride regulation remains experimentally uncharacterized, deleting lztP led to the complete loss of petrichorin production (JV755) (SI Appendix, Fig. S57), as well as any LC/MS/MS detectable piperazyl intermediates, supporting the idea that LtzP encodes for an essential petrichorin regulator. The mutant was rescued by lztP ectopic expression (JV825), but not kutzeride homolog ktzK (JV826), suggesting these regulators have host-specific functions, possibly stemming from different regulator-DNA sequence tropisms.

Himastatin regulatory analyses also suggested that hmtG, encoding an acetylglutamate kinase type protein may act as a metabolic regulator (56). While the lzt locus lacks a homolog of this protein, we found that deleting lztB (encoding a protein similar to acyl-CoA oxidoreductases) led to the complete abrogation of petrichorin production and any detectable intermediates (SI Appendix, Fig. S57). Similar to the lztP regulatory phenotype above, this suggested the lzt locus may also employ metabolic regulatory mechanisms (similar to that inferred for hmtB). Ectopic expression of lztB weakly rescued the ∆lztB phenotype, indicating that polarity is not likely causal (SI Appendix, Fig. S57).

Interestingly, Ma et al. (31) found that deleting the lztB homolog encoded within the himastatin locus (hmtG) led to complete compound loss, leading those authors to speculate that hmtG may be involved in piz production. Because piz biosynthesis is now solved, this explanation seems less likely. However, the strong phenotypes accompanying LztB/HmtG loss indicates a critical role must exist. Interestingly, both proteins are encoded within syntenic cassettes, characterized an NRPS gene encoding a predicted X-A-T domain arrangement (ltzA/hmtF), an acyl-CoA oxidoreductase (ltzB/hmtG), then a type II thioesterase (ltzChmtH). This conserved arrangement, in combination with our proposed functions for LtzA, led us to infer that LtzB/HmtG proteins may be involved in modulating NRPS protein-protein interactions or a related role. How the oxidoreductase functionality of these proteins might integrate into such a system also needs investigation, but trans-acting proteins are widely known to modulate NRPS assembly lines, including MbtH-type proteins (57) (encoded by lztG in the petrichorin locus).

Petrichorin Cytotoxicity Assays. The dimeric cyclo(depsi)peptide clotoxin, himastatin, and enzymatically cross-linked alboflavivusins show compelling cytotoxicity profiles (30, 32, 58), and their structural parallels with the petrichorins suggested the latter compounds may have similar properties. Thus, petrichorin A was evaluated for anti-proliferative activity against multiple cancer cell lines. Naturally heterodimeric petrichorin A was active against A2780 human ovarian cancer, HT1080 fibrosarcoma, the PC3 human prostate cancer, and the Jurkat human T lymphocyte cell lines (a model for acute T cell leukemia) with IC\textsubscript{50} values in the range of 20–36 nM. Petrichorins B and C were also active, but to a generally lower extent than petrichorin A. Homodimeric petrichorin B inhibited the four cell lines with IC\textsubscript{50} values of 35–73 nM, while monomeric petrichorin C was the least active with IC\textsubscript{50} values ranging from 73–274 nM (versus paclitaxel control, Table 1 and SI Appendix, Fig. S58). Prior alboflavivusin monomer vs. dimer structure-activity studies (32) led us to predict that monomeric petrichorin C would likely be less active than dimeric petrichorins A and B. However, because homodimers are far more common versus heterodimeric compounds in the context of polyvalent drug development (59), observing that petrichorin A is generally more active than petrichorin B was somewhat surprising. This suggests aryl-crosslinked cyclopeptide heterodimers like petrichorin A, whether encoded by nature or synthesized in a directed way, should be closely considered for future polyvalent molecular diversification and design.

Conclusions

Actinomycetes are a valued source of biologically active compounds. However, there is a recognized historical bias in how these organisms have been prosecuted for pharmaceutical discovery, with infrequently encountered “rare actinomycetes” remaining less-explored. The problem of molecule rediscovery is one of several significant reasons quoted to explain why large pharmaceutical companies mostly withdrew from actinomycete-based drug discovery programs (7). However, facing a broadly recognized need for new anti-infectives and other drugs, rare actinomycetes remain relatively fertile for the discovery of novel medicines (24). Despite their perceived value, the biotechnological exploitation of rare actinomycetes can be challenging. Compared to more common Streptomyces, are fewer rare actinomycetes in cultivation and they often suffer from less-developed genetic toolsets for genome engineering and other important manipulations.

In an effort to discover new bioactive compounds from rare actinomycetes, we designed and tested a bottom-up discovery approach for the targeted metabologenomic identification of yet-undiscovered piperazyl natural products produced by L. flaviverrucosa. The recent discovery of the Streptomyces incarnatapeptin piperazyl-peptides via a combined pzbB mining–\textsuperscript{15}N NMR discovery program (60) demonstrates the power of metabologenomics for targeted piperazyl molecule discovery. This work adds to prior efforts by devising a high-throughput adaptable MS/MS-based approach in place of NMR as a primary screening tool, while also demonstrating that often-used Streptomyces genetic toolsets are transferrable to L. flaviverrucosa.

Biosynthetic superclusters are chromosomally adjacent biosynthetic loci that often direct the production of synergistic pairs of molecules. This synergy results in complex pathway inhibition and robust bioactivity profiles for producing organisms (for a recent review, see 61). Here, the main product of petrichorin
supercluster (petrichorin A) is a pair structurally distinct cyclopeptides that are crosslinked by cytochrome P450 LtxS. LtxS is apparently versatile; being essential for heterodimeric petrichorin A but it also catalyzes minor homodimeric (petrichorin B) chloro-biaryl cyclopeptide crosslinking. While Guo et al. (32) reported the use of related P450s for homodimeric HPIC-piperazyl cyclopeptide crosslinking to bioengineer new cytotoxic homodimers, our findings now highlight enzymes within the LtxS/CipS/HmsS clade for (chloro)biaryl heterodimer catalysis as well. This suggests additional clade-members could be sought for diversifying HPIC-cyclopeptides via heteromultimerization.

Several mechanisms are suggested to explain why polyvalent molecules, often symmetric homodimers or higher-order multimers, have superior biological activities against monomeric analogs (59, 62–64). Given this, the substantive bioactivity activity seen for bilaterally asymmetric petrichorin A (especially in comparison to symmetric petrichorin B) is intriguing and should inspire future heterodimer structure-activity relationship and mechanistic research against additional cell lines and organisms. In sum, this study provides a framework for the exploration and directed biosynthesis of asymmetric cyclopeptide heterodimers, illustrates the utility of a mass-spectrometric-based metabologenomics approach for desirable piperazates, and highlights _L. flaviverrucosa_ as a rare actinomycete amenable to manipulation for biotechnological development.

### Materials and Methods

**Materials.** All standard laboratory chemicals were purchased from Sigma-Aldrich, Fisher Scientific, or Santa Cruz Biotechnology. Microbiological media ingredients were purchased from DIFCO. L-Ornithine was purchased from CD/N Isotopes and L-piperazic acid dihydrochloride was synthesized and purchased from WuXi AppTec. The publicly available genome sequence of _Lentzea flaviverrucosa_ (GenBank PRJNA63399) was used to guide these studies (ItaA-Ital, RDI25332.1-RDI25335.1, respectively). See _SI Appendix, Table S10_ for the oigonucleotides used in this study.

**Growth and Strains.** _Lentzea flaviverrucosa_ DSM 44664 was routinely propagated on ISP2 agar (International Streptomyces Project Medium 2, Difco) and TS8 (Trypatic Soy Broth, Difco) at 28 °C. Colony PCR templates were prepared by grinding a colony in 100 μL DMSO, essentially as noted elsewhere (65). _Escherichia coli_ was routinely propagated on lysogeny broth Agar and broth at 37 °C according to standard methods. _SI Appendix_ and Tables S11 and S12 list plasmids and strains used in this work. For details on intergenic conjugation, and deletion mutant construction, and bioactivity assays (essentially as in 66–71), see _the SI Appendix._

**Petrichorin Detection.** _Lentzea flaviverrucosa_ colonies were inoculated to 15 mL of TS8 liquid media in a 125-mL Erlenmeyer supported by NSF-CAREER 1846005 to J.A.V.B. Lentzea _flaviverrucosa_ DSM 44664 was cultured on ATCC172 agar plates at 28 °C. The ethyl acetate (EtOAc) crude extract (471.28 mg) was subjected to preparative HPLC (phenyl-hexyl column, 5 μm; 100.0 × 21.2 mm; 10 mL/min; with 0.1% formic acid in mobile phases) eluted at 20–100% acetonitrile/H2O in 40 min to obtain 40 fractions (SI-1–SI10). F17 (57.2 mg) and F18 (32.8 mg) were further separated by semipreparative HPLC (C18 column, 5 μm; 250.0 mm × 10.0 mm; 10 mL/min; with 0.1% formic acid in 78% CH3OH/H2O) to yield petrichorin A (35.7 mg, tR 11.4 min) and petrichorin B (3.61 mg, tR 14.7 min). Mutant strain JV757 (ΔltzD) was also cultured on 5% _Leuconostoc_ aga plates at 28 °C for 6 d. The EtOAc crude extract (471.28 mg) was subjected to preparative HPLC (phenyl-hexyl column, 5 μm; 100.0 × 21.2 mm; 10 mL/min; with 0.1% formic acid in mobile phases) eluted at 20–100% methanol/H2O in 35 min to get 35 fractions (F1–F35). All the fractions were analyzed by LCMS and the target compound with the molecule weight of 717 Da was detected in F28. Further separation of F28 (9.68 mg) with semipreparative HPLC (C18 column, 5 μm; 250.0 mm × 10.0 mm; 4 mL/min; 20-40% CH3CN/H2O in 60 min) led to the isolation of petrichorin C (3.25 mg, tR 11.4 min). Details on the hydrolysis and analysis of the petrichorins via Marfey ( _SI Appendix, Table S13_ ) and Mosher derivation (essentially as previously described) (37, 38) are also in _the SI Appendix_. Also see _SI Appendix_ for details of DP4 NMR calculations (73).

**Data Availability.** All study data are included in the article and/or _SI Appendix._

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