Directed Evolution of the Nonribosomal Peptide Synthetase AdmK Generates New Andrimid Derivatives In Vivo

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SUMMARY

Many lead compounds in the search for new drugs derive from peptides and polyketides whose similar biosynthetic enzymes have been difficult to engineer for production of new derivatives. Problems with generating multiple analogs in a single experiment along with lack of high-throughput methods for structure-based screening have slowed progress in this area. Here, we use directed evolution and a multiplexed assay to screen a library of >14,330 clones for derivatives of the antibiotic andrimid (I). Focusing on the site of valine incorporation, three new chemical derivatives have been created with similar or improved biological activity.

INTRODUCTION

Nonribosomal peptides are a proven source of antibacterial (Recktenwald et al., 2002), antifungal (Denning, 1997), and anticancer drugs (Du et al., 2000). These peptides often contain nonproteogenic amino acids as well as fatty acid, polyketide, carbohydrate, and isoprenoid appendages. The enzymes that build these modified peptides, nonribosomal peptide synthetases (NRPSs), are often large enzymes with multiple active sites and are characterized by keeping intermediates covalently tethered by a series of phosphopantetheine (PPant) linked thioester bonds at the thiolation (T) domains during biosynthesis. The gatekeepers of these molecular “assembly lines” are the adenylation (A) domains which select an amino acid building block from the pool available within producing cells. The modular structure of NRPSs make them attractive targets for protein engineering, but realization of this goal over the last few decades has been far more difficult than many had hoped. Here we screen a library of ~14,330 clones for derivatives of the antibiotic andrimid (I). Focusing on the site of valine incorporation, three new chemical derivatives have been created with similar or improved biological activity.

Even though amino acid substrates for adenylation domains may be predicted with some confidence via the “NRPS Code” (Challis et al., 2000; Rausch et al., 2005; Stachelhaus et al., 1999), engineering of these domains to accept noncognate substrates has been elusive. Often referred to as combinatorial biosynthesis (Khosla et al., 1999; Khosla and Zawada, 1996; Tsoi and Khosla, 1995; Walsh, 2002; Walsh et al., 2003), a main approach has employed chimeric synthetases where an adenylation domain of one NRPS is replaced by an adenylation domain from another NRPS which activates a different substrate. This so-called “domain swapping” strategy has proved successful in terms of generating the desired product, but has been plagued by decreased product yields and outright dysfunction presumably due to interruption of protein-protein interactions caused by this cut and paste approach (Baltz et al., 2006; Doekel and Marahiel, 2000; Mootz et al., 2000, 2002; Nguyen et al., 2006; Schneider et al., 1998; Stachelhaus and Marahiel, 1995a, 1995b). The activity of chimeric synthetases could be restored in the first reported use of directed evolution for reengineering NRPS systems (Fischbach et al., 2007). Domain swapping, however, is still not ideal for true combinatorial biosynthesis in that at most one variant of an NRPS can be generated per swap. Here, we present the second example of using directed evolution to alter thiopentametered biosynthetic systems. Instead of rescuing activity of a chimeric synthetase using error-prone PCR, we directly targeted active site residues for evolution using saturation mutagenesis (Chockalingam et al., 2005). This orthogonal approach to NRPS reengineering focuses on modifying the sites of substrate specificity,
CoA, the precursor for fatty acids and some polyketides (Freiberg et al., 2006; Singh et al., 1997). Andrimid is a broad spectrum antibiotic and acts by inhibiting fatty acid biosynthesis at the acetyl-CoA carboxylase step, preventing formation of malonyl-CoA and leading to the production of andrimid derivatives in vivo (Fischbach et al., 2007).

**RESULTS**

**Library Design and Construction**

We sought to express a library of mutant enzymes and monitor the effectiveness of the mutations by screening for end products produced by the pathway in vivo. To accomplish this, removal of the wild-type (WT) enzyme activity was necessary. We employed an allele replacement strategy based on homologous recombination that has proved successful in a number of organisms, namely the streptomycin resistance counterselection (Russell and Dahlquist, 1989). Using this strategy, we made a scarless deletion of the WT andrimid gene in P. agglomerans. The resulting strain served as a background strain for expression of an admK mutant library (see Figure S1A available online). This result was validated by PCR (Figure S1B). Also, we found that the E. coli expression plasmid pQE60 was capable of supplying admK and restoring andrimid production levels comparable to WT in P. agglomerans (Figure S1C).

In order to select residues in admK for mutagenesis, we turned to sequence alignments of AdmK with A-domains known to activate substrates different than the valine found in andrimid (Figure S3). We chose A-domains that activate nonpolar amino acids because SAR studies (Freiberg et al., 2006) showed that this type of residue could substitute for valine and increase the potency and selectivity of the andrimid scaffold. From the sequence alignment with the ten residues used for substrate prediction of NRPS A-domains (Stachelhaus et al., 1999), we chose the three most highly variant of the ten for saturation mutagenesis (Miyazaki and Arnold, 1999) (Figure 3). Our three-site saturation mutagenesis library was designed to create 1404 distinct mutants, and was limited by selection of codons for synthetase residues likely to select nonpolar substrates (Figure 3) for incorporation into the andrimid backbone. The mutant library was constructed by utilizing sequential megaprimer and overlap extension PCRs (Horton, 1997; Sarkar and Sommer, 1990). In

**Figure 2. Andrimid Biosynthetic Pathway**

Andrimid is biosynthesized by a highly disconnected and hybrid NRPS/PKS pathway. Adenylation domain substrate specificity is indicated by single letter amino acid abbreviation subscript. AdmK incorporates the valyl subunit (highlighted) of andrimid and has been targeted for mutagenesis. KS- ketosynthase, CLF- chain length factor, DH-dehydrogenase, KR- ketoreductase, T-thiolation, TG-transglutaminase, A-adenylation, Mut- aminomutase, C- condensation, TE-thioesterase.
Evolution of AdmK Generates Andrimid Derivatives

Assay Development and Library Screening

To screen our library of andrimid producing clones, we employed a structure-based assay. We reasoned that using an antibiotic bioassay we would not be able to distinguish between andrimid and derivatives of andrimid. Also, we would be incapable of identifying which precise andrimid derivative had been produced because most derivatives would likely be active antibiotics (Freberg et al., 2006). During evaluation, mass spectrometric detection of andrimid showed ~23,000-fold greater sensitivity than a bioassay, and we noticed a fragmentation pattern that cleanly dissected the molecule into regions to be conserved or variant (Figure 1, lower right panel). This calculation of relative sensitivity for MS versus classic halo assays is based on the limit of detection (LOD) during MS of andrimid, which is 20 fmol. Given that 96 samples are mixed per LC-MS injection, this translates to a LOD of 1.9 pmol for the pooled assay. The MIC data for andrimid against E. coli show that 0.47 nmol can be detected in a 10 μl spot in the agar overlay assay. If this assay were diluted 96-fold as in the LC-MS assay this translates to a 45.1 nmol LOD. These attributes of the MS-based screen allowed us to identify which andrimid derivative was produced and conduct the screening of 96 clones in a single LC-MS run. Single clones arrayed into 96-well plates, were inoculated into 2×YT microbial medium supplemented only with antibiotic and grown for 24 hr prior to pooled sampling of each 96-well plate. Each pooled sample representing 96 individual clones constituted one LC-MS sample. Four clones producing andrimid derivatives were isolated. Twenty-four percent of assay plates (i.e., 96 samples combined into one) still showed production of andrimid while 41% of plates showed no production beyond the octatrienyl-β-phenylalanine. See also Figure S4.

Characterization of Mutants and Derivative Compounds

The four mutant clones were identified subsequently (by pooled -row and -column searching of assay plates), sequenced and designated A2 (W240L, G291V), A7 (W240S, I265T, G291E), A9 (W240T, I265T, G291E), and B12 (R235K, W240L, I265L, G291E). Together, these clones produced three new andrimid derivatives and another that has been described previously (Figure 4; Figure S4A, i-v). Clones A2 and B12 were found to
produce an andrimid related peak at m/z 494.26, corresponding to either isoleucine- or leucine-substituted andrimid (4, 5) (Figure S4A, i, ii). Clones A7 and A9 produced a mixture of alanine- and phenylalanine-substituted andrimid (6, 7) (Figure S4A, iii, iv). In order to resolve the L-Ile versus L-Leu ambiguity for clones A2 and B12, an isotope feeding study was conducted. Growing clones A2 and B12 in media supplemented with a 50:50 mixture of $^{13}$C$_2$-Leu and $^{13}$C$_6$-Ile demonstrated that both clones A2 and B12 have preference for Ile over Leu, but at different levels of discrimination (Figure S4B). Clone A2 showed a 4:1 preference for Ile to Leu, whereas clone B12 showed a 20:1 preference for Ile to Leu. Control experiments where only one isotope labeled amino acid was supplemented clearly showed that when one amino acid is supplied in 50 mM excess, incorporation of that amino acid is efficient (Figure S4C). For that reason, we employed a mutasynthesis strategy where the desired amino acid to be incorporated was supplied in excess for scale up of the amino acid to be incorporated to be supplied in order to determine yield and bioactivity. We found that applying this mutasynthesis strategy also improved the yield for clones A2 and A9. Compounds 1, 4, 5, and 7 were all found to be bioactive in disk diffusion assays (Figure 5). When using the mutasynthesis, we were able to significantly improve the yields, in some cases approaching WT levels (Table 1).

**DISCUSSION**

This work takes on the challenges associated with engineering enzymes of secondary metabolism with a technological approach that applies generally to metabolites detectable by tandem mass spectrometry. When combined with high level expression in the native host, *P. agglomerans*, the reduced metabolic production from active site mutation of admK (largely from poor solubility of variants), did not prevent four successes from being found in a library pool where ~80% were unaffected, ~20% were dead, and ~0.03% had altered production.

Creating a mutant library of admK rather than performing several domain-swapping experiments allowed us to generate four andrimid derivatives in the same overall experiment while examining many solutions for each derivative simultaneously. The key aspect of our work that allowed for this combinatorial biosynthesis experiment is the structure-based assay that provided a direct readout of not just the AdmK variants’ activity, but also the combined output of downstream enzymes in the assembly line. Unlike many indirect assays of enzyme activity (bioactivity or coupled assays), direct structural readout of mutant activity in the integrated pathway tightly connects the overall impact of the mutagenesis with a main goal of the exercise: production of new derivatives.

We found four mutants that exhibited altered substrate specificity, A2, A7, A9, and B12. All showed a broadening of substrate specificity relative to the WT, with increased preference to substrates other than the cognate substrate, valine. To our surprise, the overall best performing mutant was actually a serendipitous quadruple mutant. The fourth mutation in B12, R235K is likely an artifact of PCR; however, it is unknown if this stray mutation is required for robust production of the Ile/Leu analogs. Structures for NRPS A-domains available exhibit low sequence identity to AdmK, 25% for 1AMU and 24% for 2VSQ. However, based on alignment of AdmK to the structures above, R235 corresponds to N697 of 2VSQ and E272 of 1AMU. These positions in the respective crystal structures are remote from the active site and on the surface of the protein. We can only speculate that R235K may change a protein-protein interaction with the catalytic domain or on the surface of the protein. We can only speculate that R235K may change a protein-protein interaction with the catalytic domain or on the surface of the protein. The overall best performing mutant was actually a serendipitous quadruple mutant. The fourth mutation in B12, R235K is likely an artifact of PCR; however, it is unknown if this stray mutation is required for robust production of the Ile/Leu analogs.

**Table 1.** Mutations in *admK* and Corresponding Products Compared with WT Production Levels

<table>
<thead>
<tr>
<th>Clone</th>
<th>Mutations</th>
<th>Compound Produced</th>
<th>Medium</th>
<th>Production per Liter (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>N/A</td>
<td>1 2xYT</td>
<td></td>
<td>378.16</td>
</tr>
<tr>
<td>A2</td>
<td>W240L, G291V</td>
<td>4 2xYT + Ile</td>
<td></td>
<td>4.94</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2xYT + Leu</td>
<td></td>
<td>0.99</td>
</tr>
<tr>
<td>B12</td>
<td>R235K, W240L, I265L, G291E</td>
<td>4 2xYT + Ile</td>
<td></td>
<td>466.39</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2xYT + Leu</td>
<td></td>
<td>93.83</td>
</tr>
<tr>
<td>A7</td>
<td>W240S, I265T, G291E</td>
<td>6 2xYT + Ala</td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2xYT + Phe</td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>A9</td>
<td>W240T, I265T, G291E</td>
<td>6 2xYT + Ala</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2xYT + Phe</td>
<td></td>
<td>0.49</td>
</tr>
</tbody>
</table>

**Table 2.** MIC Data for Andrimid and Derivatives against Representative Gram-Negative and Gram-Positive Bacterial Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (nmol)</th>
<th>1</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> imp ASR</td>
<td>0.47</td>
<td>2.72</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 13883</td>
<td>9.05</td>
<td>2.93</td>
<td>NT*</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>7.71</td>
<td>0.39</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> ATCC 19433</td>
<td>36.35</td>
<td>42.76</td>
<td>NT*</td>
<td></td>
</tr>
</tbody>
</table>

* Not tested.
outside the selectivity conferring code should be considered for future A-domain mutant libraries. A second round of mutagenesis and screening was performed on the four isolated clones using an error-prone PCR approach in order to increase the activity of the first round mutants, but no improvement was observed. MIC values for the derivative compounds 4 and 5 showed increased bioactivity toward *Staphylococcus aureus* relative to 1, and unchanged or decreased bioactivity toward *E. coli* consistent with previous SAR studies (Freiberg et al., 2006) (Table 2). Prediction of A-domain substrates is fairly robust (Rausch et al., 2005). Despite this, prescription of what active site mutations to make for a particular metabolite structure is far less developed. This study provides a large-scale survey of what amino acid substitutions could be tolerated in the A-domain active site. While many of the triple mutants that still yielded andrimid were not sequenced here, one can envisage a large-scale study specifically aimed at determining empirically what sites are stable to mutation. This would effectively map functional outcomes for many positions and sharply increase our predictive ability for creation of new NRPS derivatives.

One reason the andrimid system was chosen is because its biosynthetic enzymes are mostly < < 100 kDa (i.e., relatively small for NRPS or PKS proteins). There is no reason why our methods cannot be extended to larger multimodule NRPSs, provided the host is genetically tractable, or the pathway is amenable to heterologous expression with robust yields. We envision that the general approach used here could be extended to other domains within NRPS systems to probe substrate specificity of condensation domains. We estimate that a ~100-fold improvement in screening speed could be achieved, leading to an ability to screen 4 or even 5 site mutant libraries with similar coverage within the same approximate scope of time and resources used in this work.

Peptide natural products from NRPS systems draw high interest because of their medical uses, but harnessing their rather predictable correlation between enzyme primary structure and metabolite structure has proved elusive over the past two decades (Khosla et al., 1999). Reengineering strategies to achieve combinatorial biosynthesis (Walsh, 2002) compete conceptually with organic synthesis as a strategy for enhancing the diversity of natural molecular scaffolds. Challenges to attaining the goal of true combinatorial biosynthesis include not only the general challenges of enzyme engineering, but also the methods used to evaluate the modified enzymes and pathways. The field of NRPS reengineering is making strides through better understanding of protein-protein recognition motifs (Li and Vederas, 2009) and the use of directed evolution (Fischbach et al., 2007) to generate libraries of A-domains capable of activating a variety of substrates. Alternative approaches to making mutant libraries such as family shuffling (Cramer et al., 1998) could be employed in the future to generate even larger libraries of large active site fragments, perhaps leading to more stable and active mutant enzymes.

**SIGNIFICANCE**

Combinatorial biosynthesis is an attractive alternative to chemical synthesis for creating analogs of complex natural products. A major hurdle to realizing the potential of combinatorial biosynthesis includes the lack of selections or screens to process mutant libraries of enzymes in the context of their entire biosynthetic pathway. This work demonstrates a general method for rapidly generating and screening mutant libraries of nonribosomal peptide synthetases. The unbiased nature of structure-based screening allows for evaluation of the output from entire mutant pathways on a basis largely disconnected from total yield or specific bioactivity. Using the high-throughput multiplexed LC-MS/MS screening method outlined here, four derivatives of the antibiotic andrimid were generated, three of them not previously reported. The relative bioactivities of the derivatives were measured and it was found that two of the derivatives exhibited a shift in antibacterial specificity with concomitant decreases in their MIC values, consistent with previous synthetic chemistry driven SAR studies. Mutagenesis combined with LC-MS/MS for a structure-based readout is a general approach that can be readily extended to other nonribosomal peptide synthetase and polyketide synthase pathways as well as to other biosynthetic pathway paradigms.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**

Restriction enzymes were from Invitrogen (Carlsbad, CA), T4 DNA ligase was from New England Biolabs (Ipswich, MA), Phusion polymerase from Finnzymes (Woburn, MA) was used for PCR. Growth medium was from BD Biosciences (San Jose, CA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and are listed in Supplemental Experimental Procedures. HPLC solvents were from Fisher (Pittsburgh, PA). Formic Acid was from Acros Organics (Geel, Belgium). PCR cleanup, plasmid miniprep and gel extraction kits were from Qiagen (California, VA). Stable-isotope labeled amino acids were from Cambridge Isotopes (Andover, MA). All other reagents, chemical and consumables were from Sigma unless otherwise specified.

**Strains and Plasmids**

*E. coli* DH5α λ pir was used for all cloning steps for plasmids containing oriR6K. *E. coli* DH15 and BL21(DE3) were obtained from the UIUC Cell Media Facility. *E. coli* imp ASR, Klebsiella pneumoniae ATCC 13883, *Staphylococcus aureus* ATCC 25923, and Enterococcus faecalis ATCC 19433 were used as the indicator strains for agar overlay assays. The andrimid producer, *P. agglomerans* Eh 335 and *E. coli* imp ASR were a gift from Christopher T. Walsh. *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 25923, and *E. faecalis* ATCC 19433 were obtained from ATCC (Manassas, VA). All strains were cultured in LB or 2xYT at 37°C (E. coli, K. pneumoniae, S. aureus, and E. faecalis) or 28°C (P. agglomerans). Plasmid pQE60 (Qiagen) was used for expression of admK in *P. agglomerans*. Plasmids pACYC-Duet, pET-Duet, pET28a, and pUC-19 were obtained from Novagen (Darmstadt, Germany). The suicide plasmid pMQ118 was obtained from Presque Isle Cultures (Erie, PA). All strains and plasmids are listed with relevant characteristics in Supplemental Experimental Procedures.

**Antibiotic Bioassays**

LB agar plates (100 mm diameter) were overlaid with 4 ml molten LB agar inoculated with 75 μl of an overnight culture of the indicator strain. The inoculated top agar was allowed to solidify before sterile paper disks were laid onto the assay plates. Onto each disk 10–20 μl of sterile filtered culture supernatant or control was spotted before incubation at 37°C overnight or until zones of inhibition were visible.

**DNA Sequencing**

DNA sequencing was carried out at the University of Illinois core sequencing facility on an ABI 3730XL capillary sequencer on reactions using BigDye
Andrimid was produced from liquid culture of Isolation and Quantification of Andrimid and Analogues for construction and screening of the admK mutant library. Several adenylation domains with specificity for the desired activity in the AdmK (GenBank accession number AA391051.1) were aligned: GrsA (GenBank accession number CA036031.1, phenylalanine specific), YebB M3 (GenBank accession number AAC54929.1, phenylalanine specific), Mccy M2 (GenBank accession number CA090227.1, alanine specific), JamQ (GenBank accession number AA398786.1, alanine specific), Baca M1 (GenBank accession number CA063461.1, isoleucine specific), FubB (GenBank accession number AAB00093.1, isoleucine specific), SrFab M3 (GenBank accession number BAA08983.1, leucine specific), GrsB M4 (GenBank accession number CA438381.1, leucine specific) and LicB M1 (GenBank accession number AAD04758.1, valine specific). Adenylation domains were aligned using the program CLUSTALX (Thompson et al., 1997) (Figure S3). Adenylation domains were aligned using the program CLUSTALX (Thompson et al., 1997) (Figure S3). Adenylation domains were aligned using the program CLUSTALX (Thompson et al., 1997) (Figure S3). Adenylation domains were aligned using the program CLUSTALX (Thompson et al., 1997) (Figure S3). Adenylation domains were aligned using the program CLUSTALX (Thompson et al., 1997) (Figure S3).

**Alignment of AdmK to Other Adenylation Domains and Extraction of Binding Pocket Residues**

Several adenylation domains with specificity for the desired activity in the AdmK (GenBank accession number AA391051.1) were aligned: GrsA (GenBank accession number CA036031.1, phenylalanine specific), YebB M3 (GenBank accession number AAC54929.1, phenylalanine specific), Mccy M2 (GenBank accession number CA090227.1, alanine specific), JamQ (GenBank accession number AA398786.1, alanine specific), Baca M1 (GenBank accession number CA063461.1, isoleucine specific), FubB (GenBank accession number AAB00093.1, isoleucine specific), SrFab M3 (GenBank accession number BAA08983.1, leucine specific), GrsB M4 (GenBank accession number CA438381.1, leucine specific) and LicB M1 (GenBank accession number AAD04758.1, valine specific). Adenylation domains were aligned using the program CLUSTALX (Thompson et al., 1997) (Figure S3). Positions aligning to D235, A236, W239, T278, I299, A301, A322, I330, C331, and K517 of GrsA were extracted as the putative binding pocket residues (Stachelhaus et al., 1999). The binding pocket residues were inspected for their variation across the aligned sequences and three positions corresponding to T278, I299, and A322 in GrsA were chosen for mutagenesis. Several adenylation domains with specificity for the desired activity in the AdmK (GenBank accession number AA391051.1) were aligned: GrsA (GenBank accession number CA036031.1, phenylalanine specific), YebB M3 (GenBank accession number AAC54929.1, phenylalanine specific), Mccy M2 (GenBank accession number CA090227.1, alanine specific), JamQ (GenBank accession number AA398786.1, alanine specific), Baca M1 (GenBank accession number CA063461.1, isoleucine specific), FubB (GenBank accession number AAB00093.1, isoleucine specific), SrFab M3 (GenBank accession number BAA08983.1, leucine specific), GrsB M4 (GenBank accession number CA438381.1, leucine specific) and LicB M1 (GenBank accession number AAD04758.1, valine specific). Adenylation domains were aligned using the program CLUSTALX (Thompson et al., 1997) (Figure S3). Positions aligning to D235, A236, W239, T278, I299, A301, A322, I330, C331, and K517 of GrsA were extracted as the putative binding pocket residues (Stachelhaus et al., 1999). The binding pocket residues were inspected for their variation across the aligned sequences and three positions corresponding to T278, I299, and A322 in GrsA were chosen for mutagenesis.

**Construction and Screening of admK Mutant Library**

Please refer to Supplemental Experimental Procedures for detailed methods for construction and screening of the admK mutant library.

**Isolation and Quantification of Andrimid and Analogues**

Andrimid was produced from liquid culture of P. agglomerans in 2xYT supplemented with antibiotics and amino acids as required for 28°C for 24 hr. Cells were removed by centrifugation followed by filtration through a 0.45 μm membrane (Millipore, Billerica, MA). Filtered culture supernatant was extracted with ethyl acetate three times with one-half volume ethyl acetate. Ethyl acetate fractions were dried under vacuum and then dissolved in 13C6 isoleucine (Cambridge Isotopes) for 24 hr at 30°C. Stable Isotope Feeding Study Mutants A2 and B12 were grown in M9 medium supplemented with 0.1% yeast extract, 0.1% casamino acids and 50 mM each of 13C6 leucine and/or 13C6 isoleucine (Cambridge Isotopes) for 24 hr at 30°C. The culture supernatants were syringe filtered and analyzed using the LC-MS/MS method outlined above.

**Molecular Genetics**

Please refer to Supplemental Experimental Procedures for detailed methods for genetic manipulation of P. agglomerans.

**Mass Spectrometry**

A 7 Tesla LTQ-FT equipped with a Surveyor autosampler and a MS pump was used for all mass spectrometric analysis (Thermo-Fisher Scientific). Andrimid and its derivatives were analyzed using a Jupiter 4.6 x 150 mm C18 HPLC column (Phenomenex) and a gradient of 0%–75% ACN over 35 min. LC solvents (water and acetonitrile) contained 0.1% formic acid. Initial screening was performed at low (ion-trap) resolution with a selected ion monitoring (SIM) window from m/z 435–535 as the full scan and data-dependent fragmentation of the top 10 peaks from the full scan. Confirmation of derivative production was carried out using the same scans but in high (FT, 100,000) resolution. Mass spectrometric data were analyzed using Qual Browser software (Thermo-Fisher Scientific).

**MIC Determination**

LB agar plates (140 mm diameter) were overlaid with 12 ml molten LB agar inoculated with 225 μl of OD600 = 0.8 indicator strain. The inoculated top agar was allowed to solidify before sterile paper disks were laid onto the assay plates. Ten microliters of antibiotic was spotted onto the disks and plates were incubated 8 hr at 37°C. Zones of inhibition were defined as the radius and measured using calipers. Zones of inhibition were plotted against Log10 of the concentration of spotted antibiotic. MICs were defined as the minimum concentration required to yield a 1 mm zone of inhibition and calculated by nonlinear regression using the software package GraphPad Prism (La Jolla, CA).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.chembiol.2011.03.008.

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