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Cebulantin, a Cryptic Lanthipeptide Antibiotic Uncovered via Bioactivity-Coupled HiTES

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Abstract

The majority of natural product biosynthetic gene clusters in bacteria are silent under standard laboratory growth conditions, making it challenging to uncover any antibiotics that they may encode. Herein we combine bioactivity assays with high-throughput elicitor screening (HiTES) to access cryptic, bioactive metabolites. Application of this strategy in *Saccharopolyspora cebuensis* and growth-inhibition against *E. coli* as a read-out led to the identification of a novel lanthipeptide, cebulantin. Extensive NMR analysis allowed us to elucidate the structure of cebulantin. Subsequent bioactivity assays revealed it to be an antibiotic selective for Gram-negative bacteria, especially against *Vibrio* species. This approach, which we refer to as bioactivity-HiTES, has the potential to uncover cryptic metabolites with desired biological activities that are hidden in microbial genomes.

Graphical Abstract

Cryptic Lantibiotic Unveiled: A new natural product discovery method, bioactivity-HiTES, facilitates the identification of cryptic, bioactive metabolites. Using the approach, a new lanthipeptide antibiotic, cryptic under normal growth conditions, was characterized with selective inhibitory activity against Gram-negative bacteria.

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Keywords

natural products; silent gene cluster; biosynthesis; HiTES; lanthipeptide; cebulantin

Antibiotics have saved countless lives and driven science and innovation in chemistry and biology. After a productive period spanning the late 1930s and the early 1960s during which most of the current antibiotic scaffolds were identified, the pace of discovery has slowed down significantly.^[1,2] Today we know that one of the culprits is a feature that is inherent to microbial genomes: most biosynthetic gene clusters (BGCs) – the sets of genes responsible for the biogenesis of a natural product – are not expressed or, at best, sparingly expressed under standard growth conditions in the laboratory.^[3–5] Consequently, their products are not interrogated in routine bioactivity assays. These so-called silent or cryptic BGCs outnumber constitutively-expressed ones by a factor of 5–10 and represent a treasure trove of new natural products, and possibly antibiotics. To access the products of silent BGCs, several approaches have been developed.^[4–10] A key drawback of nearly all available methods is that they rely on genetic or complex molecular biology manipulations and/or do not report on the bioactivity of cryptic metabolites. These aspects slow down the pace and throughput by which bioactive, cryptic metabolites can be found. Herein, we combine high-throughput elicitor screening (HiTES)^[11] with biological activity assays for the facile, genetics-free discovery of cryptic metabolites with the desired biological properties. Application of this method to an actinomycete strain allowed us to identify a novel lantibiotic that appears to be selective for Gram-negative bacteria.

We previously devised HiTES as a means of activating silent BGCs in genetically tractable bacteria.^[11–13] In HiTES, a reporter gene is introduced into a silent gene cluster of interest to provide a rapid read-out for expression. Subsequently, small molecule libraries are screened against the reporter strain to identify elicitors, that is, molecules that induce silent BGCs. To eliminate the reliance on genetic manipulations and factor in the biological activity of the cryptic metabolite, we utilized the bioactivity-HiTES workflow shown (Fig. 1).^[14] In this approach, a wild-type microorganism is subjected to HiTES in the absence of any genetic alterations. The resulting induced cultures are then screened directly for biological activity. Elicitors that trigger production of the desired bioactivity are examined further to identify the active component. We have applied this idea to several actinomycetes; reported herein are results with *Saccharopolyspora cebuensis*.^[14–16] As a rare actinomycete, genetic manipulations are challenging at best and none have thus far been reported in this organism. Moreover, the genome sequence of *S. cebuensis* has not been published either, another advantage as we imagined that bioactivity-HiTES could be applied broadly to diverse microbes that have not been sequenced. Because of the need for antibiotics against Gram-negative bacteria, we used inhibitory activity against cell wall-weakened *E. coli* (*lptD*), as a read-out.^[17]

We began by asserting that *S. cebuensis* does not synthesize antibacterial compounds under standard laboratory conditions that inhibit *E. coli* growth. Next, wild-type *S. cebuensis* cultures were transferred to 96 well-plates and each well was robotically supplemented with a compound from a commercial small molecule library as a source of elicitors (Fig. S1). The

library consisted of 950 bioactive compounds of diverse structures and origins, which at the concentration used did not exhibit antibiotic activity against *E. coli*. The plates were incubated for a defined period, the supernatants transferred to 96-well plates carrying *E. coli* cultures, and optical density at 600 nm ($OD_{600\text{ nm}}$) was then measured as a proxy for production of cryptic, growth-inhibitory compounds. The $OD_{600\text{ nm}}$ from each well was normalized and the % *E. coli* growth thus obtained was plotted as a function of each elicitor (Fig. 2a). Most wells gave a value close to $100 \pm 30\%$, suggesting minimal effect on *E. coli* growth. Some induced metabolomes enhanced *E. coli* growth, while others inhibited it. For example, metabolomes induced by the antithyroid agent methimazole (**1**, Fig. 2b), the laxative Na-docusate (**2**), and the nootropic piracetam (**3**) stimulated *E. coli* growth best, while those induced by the anesthetic procaine (**4**), the clinical diuretic furosemide (**5**), and the cholesterol-lowering agent fenofibrate (**6**) inhibited it. These results provide opportunities for follow-up studies to investigate the effects of these unsuspected modulators on the physiology of *S. cebuensis*, which in turn leads to significant growth alterations in *E. coli*. For the present study, we focused further efforts on the production of a potential antibiotic induced by elicitors **4–6**.

Due to restrictions, we were not able to procure procaine. The effect of **5** and **6** on the secondary metabolome of *S. cebuensis* was investigated using agar disc diffusion assays and differential metabolic profiling. *S. cebuensis* cultures were grown in the presence of DMSO (control) or **5** and the filtered supernatants were subjected to antibacterial assays or assessed by HPLC-MS. The former showed a halo of growth-inhibition only from supernatants generated with **5**, consistent with the production of a cryptic antibiotic (Fig. 3a). In line with this conclusion, HPLC-MS profiling revealed appearance of several induced metabolites in the cultures treated with **5** or **6** (Fig. S2). Iterative activity-guided fractionation of the induced supernatants identified one peak as the bioactive component with an $[M+H]^+_{\text{obs}}$ of 2107.8798 and a UV spectrum consistent with the presence of a Trp residue (Table S1). Searches in a database of known natural products did not reveal any hits suggesting that this compound, which we name cebulantin, was a novel natural product. HPLC-MS assays of *S. cebuensis* cultures grown in the presence of **5**, **6**, or DMSO control, revealed cebulantin only in the presence of the elicitors, consistent with the screening results and the cryptic nature of the cebulantin BGC (Fig. 3b).

Cebulantin was purified from large-scale production cultures of *S. cebuensis* grown in the presence of **5**, which allowed us to isolate ~3 mg of the antibiotic (from 6 L) and to elucidate its structure using 1D/2D NMR spectroscopy and HR-MS. Analysis of ^1H , TOCSY, and COSY spectra revealed spin systems consistent with canonical amino acids, an unusual 4-hydroxy-Pro, and several dehydrated Thr residues (dehydro-butyrine, Dhb), which were identified using the characteristic olefin ^1H and ^{13}C shifts, suggesting that cebulantin was a new, cryptic lanthipeptide (Fig. 3c, Figs. S3–S4).^[18–21] COSY (NH–CH α) and HMBC (CH α –C=O or CH β –C=O) cross-peaks allowed us to map the sequence of the entire 22mer peptide. HMBC and NOESY data indicated that residues 4–9, 8–14, and 16–22 were connected via β -thioether bonds, thus completing the 2D structure of cebulantin (**7**, Figs. 3d, 3e, Table S2), which was in line with HR-MS/MS data (Fig. S5). The stereochemistry at the methyllanthionines is based on NOESY data analysis (Fig. S6). While consistent with the

(2*S*,3*S*)-configuration in previous lanthipeptides,^[19] additional experiments are needed to confirm it for **7**. Together, our results establish cebulantin as a cryptic lanthipeptide induced by the clinical drugs **5** and **6**.

Bioactivity screens gave half-maximal inhibitory concentrations (IC₅₀s) of 9.7 μM against *E. coli* (*lptD*) and between 8.8–29.1 μM against several *Vibrio* strains (Table 1). On the other hand, **7** exhibited no growth-inhibitory activity toward the Gram-positive bacteria tested, providing an unusual example of an antibiotic that appears to be selective for Gram-negative strains, especially the various pathogens of the *Vibrio* genus.

The structure of **7** should facilitate the identification of its BGC. Using Illumina sequencing technology, we determined a draft sequence of the *S. cebuensis* genome with 100-fold coverage resulting in a single chromosome of 6.7 Mbp. Analysis by antiSMASH gave a total of 23 BGCs (Table S3), almost half of these in the RiPP (ribosomally-synthesized and posttranslationally modified peptide) subfamily, suggesting that, aside from **7**, *S. cebuensis* is a rich source of potentially new RiPPs.^[21,22] By scanning the precursor peptide sequences of lanthipeptide BGCs, we found one gene that gave a perfect match to the C-terminal 22 residues of **7**. We annotate the corresponding locus as *ceb*, a class I lanthipeptide BGC (Fig. 4, Tables S4).^[18–20] It encodes two precursor peptides, *cebA1* and *cebA2*, the canonical LanB-like dehydratase (*cebB*) and LanC-like cyclase (*cebC*), which have been shown to install lanthionine bridges, a luciferase-like monooxygenase (*cebO*), which may be involved in the biosynthesis of 4-OH-Pro, and an ATP-dependent transporter (*cebT*).^[23–25] The *ceb* cluster appears to be under the control of a two-component regulatory system (Fig. 4). Given that *ceb* encodes two precursor peptides, we repeatedly attempted to find a product corresponding to CebA1, without success. Nonetheless, the identification of the *ceb* BGC as well as characterization of its bioactivity can facilitate future engineering efforts to hone the anti-*Vibrio* properties of cebulantin via recently reported methods.^[26–28]

In summary, we have combined a tried-and-true approach in natural product discovery, bioactivity-guided fractionation, with a new method for awakening silent gene clusters, HiTES, to devise a strategy that can deliver cryptic, bioactive molecules encoded in the genomes of bacteria. Previously, HiTES has been used in conjunction with a colorimetric read-out to detect production of pigments,²⁹ with a genetic read-out to more broadly assess expression of silent BGCs,^{11,13} and with MS-, MS/MS-networking-, or imaging-MS-based methods,^{30,31} all of which facilitate an activity-independent discovery approach. In the current study, we have prioritized function and employed antibiotic activity assays as a read-out to search for bioactive, cryptic metabolites. We report the structural and partial functional characterization of cebulantin, a new lanthipeptide with an unusual 4-OH-Pro that appears to selectively inhibit Gram-negative bacterial growth, notably that of diverse *Vibrio* pathogens. In the future, any bioactivity assays that can be conducted in a high-throughput fashion may be used to find cryptic metabolites with the desired biological properties using bioactivity-HiTES.

Experimental Section

Detailed Materials and Methods for bioactivity-HiTES, identification of cebulantin, purification of **7** from large-scale production cultures, structural elucidation of **7**, bioactivity assays, sequencing of the *S. cebuensis* genome, and identification of the *ceb* gene cluster; HR-MS and NMR data for **7**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Workflow for bioactivity-HiTES. A bacterium that does not produce antibiotics under normal growth conditions is subjected to HiTES. The resulting supernatants are used directly in high-throughput bioassays. The elicitor that yields the best antibiotic activity is then employed in large-culture fermentation to identify and characterize a cryptic antibiotic using activity-guided isolation. Any bioassay may be used; we have conducted antibiotic assays in the current study.

Figure 2.

Results of bioactivity-HiTES with *S. cebuensis*. (a) Effect of *S. cebuensis* supernatants – generated by challenging the organism with 950 different small molecules – on the growth of *E. coli* (*lptD*). Elicitors, which gave rise to *S. cebuensis* supernatants that enhanced *E. coli* growth best, are shown in blue. Those that gave rise to *S. cebuensis* metabolomes, which inhibited *E. coli* growth best, are shown in red. (b) Structures of the elicitors identified in panel (a).

Figure 3.

Characterization of cebulantin. (a) Induction of a cryptic antibiotic by furosemide. A halo of growth-inhibition is only observed when *S. cebuensis* is cultured in the presence of **5**. (b) HPLC-MS profiling reveals production of cebulantin only in the presence of **5** and **6**, verifying that its BGC is otherwise silent. (c, d) Relevant NMR data used to solve the structure of cebulantin (**7**). (e) Cartoon depiction of **7** emphasizing the location of (methyl)lanthionine bridges. Pro* denotes 4-OH-Pro.

Figure 4.

The cebulantin (*ceb*) BGC. The *ceb* cluster is under the control of a LuxR-type regulator (*cebR*). Genes are color-coded as shown. The sequences of CebA1 and CebA2 are displayed with the leader and core sequences at the top and bottom, respectively. Methyllanthionine crosslinks installed onto CebA2 are marked by arrows. The modified Pro residue is rendered in orange.

Table 1.IC₅₀ values (in μM) of cebulantin against select bacteria.

Bacterium	IC ₅₀
<u>Gram-Negative</u>	
<i>E. coli</i> (<i>lptD</i>)	9.7 $\pm$ 0.4
<i>V. parahaemolyticus</i>	8.8 $\pm$ 2.1
<i>V. cholerae</i>	14.1 $\pm$ 2.4
<i>V. alginolyticus</i>	24.5 $\pm$ 3.2
<i>V. ordalii</i>	27.0 $\pm$ 5.4
<i>V. anguillarum</i>	29.1 $\pm$ 8.2
<i>P. aeruginosa</i>	47.0 $\pm$ 11.4
<u>Gram-Positive</u>	
<i>S. aureus</i>	>100
<i>B. subtilis</i>	>100