Lapcin, a potent dual topoisomerase I/II inhibitor discovered by soil metagenome guided total chemical synthesis

Zongqiang Wang1,2, Nicholas Forelli1,2, Yozen Hernandez1, Melinda Ternei1 & Sean F. Brady1✉

In natural product discovery programs, the power of synthetic chemistry is often leveraged for the total synthesis and diversification of characterized metabolites. The synthesis of structures that are bioinformatically predicted to arise from uncharacterized biosynthetic gene clusters (BGCs) provides a means for synthetic chemistry to enter this process at an early stage. The recent identification of non-ribosomal peptides (NRPs) containing multiple p-aminobenzoic acids (PABAs) led us to search soil metagenomes for BGCs that polymerize PABA. Here, we use PABA-specific adenylation-domain sequences to guide the cloning of the lap BGC directly from soil. This BGC was predicted to encode a unique N-acylated PABA and thiazole containing structure. Chemical synthesis of this structure gave lapcin, a dual topoisomerase I/II inhibitor with nM to pM IC50s against diverse cancer cell lines. The discovery of lapcin highlights the power of coupling metagenomics, bioinformatics and total chemical synthesis to unlock the biosynthetic potential contained in even complex uncharacterized BGCs.

1Laboratory of Genetically Encoded Small Molecules, The Rockefeller University, New York, NY, USA. 2These authors contributed equally: Zongqiang Wang, Nicholas Forelli. ✉email: sbrady@rockefeller.edu
Biologically active bacterial metabolites have been a principal source of inspiration for the development of diverse small molecule therapeutics. A key role for synthetic chemistry in this discovery process is the total synthesis and synthetic derivatization of natural products that have been physically isolated and structurally characterized from bacterial fermentation broths. The focus on physically characterized structures significantly limits the use of synthetic chemistry in the study of natural chemical diversity as most natural product biosynthetic gene clusters (BGCs) are not expressed (i.e., silent) in the laboratory; and therefore the metabolites they encode remain a mystery. We believe that, in a growing number of instances, the ability to bioinformatically predict the output of a BGC has developed to the extent where the chemical synthesis of a bioinformatically predicted structure (i.e., a synthetic-Bioinformatic Natural Product or syn-BNP) now provides an alternative, purely in vitro method for converting the genetic information encoded in a BGC into a bioactive small molecule. The application of total synthesis methods to bioinformatically predicted small molecules provides an opportunity for synthetic chemistry to enter the natural product discovery pipeline at a much earlier phase. By focusing on the synthesis of previously inaccessible natural structures instead of already discovered natural products, synthetic chemistry could significantly expand its impact on the natural products drug discovery process.

Uncovering unexploited biosynthetic diversity is key to the identification of BGCs whose bioinformatic structure predictions can serve as appealing starting points for the total synthesis of bioactive syn-BNPs. One of the most common mechanisms by which bacteria generate biologically active small molecules is the polymerization of alpha amino acids using non-ribosomal peptide synthetases (NRPSs). The recent discovery of two structurally related antibiotics, albicidin and cystobactamid, that arise from NRPSs that polymerize p-aminobenzoic acid (PABA) monomers suggests that bacteria might produce a previously undiscovered collection of bioactive metabolites using an alternate substrate polymerization strategy than has been seen in most NRPS derived natural products characterized to date. Publicly available sequenced bacterial genomes do not appear to contain PABA polymerizing BGCs beyond those that are predicted to encode albidicin or cystobactamid. As the majority of environmental bacteria are still not readily cultured in the laboratory, we postulated that PABA polymerizing BGCs might be more commonly associated with the uncultured bacteria present in the environment.

Here we track PABA specific adenylation (A) domain sequences in soil metagenomic libraries and find that NRPS BGCs that are predicted to utilize PABA are common in these environments. Detailed bioinformatic analysis of one such BGC, the lap BGC, predicted that it encodes an N-acylated mixed PABA thiazole-based structure. Total chemical synthesis of the bioinformatically predicted lap BGC product gave a syn-BNP that we have called lapcin. Lapcin is a potent dual topoisomerase I/II inhibitor that shows low nM to pM cytotoxicity against diverse cancer cell lines and represents a distinct structural class of topoisomerase inhibitors. The discovery of lapcin represents a compelling, structurally complex, example of the potential power of linking synthetic chemistry and bioinformatics to unlock the biosynthetic instructions hidden in complex silent BGCs. Furthermore, this work shows that coupling metagenome BGC discovery methods with a syn-BNP approach provides a method for circumventing difficulties associated with both culturing bacteria and activating BGCs, two key bottlenecks that have hampered the discovery of bioactive small molecules encoded by many bacterial BGCs.

Results

Discovery of the lapcin BGC. In an effort to expand the biosynthetic diversity we can interrogate for BGCs that might encode interesting bioactive natural products we have created a collection of cosmid clone libraries containing DNA extracted directly from diverse soil samples (environmental DNA, eDNA). In total, this collection contains almost $1 \times 10^{10}$ to $40$ kb fragments of cloned eDNA. To simplify the screening and recovery of clones containing BGCs of interest, soil eDNA libraries were divided into sub-pools of $\approx 25,000$ unique clones each. In addition, to facilitate the search for NRPS BGCs in these libraries, cosmid DNA isolated from each library sub-pool was used as the template in PCR reactions with barcoded A-domain specific degenerate primers. A-domain amplicons were sequenced and the resulting reads were clustered to generate A-domain markers (natural product sequence tags, NPSTs) for NRPS BGCs captured in each library sub-pool (Fig. 1a-i). We used a two-step screening process to identify PABA-specific A-domain NPSTs. Initially, NPSTs were compared to characterized A-domain sequences using the environmental surveyor of natural product diversity (eSNaPD) software package (Fig. 1a-ii). eSNaPD was designed to identify sequences that are more closely related to a target domain sequence than any other sequences in GenBank, suggesting a common evolutionary ancestor and therefore a common biosynthetic product. NPSTs that showed the highest sequence identity to a known PABA-specific A-domain were retained. In a second round of screening, we took advantage of sequence differences seen in PABA-specific A-domains. NPSTs identified by eSNaPD were examined for the presence of three conserved sequences found in known PABA-specific A-domains (Fig. 1a-iii), including the presence of an alanine at position 235 in place of the aspartic acid that normally interacts with the a-amino group of an amino acid. NPSTs that passed both filters were considered PABA-specific NPSTs and were used to generate a phylogenetic tree to guide our discovery of previously uncharacterized PABA-containing BGCs.

NPSTs that were very closely related to PABA-specific A-domains from characterized BGCs formed a clade that has representatives from many of the soils in our cosmid library (Fig. 1a-iv). In addition to this large and common clade, we identified a second smaller, soil metagenome derived clade. We predicted that NPSTs from this clade likely arose from a novel, and potentially rare, family of PABA encoding BGCs. eDNA cosmids associated with a representative NPST in this clade were recovered from the appropriate library sub-pools. Sequencing of the isolated cosmids revealed a BGC (lap) with five NRPS genes (lap b, h, k, l, m) that encode 10 modules, suggesting the production of a decapeptide (Figs. 1b and 2; Supplementary Fig. 1 and Supplementary Table 1). The edge of the lap BGC was defined by the appearance of genes predicted to be involved in primary, instead of secondary, metabolism (Supplementary Table 2).

Bioinformatic prediction. The functional order of the 10 modules in lap BGC can be inferred from an analysis of domains present in each NRPS protein. Module 1 in LapM (NRPS5) contains a condensation starter (Cs) domain that is predicted to initiate peptide biosynthesis with a lipid (Supplementary Fig. 2). Almost all characterized close relatives of this Cs- domain use β-hydroxy C10 to C14 lipids (Supplementary Fig. 3). We propose that a similar lipid would be used to initiate the biosynthesis of the lap BGC product. The presence of the thioesterase (TE) domain at the end of LapH (NRPS2) indicates the lap peptide terminates at this module. The domain content of the terminal modules in the lap NRPS proteins allow us to place LapK
(NRPS3) and LapL (NRPS4) between the initiating mega-
synthetase LapM and the terminal megasynthetase LapH. The
substrate binding pocket in the penultimate A-domain of LapK is
missing key conserved residues, suggesting that it is not active
(Supplementary Fig. 4)\(^8,9,14\). As is seen in other BGCs with
inactive A-domains, including other PABA-encoding BGCs, the
isolated A-domain in LapB (NRPS1) was predicted to function in
trans with LapK\(^8,14\).

The substrate specificity of each NRPS module was predicted
based on the 10 amino acids that makeup an A-domain substrate
binding pocket\(^13\). Each lap A-domain code has an identical, or
nearly identical, match among A-domains from characterized
BGCs, thus allowing for a high confidence substrate prediction to
be made for each A-domain (Fig. 2a). The only disagreements
were at position 299 which is known to be the most variable
position in the A-domain substrate binding pocket\(^13\). Four
A-domains (AD4, AD8, AD9, AD10) were predicted to use
PABA-like substrates. ADs 4, 8 and 10 were predicted to be
specific for PABA, while AD9 was predicted to use the modi-
fied PABA substrate, 4-amino-2-hydroxy-3-isopropoxybenzoic acid
(AHIBA). The use of AHIBA by at least one A-domain is
supported by the presence of lap D, F, G, and C, which were
predicted to encode for the hydroxylation, methylation and
isomethylation of PABA (Fig. 2b). Two modules were predicted

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**Fig. 1 Discovery of the lap gene cluster.** a Overview of the PABA-specific A-domain guided discovery of the lapcin (lap) BGC from the soil metagenome. i DNA extracted from soil was used to construct metagenomic libraries. ii NPSTs generated from arrayed metagenomic libraries and iii the resulting NPSTs were searched for PABA-specific A-domains based on a signature sequence derived from known PABA A-domains. iv Phylogenetic analysis of predicted PABA NPSTs was used to identify sequences that arose from a new family of BGCs. v Clones containing NRPS BGCs of interest were recovered from the arrayed library subpools and fully sequenced to reveal BGCs that encode PABA-based natural products. b The lap BGC, which is shown here, was recovered from an archived soil metagenome library using this process. olive, resistance gene; red, NRPS biosynthesis; green, PABA tailoring; blue, thiazole formation; pink, PABA core.
to encode cysteine-specific A-domains (AD5 and AD6). Both of these modules contain heterocyclization condensation (Cy) domains, suggesting the formation of two thiazoline rings. Furthermore, the presence of a predicted FMN-dependent dehydrogenase (LapI), suggested that ultimately the two cysteines are converted to two thiazole rings. The remaining 4 modules were predicted to introduce 4 additional proteinogenic amino acids: Ala (AD1), Ser (AD2), Ala (AD3), Asn (AD7). In the case of cystobactamid and albicidin, LapB homologs that install Asn-7, together with other tailoring enzymes that are not encoded by the lapBGC, are thought to be responsible for generating a number of different L-asparagine modifications. As the naturally produced collections of cystobactamid and albicidin both include simple L-asparagine containing congeners that show potent activity, we included L-asparagine in our structure prediction of lapcin. 

Taken together, this analysis allowed us to predict the product of the lapBGC as an N-acylated decapeptide containing two thiazoles, four PABAs and four proteinogenic amino acids. We have called this structure lapcin. While the right-hand tri-PABA substructure is similar to that seen in the antibiotics albicidin and cystobactamid, the majority of the structure is completely distinct from previously characterized natural products (Supplementary Table 3). In fact, no N-acylated or thiazole containing NPRS-derived PABA-based natural products have been identified in traditional natural product screening programs.

As the lapBGC was cloned directly from the complex mixture of bacteria present in a soil metagenome, the exact organism from which it was cloned is not known. A BLAST search indicated that the closest relative of each individual gene found in the lap gene cluster most often arose from the genome of a myxobacterium (Supplementary Table 1). Cultured Myxobacteria are often rich in secondary metabolite BGCs. Unfortunately, most members of this group of bacteria are believed to remain uncultured. Direct cloning of DNA from environmental samples as we have done here circumvents this culture bottleneck; however, it introduces the challenge of accessing metabolites encoded by captured BGCs. As our understanding of natural product biosynthesis has matured, it has become possible to make increasingly accurate predictions about the structure encoded by a BGC. Our analysis of the lapBGC suggested that lapcin was likely an accurate representation of the intended product of this BGC and that total chemical synthesis was therefore a viable method for accessing the metabolite, or at least a close analog of the metabolite encoded by the lapBGC. To be successful, a syn-BNP does not need to be a perfect copy of a natural product, only an analog that is close enough to mimic its natural biological activity.

Total chemical synthesis. Our retrosynthetic analysis of lapcin suggested two amide bond disconnections to give 3 fragments (A-C) that could be readily synthesized and coupled (Fig. 3). Firstly, the preparation of fragment A began with the synthesis of the tripeptide portion on 2-chlorotrityl chloride (CTC) resin using standard Fmoc-based solid-phase peptide synthesis (SPPS) methods (Fig. 3, Supplementary Fig. 5). With the tripeptide complete, DL-3-hydroxy myristic acid was appended to its N-terminus. NRPS-derived lipopeptides are often found naturally as mixtures with different fatty acids. As there is unlikely to be one correct answer to the exact lipid found naturally on lapcin, it was synthesized using a racemic version of one of the most frequently seen lipids in NRPS-derived lipopeptides, DL-3-hydroxy...
myristic acid. The resulting fatty-acyl tripeptide was released from the CTC resin using 20% hexafluoroisopropanol (HFIP) to give a protected product ready for amide coupling (1). To obtain the two thiazole rings in fragment B (Fig. 3, Supplementary Fig. 6), 4-(N-Boc-amino)phenylboronic acid (2) was initially linked to ethyl 2-bromothiazole-4-carboxylate (2-1) using a Suzuki-Miyaura coupling. After conversion to the corresponding amide (4) and thionation by Lawesson’s reagent, a second thiazole was installed through a Hantzsch thiazole synthesis (7). Finally, the synthesis of fragment C (Fig. 3, Supplementary Fig. 7) was started with synthesis of the alloc-protected PABA subunits from 2-hydroxy-3-isopropoxy-4-nitrobenzaldehyde (9). The exposed ortho hydroxyl was alloc-protected prior to oxidation of the aldehyde to a carboxylic acid of compound 11. A second alloc-protected PABA subunit (11-2) was coupled to this carboxylic acid using phosphoryl chloride (POCl₃) and N,N-diisopropylethylamine (DIPEA). Tin(II) chloride (SnCl₂) was used to reduce the nitro group to the free amine of compound 13, which was then coupled to 4-nitrobenzoic acid. Subsequent nitro-reduction to compound 15 followed by solution phase coupling (T₃P, Py) of Boc-Asn(Trt)-OH and deprotection yielded the complete fragment C (16). Fragments A and B were connected by activating the free carboxylic acid on A with isobutyl chloroformate. After hydrolysis to the carboxylic acid, the resulting AB fragment was coupled to fragment C using HBTU-mediated amide bond formation to give the desired final protected product (S19, Supplementary Fig. 8). After deprotection, lapcin was purified by high performance liquid chromatography (HPLC) and its structure confirmed by 1 and 2D NMR spectroscopy as well as HRMS (Supplementary Fig. 9–30).

**Heterologous expression.** In addition to synthesizing lapcin, we also tried to access the metabolite encoded by the lap gene cluster using a biological system (Supplementary Fig. 31). The two overlapping eDNA cosmids containing the lap BGC were assembled into a contiguous fragment of DNA using transformation association recombination (TAR) in yeast. The TAR assembly reaction was carried out using pTARa4, a broad host range yeast artificial chromosome (YAC): bacterial artificial chromosome (BAC) shuttle vector that is capable of being introduced into a wide range of bacterial taxa. For heterologous expression purposes, the lap BGC-containing BAC, pTARa4-lap, was either electroporated or conjugated into Myxococcus xanthus DK1622, Streptomyces albus J1074, Streptomyces coelicolor M1152, and Pseudomonas putida KT2440. Culture broth extracts from strains transformed with either pTARa4-lap or the empty pTARa4 shuttle vector were compared by high resolution liquid chromatography mass spectrometry (HR-LCMS) to look for lap BGC-specific metabolites. Unfortunately, none of these strains, even when grown under multiple culture conditions, produced any detectable lap BGC-specific metabolites. This was not particularly surprising in light of the fact that most natural product BGCs are silent in the laboratory and that is the key reason for exploring a syn-BNP approach for accessing bioactive small molecules from the genetic instructions contained in bacterial BGCs.

**Biological activity and model of action.** As an initial step to assess lapcin’s bioactivity, we assayed lapcin for toxicity against diverse microbial pathogens and human cancer cell lines. At the
Table 1 Lapcin activity against human cell lines.

| Cancer type | Lapcin (nM) | Etoposide | Camptothecin |
|-------------|-------------|-----------|--------------|
| Colon cancer|             |           |              |
| HT29        | 0.516       | 9,610     | 22.3         |
| Colo205     | 0.504       | >54,400   | 95.7         |
| HCT116      | 923         | >54,400   | 129          |
| SW480       | 37.2        | 26,900    | 284          |
| Breast cancer|            |           |              |
| MCF 7       | 35.1        | 333       | >29,200      |
| HCC1806     | 212         | 3,330     | 19.6         |
| Lung cancer |             |           |              |
| A549        | 2.66        | 79.9      | 17.6         |
| NCI-H1299   | 0.0168      | 716       | 15.0         |
| NCI-H226    | 241         | 217       | 28.5         |
| Other       |             |           |              |
| Hela (Cervical) | 0.986   | 1,510     | 62.6         |
| U2OS (Bone) | 1.29        | 1,840     | 172          |
| Normal cell |             |           |              |
| HEK293      | 48.4        | 729       | 19.2         |

Note: IC50s were rounded to three significant figures. n = 3 biologically independent cells.

Discussion

Sequencing of A-domain PCR amplicons from soil metagenomic libraries identified a number of sequences that we predicted arose from BGCs that use PABA monomers instead of alpha amino acids. The cloning and sequencing of one such BGC revealed the lap BGC, which we bioinformatically predicted would encode a N-acylated thiazole and PABA containing decapetide, lapcin. To circumvent the challenge of decoding the lap BGC using biological processes, we produced lapcin by total chemical synthesis. Lapcin is a dual topoisomerase I/II inhibitor that inhibits the growth of diverse cancer cells. Topoisomerase inhibitors are clinically validated targets for anticancer therapy. While there are currently no topoisomerase inhibitors in clinical use, topoisomerase poisons are used in the treatment of a number of cancers including, breast, lung, testicular, and prostate, with a number of additional candidates in clinical trials. Lapcin is structurally distinct from any previously identified topoisomerase inhibitor (including poisons and catalytic inhibitors), providing a different structural class to investigate as antineoplastic agents. Between the non-cancerous HEK293 cell line control and most susceptible cell lines we tested, there is over 3 orders of magnitude difference in IC50 (Table 1), suggesting that lapcin has a sufficient therapeutic window to explore its utility as an antineoplastic agent in vivo. To the best of our knowledge, lapcin is the first NPRS derived N-acylated or thiazole containing PABA-based natural product.

The number of uncharacterized sequenced BGCs, whether derived from cultured bacteria or metagenomes, is rapidly increasing. Unfortunately, the rate at which the instructions contained in these BGCs are converted into chemical entities...
remains very low. Our discovery of lapcin confirms that a syn-BNP approach represents a viable alternative strategy for generating even complex classes of biomedically relevant molecules from uncharacterized BGCs. Traditional natural product total synthesis efforts have almost exclusively focused on characterized bioactive natural products. Syn-BNP methods provide an alternative paradigm where targets for total synthesis are structures that are bioinformatically predicted from silent BGCs. Lapcin provides a key proof of principle example of a syn-BNP approach being able to generate a previously unknown small molecule whose potency rivals that of natural products produced biologically.

Methods

Identification of the lap biosynthetic gene cluster in the soil metagenome.

Archived eDNA cosmid libraries were used to screen for BGCs that encode PABA-containing natural products. Procedures for library construction and A-domain screening to facilitate BGC discovery have been described in detail previously. Briefly, crude environmental DNA (eDNA) was obtained from ~0.5 kg of soil by heating (70 °C) in lysis buffer (100 mM Tris-HCl, 100 mM ethylenediaminetetraacetic acid, 1.5 M NaCl, 1% (w/v) hexadecyltrimethylammonium bromide, 2% (w/v) sodium dodecyl sulfate, pH 8.0) for 2 h. Soil particulates were removed from the crude lysate by centrifugation, and eDNA was precipitated from the resulting supernatant with the addition of 0.7 volumes isopropanol. Crude eDNA was collected by centrifugation, washed with 70% ethanol and resuspended in TE buffer. Crude eDNA was purified by preparative agarose gel electrophoresis to yield pure high-molecular-weight (HMW) eDNA. HMW eDNA was blunt ended (Epicentre, End-It), ligated into pWEB-TNC, packaged into lambda phage and transfected into Escherichia coli EC100. Following recovery, transfected cells were selected using chloramphenicol (12.5 μg/mL). The resulting clones were arrayed at a density of ~25,000 clones per pool. Matching glycerol stocks and cosmid DNA minipreps were prepared from each pool. Pool-specific barcoded A-domain degenerate primers (AD-FW: 5′-GCSTACYSATSTACACSTCGG-3′ and AD-RV: 5′-SASGTCVCCSGTSCGGTA-3′) were used to amplify A-domain sequences from each library sub-pool. PCR reactions: 12 μl
sequences were aligned by MUSCLE algorithm using Macvector 18.0.242. The compounds were considered to be associated with potential PABA-specific A-domain (N7) AARGAR, Y

controls (Graphpad Prism 9.0).

compound required for 50% inhibition of cell growth relative to the no compound

dilution PCR method38. These clones were sequenced using Illumina MiSeq technology. A single continuous eDNA contig containing the lap BGC was assembled from this data using Newbler 2.6 (Roche).

In silico analysis of lap BGC. The lap BGC was annotated using a pipeline consisting of open reading frame (ORF) prediction and BLAST searches. To predict the amino acid specificity of each A-domain sequence in the lap BGC, the sequence was analyzed using the online version of antimash v5.1.2 (bacterial)39. The 10 amino acids (positions 235, 236, 239, 278, 299, 301, 322, 330, 331, 517) making up each A-D domain substrate binding site were compared to the corresponding amino acids from A-domains found in characterized natural product BGCs to predict the substrate of each A-domain35. This information combined with the predicted functions of the tailoring enzymes found in the lap BGC was used to determine the final structure of lapcin.

Cell viability assay. An MTT (2-(4,5-dimethylthiazolyl)-2-5-diphenyl-tetrazolium bromide) assay was used to determine the cytotoxicity of lapcin towards diverse cancer cell lines45. Lapcin was dissolved in DMSO to make a 3.2 mg/mL working solution. For each cancer line detailed in Supplementary Table 5, cells at 80-90% confluency were counted and seeded in 96-well, flat bottom microplates and incubated at 37 °C with a 5% CO2 atmosphere. Outer wells were unused to avoid edge effects. After adhering for 24 h, the medium was steriley aspirated and replaced with 100 μL of fresh medium containing lapcin or a control compound serially diluted at concentrations ranging from 8,000 to 0.00382 ng/mL. After 48 h (37 °C, 5% CO2), the medium was carefully removed and 110 μL of freshly prepared MTT solution (5 mg/mL MTT in PBS (pH 7.4) premixed with 100 μL of complete medium) was added to each well. After 3 h at 37 °C with 5% CO2, 100 μL of solubilization solution (40% DMSO, 16% SDS and 2% acetic acid in H2O) was added to each well and precipitated formazan crystals were allowed to dissolve for 4 h. The absorbance of each well was then measured at 570 nm using a Tecan microplate reader. IC50 values were calculated as the concentration of each compound required for 50% inhibition of cell growth relative to the no compound controls (Graphpad Prism 9.0).

Data availability

All the characterization data and experimental protocols are provided in the article and its supplementary information. The lap BGC has been annotated and deposited in the NCBI database under deposition number MZ165589.

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**Author contributions**

S.F.B., Z.W., and N.F. designed the experiments. Z.W. conducted the metagenomic studies, bioinformatic prediction of lapcin and biological assays. N.F. and Z.W. conducted the synthetic experiments. Y.H. conducted the bioinformatic analysis. M.T. performed the Miseq sequencing. S.F.B., Z.W., and N.F. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Correspondence and requests for materials** should be addressed to Sean F. Brady.

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