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Articles

Multi-Institution Research and Education Collaboration Identifies New Antimicrobial Compounds

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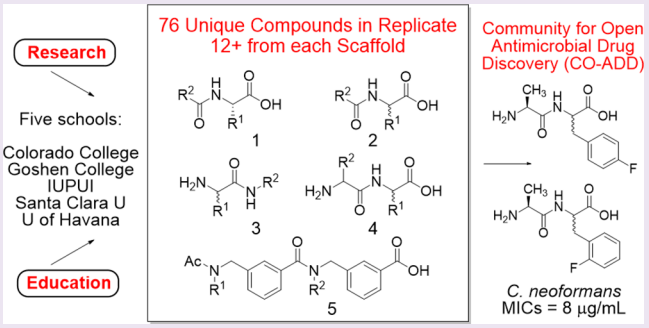
ABSTRACT: New antibiotics are urgently needed to address increasing rates of multidrug resistant infections. Seventy-six diversely functionalized compounds, comprising five structural scaffolds, were synthesized and tested for their ability to inhibit microbial growth. Twenty-six compounds showed activity in the primary phenotypic screen at the Community for Open Antimicrobial Drug Discovery (CO-ADD). Follow-up testing of active molecules confirmed that two unnatural dipeptides inhibit the growth of *Cryptococcus neoformans* with a minimum inhibitory concentration (MIC) ≤ 8 μg/mL. Syntheses were carried out by undergraduate students at five schools implementing Distributed Drug Discovery (D3) programs. This report showcases that a collaborative research and educational process is a powerful approach to discover new molecules inhibiting microbial growth. Educational gains for students engaged in this project are highlighted in parallel to the research advances. Aspects of D3 that contribute to its success, including an emphasis on reproducibility of procedures, are discussed to underscore the power of this approach to solve important research problems and to inform other coupled chemical biology research and teaching endeavors.



The increasing rate of infections by multidrug resistant (MDR) microbes constitutes a serious threat to global public health and economic output.[1] MDR infections are a consequence of overuse and misuse of existing antibiotics over the past several decades coupled with the ability of bacteria and fungi to rapidly develop varied resistance mechanisms.[2−4] There is a critical need to prioritize the identification and development of new therapeutic agents that can arrest and/or prevent microbial growth, including compounds that employ novel molecular mechanisms of action.

Phenotypic screening is an efficient approach to identify new compounds that inhibit bacterial and fungal growth without bias toward a specific molecular mechanism of action.[5,6] Addressing the need for a reliable phenotypic screening resource, the Community for Open Antimicrobial Drug Discovery (CO-ADD) was launched in 2015 and has since tested over 300,000 compounds.[7−9] It affords free access to assess the antimicrobial activity of submitted compounds (only 1 mg sample required) by testing their ability to inhibit growth of "ESKAPE" pathogens: *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA), and the yeasts *Cryptococcus neoformans* and *Candida albicans*. To leverage this powerful resource to discover antimicrobial molecules, an important synthetic need was identified: preparation of a large set of compounds wherein varied structures and functionality were prioritized.

Large numbers of molecules with diverse functionality are readily and reproducibly available through the Distributed Drug Discovery (D3) program implemented at a network of global schools.[10−15] Briefly, D3 enlists undergraduate students enrolled in organic chemistry laboratory courses to prepare combinatorial compound arrays for biological evaluation. To date, D3 has successfully synthesized analogs of antimelanoma compounds, for example.[13] Indeed, a growing body of literature reports significant research outcomes from curriculum-based undergraduate research experiences (CUREs).[16−23] In addition to providing a powerful method to achieve research goals, CUREs, including D3, benefit students' education. CUREs deepen students' understanding of scientific concepts,

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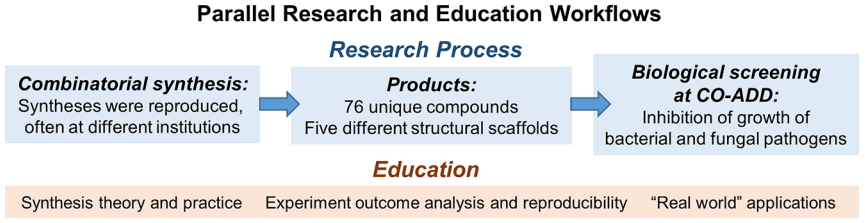

Figure 1. Schematic illustration of the parallel research process (blue) and education (tan) work flows in the D3/CO-ADD collaboration.

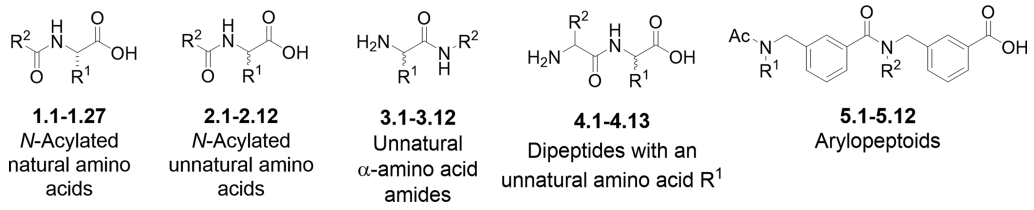

Figure 2. Structures of the five molecular scaffolds prepared and tested.

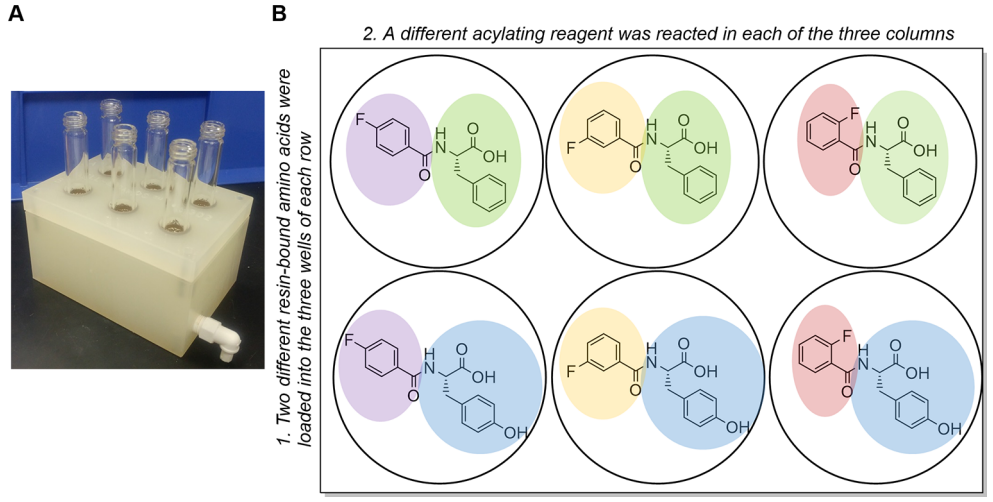

Figure 3. Bill-Board apparatus and an example combinatorial array. A) Picture of the Bill-Board equipment for parallel solid phase synthesis; B) Example combinatorial array of scaffold **1** products. Phenylalanine (top row, green) or *t*-butyl-protected tyrosine (bottom row, blue) was *N*-acylated with 4-, 3-, or 2-fluorobenzoic acid (left/violet, middle/gold, right/red columns, respectively).

enhance their confidence for future experiences, and equip them to think independently.[24,25] To meet the important challenge of identifying new antimicrobial compounds, a D3/CO-ADD collaboration integrates the critical need for new antibiotics with the educational priorities of undergraduate organic chemistry students (Figure 1).

This article details the successful identification of antimicrobial compounds through a multi-institutional collaboration involving D3 combinatorial library synthesis and CO-ADD testing. Collaborators at five international institutions—IUPUI, Santa Clara University, Colorado College, Goshen College, and the University of Havana—synthesized 76 functionally varied bio- and peptidomimetic molecules based on five structurally unique scaffolds **1**–**5** (Figure 2). Of the 76 unique structures tested in a primary screen at CO-ADD, 26 compounds met a threshold level of antimicrobial growth inhibition. Follow-up testing confirmed the ability of two unnatural dipeptides **4** to inhibit the growth of *Cryptococcus neoformans* with a minimum inhibitory concentration (MIC) ≤ 8 μg/mL. Further, the successful identification of potent antimicrobial compounds showcases the value of the D3 approach to discover new bioactive compounds while simultaneously positively impacting undergraduate educational experiences.

RESULTS AND DISCUSSION

Scaffold and Experimental Design Rationale. Access to diverse, biomimetic molecules was prioritized in library scaffold design. Each scaffold can be differently functionalized at two or more sites, thereby providing access to a wide variety of structures for screening. Recognizing that CO-ADD offered a phenotypic screen—a successful tool in the discovery of many new medicines—a specific molecular mechanism of antimicrobial action was not targeted.[5,6] Nonetheless, abundant examples of bioactive molecules that contain each scaffold exist, and expectations of antimicrobial activity were reasonable. Marketed pharmaceuticals and bioactive analogs that include these scaffolds have been comprehensively surveyed,[26] and a few examples were highlighted.

The following drugs all contain *N*-acylated amino acid motifs found in scaffolds **1** and **2**: Alvimopan (GI surgery recovery); L-DOPA (Parkinsonism); Folic Acid (Vitamin B9); Lacosamide (epilepsy); Levothyroxine (hypothyroidism); Liothyronine (hypothyroidism); Lymecycline (acne, some

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bacterial infections); Melphalan (multiple myeloma); Methotrexate (cancers); Mimosine (cancer); Pemetrexed (nonsmall cell lung cancer); Pralatrexate (peripheral T-cell lymphoma); Raltiterxed (malignant neoplasm).

Like scaffold **3**, these drugs all contained α-amino acid amides: Atazanavir (HIV); Clindamycin (anaerobic bacteria); Cobicistat (HIV); Valium (anxiety disorders); Lenalidomide (multiple myeloma); Lidocaine (anesthesia); Lisdexamfetamine (ADHD); Lopinavir (HIV); Pomalidomide (multiple myeloma); Ritonavir (HIV); Saxagliptin (type 2 diabetes mellitus); Thalidomide (erythema nodosum leprosum).

For Scaffold **4** (dipeptides), bioactive examples included Bortezomib (multiple myeloma); Ceftaroline (bacterial infections); Enalapril and Lisinopril (hypertension); Penicillin V (bacterial infections); Cialis (erectile dysfunction); Ximelagatran (deep vein thrombosis). Additionally, there are antimicrobial unnatural dipeptides that deliver their toxic payload through the microbe's specific dipeptide permease.[27]

A bioactive arylopeptoid[28−30] (Scaffold **5** analog) was reported.[31] Further, the *N*-substituents in the oligo-amide backbone of arylopeptoid scaffold **5** are analogous to those found in peptoids; many examples of anti-infective peptoids have been reported.[32]

D3 methods were identified as an efficient approach to prepare an initial screening library of widely varied analogs within each scaffold. The D3 strategy involved students enrolled in independent or course-based research experiences executing straightforward, highly reproducible combinatorial procedures on solid support. In the hands of novice student researchers, reactions on solid support are highly successful owing to the introduction of reagents in large excess, simple workups, and the absence of purification steps for synthetic intermediates. Meeting the need for large numbers of compounds, individual students or student teams synthesized arrays of six compounds using the "Bill-Board" apparatus (Figure 3A). The Bill-Board is a commercially available, affordable, and compact array of six solid-phase reaction vessels.[10] Figure 3B shows an example combinatorial array that was prepared by a student group using this equipment.

The participation of several institutions and scientists was leveraged to enhance the number and diversity of examples prepared as well as the educational experiences of the students involved in these projects. Because D3 engaged many researchers at a variety of institutions, collaborators selected the scaffold chemistry most compatible with their school's resources, expertise, and skill level. Simultaneously, students were highly motivated by the opportunity to contribute to finding solutions to an important societal problem while learning essential organic chemistry concepts and experimental techniques.

Reproducibility, a fundamental science requirement, was prioritized in the synthesis and evaluation of the initial screening libraries. Despite its recognized importance, how and when reproducibility is demonstrated is the subject of much discussion and controversy.[33−36] We demonstrated in this collaboration that meaningful replication goes well beyond a single researcher and his or her own experimental results. Except for four structures (**1.5**, **1.27**, **4.12**, and **4.13**), each of the compounds was synthesized in replicate in one or more of the varied environments shown in Table 1, always by two or more scientists, often at different levels of expertise, and sometimes at different global locations. An emphasis on meaningful replication in these experiments had tandem impacts: it strengthened synthetic and biological results and offered a robust training opportunity for students just learning the research process.

Table 1. Varied Replication Location and Environments for Scaffold Compounds

scaffold	replication location(s)	laboratory environment(s)
Scaffold 1	multisite replication (Colorado College, University of Havana, Goshen College, IUPUI)	undergraduates engaged in independent faculty-led research; large undergraduate laboratory course; large scale workshop
Scaffold 2	internal replication (IUPUI)	large scale undergraduate laboratory course
Scaffold 3	internal replication (IUPUI)	large scale undergraduate laboratory course
Scaffold 4	internal replication (IUPUI)	undergraduates in independent faculty-led research group
Scaffold 5	bilateral replication (Santa Clara University, IUPUI)	undergraduates in independent faculty-led research group

The inclusion of a "control" reaction in the six-reaction array represented a second way in which reproducibility and experimental design were emphasized. Successful synthesis of the control demonstrated students' mastery of the synthetic techniques applied to the preparation of the five new compounds. Notably, the fate of the new syntheses was not predetermined, an aspect of authentic research. When controls gave the expected result but a new synthesis did not, the unexpected observation, if replicated, became an incentive to propose alternative explanations rather than dismiss it as due to "failure of technique". Although we do not report any examples that follow this path in this work, this experimental design feature is important to teach students that properly conducted "unsuccessful" experiments has previously led to hypotheses, further research, and discoveries.[37]

Library Synthesis. *Scaffold 1.* Examples of the simplest scaffold, the *N*-acylated natural amino acids (Scheme 1, **1.1**−**1.27**), comprised diversity from the commercially available combinatorial reagents, Fmoc-amino acid-functionalized Wang resins and carboxylic acids. The 27 analogs were prepared in three synthetic steps: (1) removing the Fmoc protecting group from the amino acid-functionalized resin, (2) coupling to a carboxylic acid, and (3) liberating the product from the resin with concomitant removal of amino acid side-chain protection (Scheme 1). To access structures **1.1**−**1.27**, either phenylalanine, tyrosine, or isoleucine was acylated with benzoic acid or a substituted benzoic acid derivative. Because of the brevity of the procedure and the availability of inexpensive combinatorial reagents, a large number of student researchers prepared variants of **1** (Table 1).[15]

Scaffolds 2−4 via Intermediate A. Three scaffolds—*N*-acylated unnatural amino acids **2** (**2.1**−**2.12**), unnatural α-amino acid amides **3** (**3.1**−**3.12**), and unnatural dipeptides **4** (**4.1**−**4.13**) that contain an unnatural amino acid—were prepared from a common, racemic, Wang resin-bound unnatural amino acid intermediate, **A** (Scheme 2). Consequently, all the final products **2**-**4** were either racemic or a mixture of diastereomers. Screening of limited stereochemical mixtures was considered an advantage in preliminary identification of bioactive molecules.

To access intermediate **A**, a resin bearing the benzophenone imine of glycine was alkylated.[38−40] This step introduced considerable structural diversity from a rich pool of alkyl or benzyl halides, and stereoisomeric mixtures were formed.

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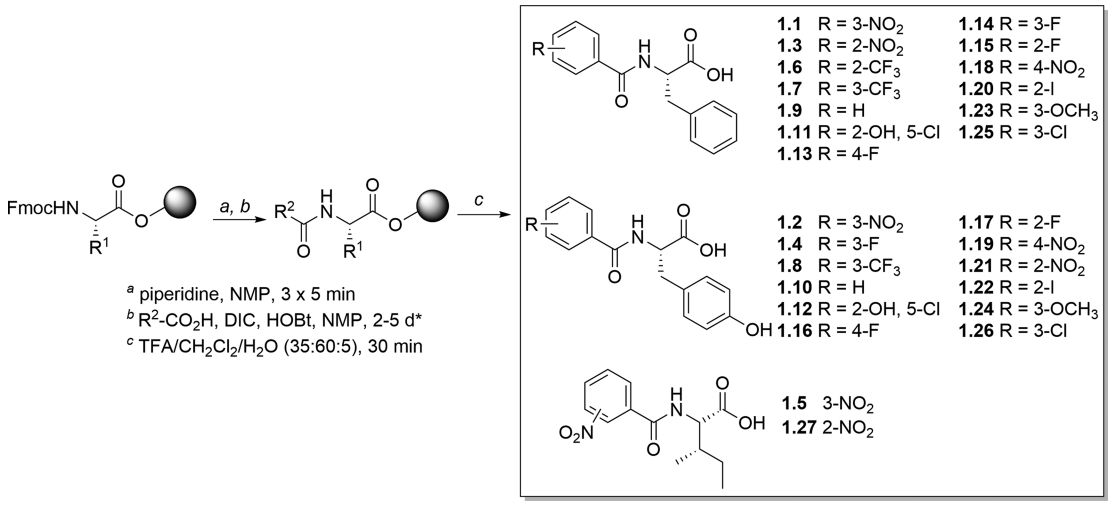
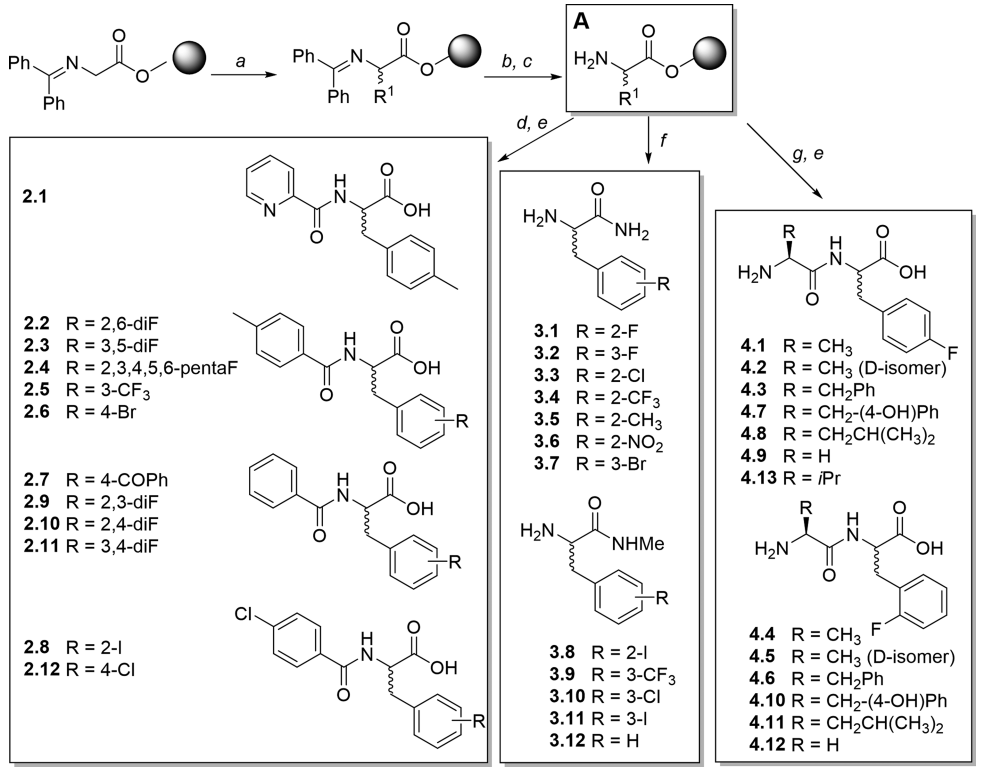
Scheme 1. Synthesis of 1.1−1.27[a]



[a]Reaction times were varied to match instructional laboratory schedules, as detailed in the methods. NMP: *N*-methyl-2-pyrrolidone, DIC: *N*,*N*′-diisopropylcarbodiimide, HOBt: hydroxybenzotriazole, TFA: trifluoroacetic acid.

Scheme 2. Synthesis of 2.1−2.12, 3.1−3.12, and 4.1−4.13 via Intermediate A[a]



[a]Reaction times were varied to match instructional laboratory schedules, as detailed in the methods. THF: tetrahydrofuran, MeOH: methanol, NMP: *N*-methyl-2-pyrrolidone, DIC: *N*,*N*′-diisopropylcarbodiimide, DIEA: *N*,*N*′-diisopropylethylamine, HOBt: hydroxybenzotriazole, TFA: trifluoroacetic acid.

Acidic hydrolysis of the imine provided the key resin-bound, racemic, unnatural amino acid intermediate **A**.[38,39]

Scaffold 2. *N*-acylated compounds **2.1−2.12** were prepared by DIC/HOBt-mediated reaction of 12 substituted phenylalanine analogs of **A** with one of four substituted benzoic or heteroaromatic carboxylic acids, followed by acidic cleavage from the resin.

Scaffold 3. Twelve substituted phenylalanine amide analogs (**3.1−3.12**) were prepared directly from aminolytic cleavage of the resin link in **A** with ammonia or methylamine.

Scaffold 4. Unnatural dipeptides **4.1−4.13** were accessed by acylating two substituted fluorophenylalanine analogs **A** with seven different BOC-protected amino acids. This was followed by acidic cleavage from the resin with concomitant *N*- and side chain functionality deprotection.

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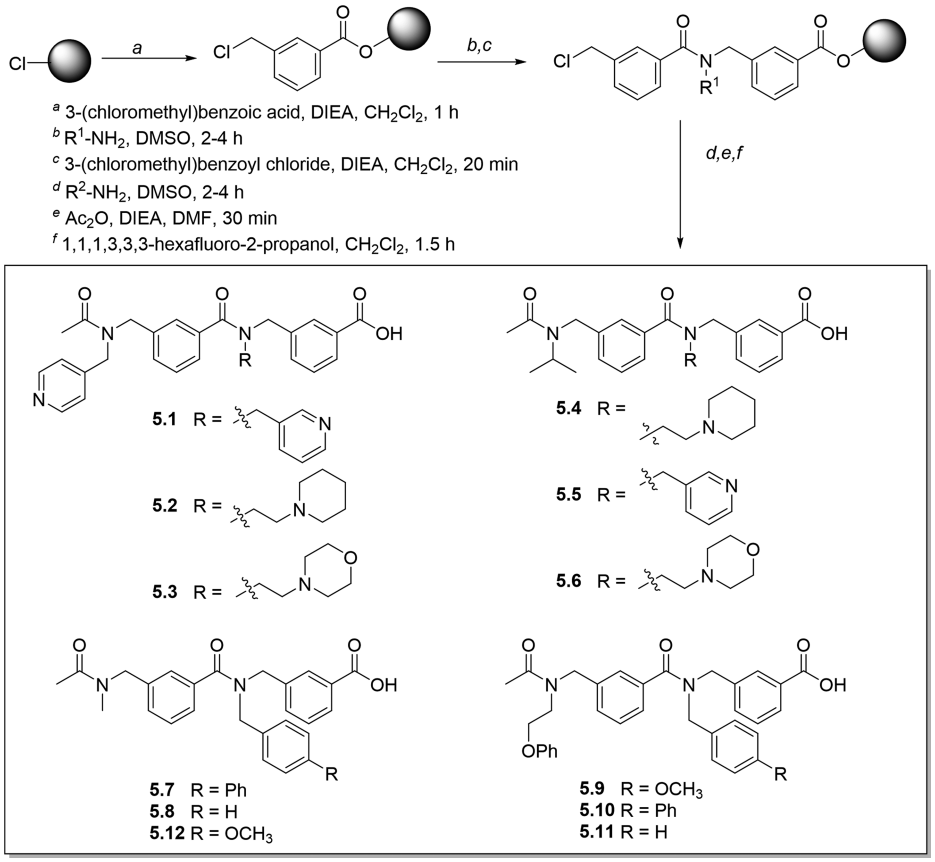
Scheme 3. Synthesis of 5.1−5.12[a]



[a]DIEA: *N,N′*-diisopropylethylamine, DMSO: dimethylsulfoxide, DMF: *N,N*-dimethylformamide.

Scaffold 5. To prepare the final scaffold, the arylopeptoids (Scheme 3, **5.1−5.12**),[28−30] a chlorotrityl chloride resin was functionalized with 3-(chloromethyl)benzoic acid, and the primary chloride was subsequently displaced by one of six primary amines. The resultant secondary amine was acylated with 3-(chloromethyl)benzoyl chloride. Again the primary chloride was displaced by one of four primary amines, and the *N*-terminus was capped with acetic anhydride before cleaving the molecule from the resin under acidic conditions.[30] Arylopeptoids **5.1−5.12** derived structural diversity from the alkyl, heterocyclic, and aromatic primary amines incorporated.

Synthesis summary. For each scaffold, at least 12 representative compounds were prepared in duplicate or quadruplicate (with the exception of **1.5**, **1.27**, **4.12** and **4.13**, which were prepared only once). These replications were always by separate researchers. Crude purities (typically >85%, Supplementary Table S2) and identities of products were assessed by liquid chromatography−mass spectrometry (LC-MS). These crude products were suitable for biological evaluation without further purification. Select compounds were purified by chromatography, most commonly because the curriculum plan included a purification step.

The easy synthetic access to a diverse set of analogs, showcased by the 76 compounds prepared here, enhances the value of these scaffolds as a continuing source of potential drug candidates. The procedures for their preparation are rigorously validated and accessible to chemists working in varied laboratory conditions,[12] enabling ready design and synthesis of new analogs. Future synthetic targets will explore key structure−activity relationships for molecules that exhibited biological activity.

Biological evaluation. All compounds synthesized were submitted to CO-ADD for evaluation of their ability to inhibit the growth of five bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), and two yeasts (*Candida albicans* and *Cryptococcus neoformans*). The complete set of results is in Supplementary Table S2. Impressively, 26 of the 76 unique molecules inhibited growth of one or more microbes in the primary screen at 32 μg/mL. Structures of all molecules with biological activity are shown in Figure 4. Seventeen unique molecules were identified as being "partially active" (i.e., they inhibited 50−80% of growth of one or more pathogens in the assay, Figure 4A). The partially active molecules comprised examples from all five scaffolds, suggesting that any of these is a promising template for further synthesis and evaluation. Five compounds showed partial activity replicated across duplicate lots (**2.8**, **3.2**, **4.8**, **4.11**, and **5.4**). Nine unique structures were identified as "active" (i.e., they inhibited >80% of pathogen growth, Figure 4B), and the activity of five of these (**3.10**, **4.1**, **4.4**, **4.7**, **4.9**) was replicated in both sets of duplicate compounds. The active molecules include three structural scaffolds: *N*-acylated natural amino acids (**1.6** and **1.12**), an unnatural α-amino acid amide (**3.10**), and unnatural dipeptides (**4.1**, **4.4**, **4.7**, **4.9**, **4.12**, **4.13**).

Microbial growth inhibition was not always reproduced between two lots of the same compound. For example, the inhibition of the growth of three organisms observed for one

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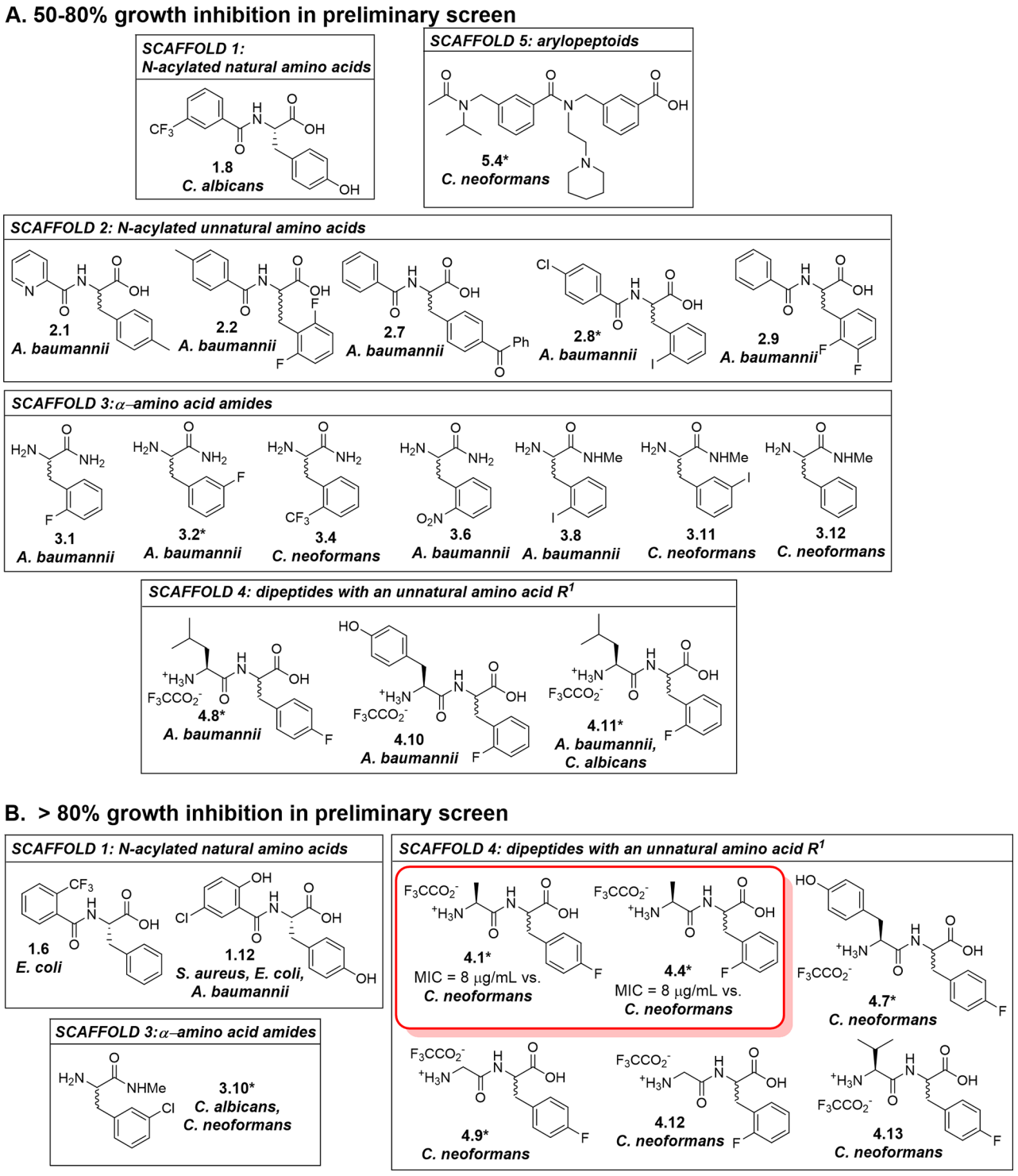

Figure 4. Structures of molecules with activity in the biological screens at CO-ADD. The pathogen inhibited is indicated below each compound. *The compound showed replicated biological activity when duplicate compounds were tested. A) Structures that inhibited 50−80% of pathogen growth ("partially active") in the primary screen at 32 μg/mL; B) Structures that inhibited >80% of pathogen growth ("active") in the primary screen. Structures **4.1** and **4.4** in the red box were active in preliminary screen, with subseqently confirmed MIC values and no cytotoxicity at the highest concentrations tested in the secondary hit confirmation screen.

lot of **1.12** was not replicated in the other three lots. There are serveral possible sources for these inconsistencies, including differences in actual concentrations and/or purity of the two or more lots of compounds submitted, errors in compound transfers, and biological assay variability. The specific causes of nonreplicated activity across duplicate lots were not pursued. Nonetheless, the variability between sets of duplicate compounds reiterated that replication at all stages of the research process is essential; decisions are not based on a single observation or experiment.

Each lot of the 26 compounds that showed at least partial activity in the preliminary screening was subsequently

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subjected to follow-up hit confirmation screening, with MICs for each microbe determined contingent upon verification of activity. Compounds were also tested for cytotoxicity to a mammalian cell line (HEK293, human embryonic kidney). From this more stringent assay, two unnatural dipeptides (**4.1**, **4.4**, Figure 4B) were confirmed to inhibit growth of *C. neoformans* with an MIC ≤ 8 μg/mL. This value is comparable to the MIC against this fungus measured for the common antifungal fluconazole under identical screening conditions.[41] It was encouraging that neither of these unnatural dipeptides inhibited the viability of HEK293 cells at the highest concentrations tested (32 μg/mL) (see Supporting Information). The relatively low number of molecules that emerged as confirmed hits underscores the importance of applying this rigorous two-step screening process to identify new bioactive molecules.

In this work, we have demonstrated that the D3/CO-ADD collaboration is a powerful strategy to couple combinatorial synthesis and phenotypic screening to identify new antimicrobial compounds. The identification of two readily accessible molecules that inhibit *Cryptococcus neoformans* growth (**4.1** and **4.4**), verified over two screening steps (preliminary and hit confirmation), is an exciting initial accomplishment. A more detailed investigation to validate and evaluate the scope of the antifungal activity of **4.1**, **4.4** and analogs, as single diastereomers, is underway. Further, the successful preparation of 76 molecules comprising five different scaffolds highlights the reliability of the procedures developed. In parallel, student researchers were equipped with valuable hands-on learning through the D3 processes.

Based on these experiences and previous reports,[11,15,28] we offer our perspectives on important factors for the success, sustainability, and future of the D3/CO-ADD collaboration. One key to sustained success is that D3/CO-ADD experiments meet the resources and needs of particular institutions (e.g., number of students, costs, available instrumentation). They employ cost-effective, operationally straightforward equipment, like the Bill-Board[42,43] to prepare numerous and diverse analogs. In doing so, both the clarity of procedures and robustness of chemistry are validated. Finally, we find that this project provides a meaningful context for learning basic organic chemistry experimental techniques and theory. The D3/CO-ADD collaboration model is well-positioned to be a template for other investigators who may adapt these strategies to design new scaffolds for synthesis and subsequent biological evaluation, thereby advancing the pace of discovery of new antimicrobial compounds.

METHODS

Chemistry. *Safety Note.* DIC is a known irritant and contact sensitizer.[44] Care with any peptide coupling reagents is advised owing to their documented health hazards.[45]

General Notes. The general procedures documented below for the syntheses of Scaffolds **2** and **5** compounds were performed using the methods previously described.[10−15,28,38] The Bill-Board apparatus[42] was used for the synthesis of all scaffolds. The utility and accessibility of this equipment has been previously described in detail.[10−12,15,28,46] Slight modifications to general procedures were implemented in different laboratory environments, and reactions outcomes were comparable despite these changes. Reaction times were varied to accommodate the frequency of laboratory meetings for students enrolled in organic chemistry classes. Reactions were performed in some laboratories with agitation provided by a motor-driven rotation assembly. However, reaction outcomes were not compromised if the Bill-Board was allowed to stand stationary after manual inversion of the Bill-Board a few times to effect mixing. Additionally, two procedures for cleavage of the reaction products from the resin have been used effectively and interchangeably. Sources of materials as well as the preparation of the benzophenone imine of glycine-Wang resin used for the synthesis of intermediate **A** are detailed in the Supporting Information. Structures, purities, masses, and school source for all replicates of all compounds prepared are tabulated in Supporting Information Table S2.

*Synthesis of N-Acylated Natural Amino Acids (**1.1**−**1.26**).* These compounds were prepared according to previously reported methods,[15] which are further detailed in the Supporting Information.

*Synthesis of Unnatural Amino Acid Intermediate **A**.* Intermediate **A** was prepared as previously reported.[10,12] Details of the synthesis are included in the Supporting Information.

*Synthesis of N-Acylated Unnatural Amino Acids (**2.1**−**2.12**).* These compounds were prepared according to previously reported methods;[10] these are further detailed in the Supporting Information.

*Synthesis of α-Amino Acid Amides (**3.1**−**3.12**).* From intermediate **A**, reaction vessels were washed 3 × 2 mL with THF, and reaction vessel bottom caps were replaced. Either ammonia (2.5 mL of 7 N solution in methanol) or methylamine (2.5 mL of a 33% solution in ethanol) were added. The Bill-Board was capped and inverted three times, then allowed to sit at RT for 12 days. The reaction vessel caps were then removed, and the solution was collected into individually labeled, tared vials. Each reaction vessel was rinsed with an additional 2 × 2 mL with THF. A small aliquot of the filtrate (100 μL) was evaporated to dryness for LC/MS analysis. The remainder was concentrated by evaporation under a stream of N_2.

*Synthesis of Dipeptides (**4.1**−**4.13**).* From intermediate **A**, the reaction vessels were capped at the bottoms. A solution was prepared of the appropriate acid (0.25 M solution in 0.25 M HOBt in NMP). To each reaction vessel, 1 mL of the appropriate solution was added (0.25 mmol acid, 5 equiv acid, 0.25 mmol HOBt, 5 equiv HOBt) followed by 0.5 mL of DIC solution (0.5 M in NMP, 0.25 mmol, 5 equiv). The tops of the reaction vessels were capped, and the Bill-Board was allowed to rotate for 2−5 days. The reaction vessels were uncapped, and each was washed twice with NMP, twice with THF, and thrice with CH_2Cl_2. Reaction vessels were capped at the bottom, then each was treated with 2 mL of a mixture of TFA/CH_2Cl_2/H_2O for 30 min. The reaction vessel caps were then removed, and the solution was collected into individually labeled, tared vials. Each reaction vessel was rinsed with an additional 2 mL of the cleavage solution, then 2 mL CH_2Cl_2. A small aliquot of the filtrate (100 μL) was evaporated to dryness and was analyzed by LC/MS. The remainder was concentrated by evaporation under a stream of N_2 (effluent TFA vapor was scrubbed in a caustic solution of sodium hydroxide).

*Synthesis of Arylopeptoids (**5.1**−**5.12**).* The preparation of these was carried out according to previously reported methods;[28] these are further detailed in the Supporting Information.

LC/MS Analysis of Synthetic Products. Molecules prepared were characterized exclusively by LC/MS to evaluate purity and identity (methods detailed in the Supporting Information). Crude purities of synthesized products were determined by integration of the chromatograms (Table S2).

Preparation of Samples for Submission to CO-ADD. Solutions of samples were prepared in known volumes of either methanol (scaffolds **2**−**4**) or acetone (scaffolds **1** and **5**). An aliquot representing 1.0 mg was then transferred to a barcoded vial provided by CO-ADD and the solvent was removed by evaporation using a stream of nitrogen gas.

Biology. All preliminary screening and hit confirmation assay results are tabulated in Table S2.

Preliminary Screening Sample Preparation. Samples received by CO-ADD were stored frozen at −20 °C. Samples were prepared in DMSO and water to a final testing concentration of 32 μg/mL or 20 μM in 384-well, nonbinding surface plate (NBS) for each bacterial/fungal strain, and in duplicate (n = 2), and keeping the final DMSO

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concentration to a maximum of 1% DMSO. All the sample preparation solution transfers were done using liquid handling robots.

Preliminary Screening Assays. Preliminary antibacterial and antifungal screening assays were carried out following reported procedures.[9] Additional details are in the Supporting Information.

Preliminary Screening Analysis of Assay Results. Percentage growth inhibition was calculated for each treated well using the absorbance readouts, in comparison with median absorbance value of untreated bacteria (positive growth control) and with median absorbance value of media only (negative growth control):

$$\text{inhibition}_i = 1 - \frac{\text{OD}_i - \text{median}(\text{OD}_{\text{NegControl}})}{\text{median}(\text{OD}_{\text{PosControl}}) - \text{median}(\text{OD}_{\text{NegControl}})}$$

$$\text{MScore}_i = \frac{0.6745(\text{OD}_i - \widetilde{\text{OD}})}{\text{MAD}}$$

The significance of the inhibition values was determined by modified Z-scores, calculated using the median and MAD of the samples (no controls) on the same plate. In the preliminary screening, samples with inhibition value above 80% and Z-Score above 2.5 for either replicate (n = 2 on different plates) were classed as actives. Samples with inhibition values between 50−80% and Z-Score above 2.5 for either replicate (n = 2 on different plates) were classed as partial actives.

Preliminary Screening, Antibiotic Standards Preparation, and Quality Control. Colistin and Vancomycin were used as positive bacterial inhibitor standards for Gram-negative and Gram-positive bacteria, respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans* and *C. neoformans*. The antibiotics were provided in four concentrations, with 2 above and 2 below their MIC values, and plated into the first eight wells of column 23 of the 384-well NBS plates. The quality control (QC) of the assays was determined by the antimicrobial controls and the Z′-factor (using positive and negative controls). Each plate was deemed to fulfill the quality criteria (pass QC), if the Z′-factor was above 0.4, and the antimicrobial standards showed full range of activity, with full growth inhibition at their highest concentration, and no growth inhibition at their lowest concentration.

Hit Confirmation Screening, MIC Determination, and Cytotoxicity Assay. Compounds identified as partially active or active in the preliminary screen were subjected to hit confirmation screening, MIC determination, and cytotoxicity assays following reported methods.[9] Additional details are provided in the Supporting Information.

Hit Confirmation Quality Control. All screenings were done as two replica (n = 2), with both replicas on two different plates, but from single plating and done in a single screening (microbial incubation). In addition, two values are used as quality control for individual plates:

$$\frac{3 \times (\text{MAD}(\text{OD}_{\text{PosControl}}) + \text{MAD}(\text{OD}_{\text{NegControl}}))}{|\text{median}(\text{OD}_{\text{PosControl}}) - \text{median}(\text{OD}_{\text{NegControl}})|}$$

and standard antibiotic controls at different concentrations (above and below their MIC). The plate passes the quality control if Z′-Factor >0.4 and standards are active and inactive at highest and lowest concentrations, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00732.

Synthetic procedures (21 pages) for preparation of analogs of previously reported scaffolds **1**, **2**, and **5**; tabulated conditions for LC/MS analyses of synthetic products; previously reported biological screening methods; tabulated synthetic and biological screening data for all compounds reported (PDF)

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Notes

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