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Concise Synthesis of Spergualin-Inspired Molecules With Broad-Spectrum Antibiotic Activity

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Abstract
There is a growing need to identify new, broad-spectrum antibiotics. The natural product spergualin was previously shown to have promising anti-bacterial activity and a privileged structure, but its challenging synthesis had limited further exploration. For example, syntheses of spergualin and its analogs have been reported in approximately 10 linear steps, with overall yields between 0.3 and 18%. Using the Ugi multi-component reaction, we assembled spergualin-inspired molecules in a single step, dramatically improving the overall yield (20% to 96%). Using this strategy, we generated 43 new analogs and tested them for anti-bacterial activity against two Gram-negative and four Gram-positive strains. We found that the most potent analogue, compound 6, had MIC values between 4 and 32 μg/mL against the six strains, which is a significant improvement on spergualin (MIC ~ 6.25 to 50 μg/mL). These studies provide a concise route to a broad-spectrum antibiotic with a novel chemical scaffold.

Keywords
convergent synthesis, Ugi reaction, 15-deoxyspergualin, anti-infective, polyamine

Introduction
Spergualin was first isolated from culture broths of *Bacillus laterosprus* in 1981 and shown to have broad-spectrum antibacterial activity.[1] This compound has a modular structure consisting of a guanidino group and a spermidine-like polyamine linked through a peptide (Figure 1).[2] Spergualin is structurally distinct from other antibiotics used in the clinic,[3] building interest in re-visiting this privileged scaffold. However, there has been limited exploration of this molecule since the 1990s.[4] One of the major reasons is that the synthesis of spergualin is protracted. Typical routes produce spergualin and its analogs in low yield (0.3 to 18%) over at least 10 steps.[5] Another issue is the poor chemical stability of spergualin, which rapidly hydrolyzes in aqueous buffers and consequently has a short lifetime *in vivo*.[6] Towards these goals, we recently reported an improved synthetic approach that features the Ugi multi-component.[7] This route improved the overall yield of spergualin derivatives to between 31 and 47%, while also reducing the number of synthetic transformations (by 4 or 5 steps) and expanding the scope of accessible analogs. Further, it was found that removing the hydroxyl at carbon 15 and installing an aromatic group at the 11 position (Figure 1) greatly increased chemical stability.[7b] While

these initial efforts were informative, we hoped to support the creation of a greater number of analogues by further reducing the number of synthetic transformations.

Here, we report a concise route to spergualin-inspired molecules, most created in a single step in yields between 20 and 96%. Using this approach, we created a library of ~40 analogues and tested each compound for the ability to inhibit growth of *Bacillus anthracis*, *Bacillus cereus, Bacillius subtilis*, *Escherichia coli, Haemophilus influenzae,* and *Staphylococcus aureus*. We found that the most potent molecule, compound 6, had broad-spectrum, anti-bacterial activity, with minimum inhibitory concentration (MIC) values between 4 and 32 μg/mL. This activity is significantly better than the natural product (MIC values between 6.25 and 50 μg/mL). These studies introduce an improved synthetic route and initial structure-activity relationships, guiding the design of more potent, broad-spectrum antibiotics.

Results and Discussion

Studies of spergualin as an antibiotic are hindered by the poor overall yield and limited reaction scope in previous syntheses, as well as the rapid hydrolysis of the products in basic buffers. The stability issue has previously been overcome through removing the hydroxyl position at position 15, to produce 15-deoxyspergualin (15-DSG)[5], and installing a bulky group at position 11 (Figure 1).[7b] However, the overall yields are still un-optimized, with the major losses coming during purification and workup. We envisioned a route to spergualin-like analogs that might improve access (Table 1). Key early studies showed that benzyl protection of the amine at position 12 dramatically improved the ease of purification without negatively impacting biological activity (data not shown), so we designed the library to include this feature. Specifically, the Ugi reaction proceeded through the condensation of benzylamine with a variety of isocyanides, carboxylic acids and aldehydes to probe the requirements in the guanidine (R-), 11 position aromatic (R1-) and polyamine (R2-) regions (Table 1). Most of the components were commercially available or accessible in a single step.

The individual components were combinatorially assembled to generate a library of 43 molecules (Table 1). Briefly, benzylamine (1 equiv) and an aldehyde (1 equiv) were mixed in methanol at room temperature until imine formation was detected by thin layer chromatography (~30 minutes). The reaction was then purified by column chromatography on silica gel using a hexane and ethyl acetate gradient, resulting in compounds 1 and 13-43. Compounds 2-12, which were derived from *tert*-butyl (4-isocyanobutyl) carbamate[8], were first subjected to a Boc deprotection prior to purification by column chromatography on basic alumia oxide using an ethyl acetate gradient and methonal gradient. The final purified yields ranged from 20 to 96% (see the Supporting Information for synthesis and characterization), representing a dramatic increase in overall yield compared to previous reports.

To explore the antibacterial activity of these compounds, we tested them for the ability to suppress bacterial growth using a 96-well, OD_{600} turbidity platform. Each library member was initially screened at a single concentration (200 μM) in triplicate against six bacterial strains, including two Gram-negatives (*E. coli and H. influenzae*) and four Gram-positives (*B. anthracis*, *B. cereus*, *B.subtilis*, and *S. aureus*). We defined the negative control as the growth of each bacterial strain in the presence of 5% DMSO and the positive control was 200 μM ampicillin (Figure 2a and Figure S1). Compounds that decreased turbidity relative to DMSO were considered “active”. We found that 21 molecules were active against at least one of the six bacterial strains. *B. anthracis* was the most susceptible, while only two compounds were active against *E. coli* (Figure 2b).

The ten molecules with activity against both Gram negatives and positives (Figure 2c) were then subject to confirmatory re-testing to determine their minimum inhibitory concentration (MIC) values. Only two compounds (6 and 12) had MIC values < 256 μg/mL against all six bacterial strains (Figure 3a,b). The best of these, compound 6, had MIC values between 4 and 32 μg/mL. These MIC values are even better than those of spergualin (MIC values 6.25 to 50 μg/mL), so it was chosen for further study (Figure 3c). This compound was first re-synthesized and its activity confirmed in the MIC assay (see Supporting Information). Next, we tested compound 6 in liquid broth cultures (Figure 3d) and found that it also dose-dependently inhibited bacterial growth of all six strains in that platform (Figures S2 and S3). In control experiments, we confirmed that none of the synthetic precursors to compound 6 (*i.e.* benzyl amine, 3-mercaptopropionic acid, 4-bromobenzaldehyde or spermidine, an alkyl polyamine) were active (Figure S4), suggesting that the functionalized peptide is the relevant pharmacophore.

To explore the potential structure-activity relationships (SAR) around compound 6, we purchased structurally similar molecules (compounds 6a-d) (Figure S5a) and tested their activity against the six strains. None of these analogues were significantly active (MIC > 128 μg/mL) (Figure S5b), but this information, combined with the previous results, helped refine our knowledge of the pharmacophore. Specifically, the minimal pharmacophore appears to be the peptide region with pendant aromatic groups at the C11 and N12 positions. Short, flexible alkyl chains terminated with electron-rich groups, such as thiols and amines, were preferred at either end (Figure S5c). Future work will explore the SAR in more detail. One goal of those studies will be to explore the effects of stereochemistry at position 11 on potency, as all the molecules reported here are racemic mixtures. Previous studies suggested that the stereochemistry at this position has modest effects on anti-tumor activity,[9] but this issue needs to be resolved for antibacterial activity.

Finally, we wanted to explore whether compound 6 was bactericidal or bacteriostatic. *S. aureus* cultures were treated for 24 hours at the MIC value (8 μg/mL) or 2x the MIC value (16 μg/mL) and the resulting samples were plated on solid agar to count colony forming units (CFUs). At both concentrations, compound 6 was clearly bactericidal (>3 $\log_{10}$ decrease in CFUs) (Figure 4). Taken together, these results suggest that compound 6 is a promising scaffold for further development.

Conclusion

The natural product spergualin has promising anti-bacterial activity, but its cumbersome synthesis and limited stability have hindered its use. Using a route that features the Ugi multi-component reaction, we generated a small library of synthetically tractable analogs and tested them for antibacterial activity. The results showed that short and flexible alkyl chains terminated with electron-rich groups at either end of the peptide-based pharmacophore were necessary for activity. Most notably, we found that compound 6 had particularly promising broad-spectrum, anti-bacterial activity against six strains. The concise and convergent assembly of compound 6 is expected to accelerate discovery of anti-bacterial molecules.

The mechanism-of-action (MOA) of spergualin is not yet known and its targets in bacteria are unclear, so these molecules could additionally serve as useful chemical probes. To this point, MOA studies with spergualin have not been practical because of its poor stability and its tendency to rapidly hydrolyze. Compound 6 represents a chance to possibly identify the target responsible for broad spectrum, bactericidal activity. This next step will be particularly powerful because the structure of compound 6 has significantly diverged from that of the natural product. After the MOA and target are identified, it will be essential to re-explore whether compound 6 and its analogs share the same profile as spergualin. Finally, although the activity of compound 6 is promising, the potency of this series needs to be further improved. Because MIC values of 4 μg/mL were obtained in a relatively small library of ~40 analogs, we

are optimistic about the possibility of additional potency after more medicinal chemistry. However, the relatively narrow scope of the SAR suggests that dramatic changes to the pharmacophore may not be tolerated. Regardless, the improved route and preliminary SAR provided here warrant further exploration.

Experimental Section

Synthesis of *tert*-butyl (4-isocyanobutyl) carbamate. The preparation of this isocyanide was based on literature precedent.[8] Specifically, to a 500 mL 3-necked RBF equipped with an addition funnel and purged with N_2(g) was added 1,4-diaminobutane (15 g, 170.2 mmol) dissolved in 60 mL of dioxane. Using the addition funnel, boc anhydride (3.7 g, 17.02 mmol) dissolved in 60 mL of dioxane was added dropwise over 1.5 hrs. The addition of boc anhydride resulted in the formation of a white precipitate. The reaction was allowed to stir overnight at RT. The next morning, the solvent was removed the under vacuum resulting in a white solid to which 100 mL of water was added. The resulting insoluble material was removed by gravity filtration. The filtrate was then extracted with DCM (3 x 100 mL). The organic layers were dried with anhydrous sodium sulfate and concentrated to give *N*-Boc-1,4-diaminobutane, a light yellow oil, in 80-89% yield. Next, to a 100 mL RBF purged with N_2(g) was added *N*-Boc-1,4-diaminobutane (2.8 g, 14.9 mmol) diluted in 25 mL in DCM. The reaction was then cooled using an ice bath. Once cooled, DIC (2.3 mL, 14.9 mmol) was added dropwise and the resulting white mixture was allowed stir overnight at RT. The next morning, the reaction was subjected to gravity filtration. The resulting filtrate was washed 2 x 50 mL with saturated sodium bicarbonate. The organic layers were pooled, dried with anhydrous sodium sulfate, concentrated to give the intermediate *tert*-butyl (4-formamidobutyl)carbamate. Next, in a 100 mL RBF purged with N_2(g) was added *tert*-butyl (4-formamidobutyl)carbamate (1g, 4.6 mmol) dissolved in 10 mL of DCM and TEA (1.9 mL, 13.8 mmol). This reaction was cooled using an ice bath. Once cooled, phosphoryl chloride (0.44 mL, 4.6 mmol) was added dropwise, causing the reaction mixture to turn orange. This mixture was allowed to stir at RT for 30 minutes. Afterwards, potassium carbonate (6.4g, 4.6 mmol) dissolved in water was added dropwise and the reaction was allowed to stir for an additional 30 minutes. The reaction was then transfer to separatory funnel, the organic layer was removed and saved. The remaining aqueous layer was extracted with 5 x 20 mL of DCM. The combined organic layers were dried with anhydrous sodium sulfate and then concentrated under vacuum. This crude product was purified by column chromatography on silica gel using a hexane:ethyl acetate gradient. The purified product eluted at 50:50 hexane:ethyl acetate. Solvent removal resulted in a yellow oil in 20-43% yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.65 (s, 1H), 3.42 (t, *J* = 6.5 Hz, 1H), 3.16 – 3.13 (m, 2H), 1.74 – 1.68 (m, 2H), 1.65 – 1.59 (m, 2H), 1.43 (s, 9H).

General synthesis for guanidylated acids. The preparation of guanidylated acids was based on literature precedent.[10] To a 3-necked RBF equipped with condenser and N_2(g) inlet, was added either pentanoic, hexanoic, or octanoic acid (1 mmol). The flask was purged with N_2(g). Afterwards, anhydrous 8 mL DCM was added and the flask was heat to 55-60°C using an oil bath. Once heated, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (0.4 mL, 2.2 mmol) was added dropwise. The resulting cloudy mixture was allowed to reflux for 2 hrs. Afterwards, the reaction was removed from heat and was allowed to cool to RT. TEA (0.15mL, 1.1 mmol) was added, followed by 1 3-di-boc-2-(trifluoromethylsulfonyl)guanidine (0.430g, 1.1 mmol) and an additional 2 mL of DCM. The reaction flask was re-purged with N_2(g) and allowed to stir for 4-5 hrs at RT. During this time the reaction mixture clarified. Afterwards, the reaction was washed in the following manner: 2 x 8 mL of brine, 1 x 8 mL water, and 1 x 8 mL 10% citric acid. The combined organic layers were dried with anhydrous sodium sulfate and then concentrated under vacuum. This crude product was purified by column

chromatography on silica gel using a hexane:ethyl acetate gradient. The purified product eluted at 50:50 hexane:ethyl acetate. (*Z*)-5-(2,3-bis(*tert*-butoxycarbonyl)guanidino)pentanoic acid. White solid in 48.3% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.48 (s, 1H), 3.48 (s, 2H), 2.41 (t, *J* = 6.9 Hz, 2H), 1.75 – 1.59 (m, 4H), 1.49 (s, 18H). (*Z*)-6-(2,3-bis(*tert*-butoxycarbonyl)guanidino)hexanoic acid. White solid in 41% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.36 (s, 1H), 3.41 (q, *J* = 8.0 Hz, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 1.71 – 1.61 (m, 2H), 1.61 – 1.54 (m, 2H), 1.48 (d, *J* = 2.7 Hz, 18H), 1.44 – 1.34 (m, 2H). (*Z*)-8-(2,3-bis(*tert*-butoxycarbonyl)guanidine)octanoic acid. White solid in 63% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.28 (s, 1H), 3.36 (q, *J* = 7.2 Hz, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.65 – 1.57 (m, 2H), 1.56 – 1.50 (m, 2H), 1.47 (d, *J* = 2.4 Hz, 18H), 1.31 (s, 6H).

General synthesis of analogs. In a 50 mL RBF, benzylamine (1 mmol), an aldehyde (1 mmol), and 5 mL of methonal were mixed at RT until imine formation was detected by thin layer chromatography (~30 minutes). Next, a carboxyl acid (1 mmol) and an isocyanide (1 mmol) were added and allowed to react overnight. This reaction was then purified by column chromatography on silica gel using a hexane and ethyl acetate gradient, resulting in compounds 1 and 13-43. Molecules 2-12, compounds that contained the *tert*-butyl (4-isocyanobutyl) carbamate starting material, were then subjected to a boc deprotection before purification. Briefly, the boc protected peptoid was dissolved in DCM (10 mL) and treated with 85% phosphoric acid (3 equiv). This mixture was allowed to stir overnight at RT. Afterwards, 10 mL of water was added and then the reaction mixture's pH was neutralized using 10% NaOH. This mixture quenched saturated sodium bicarbonate and then extracted 3 x 10 mL with ethyl acetate. The combined organic layers were dried, concentrated, and the subjected to column chromatography on basic alumina oxide using an ethyl acetate gradient and methanol gradient. Compound yields ranged from 20 – 96%. See the Supporting Information for additional details and characterization.

Laboratory Growth and Maintenance of Bacterial Strains. The following bacterial strains were used: *Bacillus anthracis* 34F2 Sterne, *Bacillus cereus* ATCC 11778, *Bacillius subtilis* 168, *Escherichia coli* K-12 (MG1655), *Haemophilus influenza* ATCC 51907, and *Staphylococcus aureus RN4220*. *H. influenzae* was grown in Brain Heart Infusion (BHI) media supplement with hemin and β-nicotinamide adenine dinucleotide hydrate.[11] Broth cultures of *H. influenzae* were prepared by scraping bacteria from agar plates and suspending into fresh supplemented BHI medium to the desired OD_{600}. All other bacterial strains were grown in Luria-Bertani (LB) medium. Inoculum for liquid culture assays was prepared by diluting an overnight LB broth culture, grown and 37 °C with shaking (200 rpm), into fresh liquid medium to the desired OD_{600}.

Antibacterial Screening Assay. Bacterial inoculum of each strain was prepared to an OD_{600} of 0.1 as described above. Next, 100 µL of each dilute culture was added in triplicate to a sterile non-treated CytoOne 96-well clear bottom plate. To each well, was added 5 µL of either compound in DMSO or DMSO alone. The final concentration of compound was 200 µM and the concentration of DMSO was 5%. The plates were covered and incubated at 37 °C with shaking (200 rpm) for 6 to 7 hours. Afterwards, bacterial growth was recorded by measuring OD_{600} using a SpectaMax M5 plate reader.

Minimum Inhibitory Concentration (MIC) Assay. MIC experiments were performed using the double dilution method. Briefly, inoculum of each strain was prepared to an OD_{600} of 0.1 and 200 µL of each dilute culture was added to a sterile non-treated CytoOne 96-well clear bottom plate. To the plated

dilute cultures, was added 10 µL of compound from a 2-fold dilution series. The final concentrations of the compounds were in the range of 256 to 2 µg/mL. Plates were covered and incubated at 37 °C with shaking (200 rpm) for 24 hours before MIC values were determined. All experiments were performed at least twice in triplicate.

Bacterial Growth Assay in Liquid Culture. Bacterial cultures were prepared to an OD_{600} of 0.1 and 200 µL of each dilute culture was added to the wells of sterile non-treated CytoOne 96-well clear bottom plates. Compounds (10 µL) from a 2-fold dilution series were then added to a final concentration between 32 and 1 µg/mL. Plates were covered and incubated at 37 °C with shaking (200 rpm). Bacterial growth was recorded every 30 minutes by measuring OD_{600} using a SpectaMax M5 plate reader. All experiments were performed at least twice in triplicate.

Assay of Bactericidal/Bacteriostatic Activity. Time-kill studies were performed using 5 mL cultures of *S. aureus* (OD_{600} = 0.1) treated with either 250 µL compound 6 or DMSO. Final compound concentrations were 8 to 16 µg/mL. Samples (100 µL) of each culture were removed after 0, 4, and 24 hours, serially diluted 10-fold in sterile phosphate buffered saline, and spotted (2 µL) on LB agar plates. Finally, colonies were counted after incubation for 24 hours at 37 °C.

Compound 6 Analogues. Compound 6a (4-cyano-*N*-(2-oxo-2-(piperazin-1-yl)ethyl-*N*-(3-phenylpropyl)benzamide) was purchased from ChemDiv. Compound 6b (N-(2-(benzylamino)-2-oxoethyl)-4-isopropylcyclohexane-1-carboxamide) was purchased from Vitas-M Laboratory. Compound 6c (*N*-benzyl-2-(2-(isopropylthio)acetamido)acetamide) and compound 6d (*N*-(2-(4-benzylpiperazin-1-yl)-2-oxoethyl)-methylbutanamide) were purchased from Enamine. Mass spectrometry was used to validate the identity of each purchased compound (see the Supporting Information).

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