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Analogue Synthesis Reveals Decoupling of Antibiofilm and β-Lactam Potentiation Activities of a Lead 2-Aminoimidazole Adjuvant Against *Mycobacterium smegmatis*.

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Abstract

Biofilm formation is one of the many mechanisms bacteria utilize to survive antibiotic treatment. It has been demonstrated that when *Mycobacterium tuberculosis* exists in a biofilm *in vitro* it expresses phenotypic resistance to antimicrobial drugs. Since the *in vivo* survival of *M. tuberculosis* following drug treatment is potentially linked to a biofilm-like expression of drug tolerance, it is hypothesized that biofilm dispersion should increase antibiotic susceptibility and reduce the duration of the current antibiotic treatment regimen. Previously, we have identified a 2-aminoimidazole (2-AI) compound capable of dispersing and inhibiting *M. tuberculosis* and *M. smegmatis* biofilms *in vitro*. Additionally, this compound potentiated the activity of carbenicillin against *M. tuberculosis* and, to a lesser degree, *M. smegmatis*. Here, we describe a SAR study on this compound evaluating each derivative for biofilm dispersion and β-lactam potentiation capabilities against *M. smegmatis*. This study identified a compound that improved upon the biofilm dispersion capabilities of the lead compound. Interestingly, a different compound was identified with an increased ability to potentiate a subset of β-lactam antibiotics. These compounds indicate that biofilm dispersion and potentiation capabilities may not be associated.

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Introduction

Antimicrobial resistance is one of the most pressing biomedical issues of the 21st century [1]. Bacteria utilize many different mechanisms to evade the effects of antibiotics, and one mechanism used by many species of bacteria is the formation of bacterial biofilms. Biofilms are defined as a surface attached community of bacteria, which are embedded within an extracellular polymeric matrix [2, 3]. The National Institutes of Health (NIH) estimates that 80% of all bacterial infections in the human body are biofilm related, which leads to approximately 550,000 annual fatalities [4]. Residing within a biofilm state confers a large increase in the ability of bacteria to withstand antibiotics due to the extra protection provided by the extracellular matrix, increased gene transfer between colonies, and differential gene expression compared to planktonic bacteria [4, 5]. It has been established that bacteria reside in a biofilm state to better protect themselves from environmental stress or sources of eradication, which include antiseptics, antibiotics, and immune responses with studies showing that biofilm associated bacteria can exhibit upwards of 1000-times less sensitivity to conventional antibiotics [3, 4, 6].

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) and has recently exceeded HIV as the number one cause of death of humans from an infectious disease [7]. According to the CDC, TB accounted for approximately 1.7 million deaths worldwide in 2016 [8], and it is believed that approximately one third of the world's population is currently infected with *M. tuberculosis* [8]. These statistics are alarming due to the complexity and length of treatment along with the emergence of resistant strains [9]. The contribution of *M. tuberculosis* biofilm formation on the pathogenesis of bacterial persistence and antimicrobial drug resistance remains controversial; however, during the chronic stages of a *M. tuberculosis* infection in humans and some animals, bacilli are released from dead cells, and colonies survive by attaching to an extracellular matrix derived by the host and pathogen. This process is similar to the survival techniques employed by biofilm forming bacteria. Previously, we showed that *in vitro* culture of extracellular bacilli attached to an extracellular matrix derived from lysed host cells expressed extreme phenotypic resistance to first line anti-TB drugs alone or in combination. Therefore, a biofilm mode of growth is thought to potentially play a role in the persistence of *M. tuberculosis* infections [10, 11].

Currently, the first-line treatment for drug-susceptible *M. tuberculosis* consists of a minimum of two months of antibiotic combination therapy with isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) followed by an additional 4-7 months of combination therapy with INH and RIF [12, 13] to eliminate persistent reservoirs of *M. tuberculosis*, which can lead to a rebound infection if not completely eradicated [11]. This treatment regimen does, however, lead to patient non-compliance due to complications and toxicity associated with long-term antibiotic therapy. Our previous work has shown that *M. tuberculosis* biofilm communities are significantly more resistant than planktonic bacteria to treatment with isoniazid [14]. Therefore, the development of small molecules that disrupt mycobacterium biofilms may find use as adjuvants that, when paired with standard TB antibiotics, could significantly reduce the TB treatment regimens and lead to better patient compliance.

Previously, we have shown that 2-aminoimidazole (2-AI) derivatives that disperse *Mycobacterium smegmatis* biofilms will also disperse *M. tuberculosis* biofilms. These compounds were shown to work synergistically with INH to effectively eradicate *M. tuberculosis* biofilms in an *in vitro* model mimicking the lesions in tuberculosis patients' lungs by employing lysed human leukocytes as a biofilm growth surface [10]. One of these active 2-AI derivatives is compound **1** (Figure 1), which exhibited an EC_{50} value of 52.8 μM against *M. smegmatis* [14]. Here, the EC_{50} value is defined as the concentration necessary to elicit 50% dispersion of a pre-formed biofilm as compared to media-only treated controls. Interestingly, in addition to its biofilm dispersion activity, this compound also sensitized *M. tuberculosis* to the effects of a certain subset of β-lactam antibiotics, effecting a 16-fold reduction in the minimum inhibitory concentration (MIC) of carbenicillin against the *M. tuberculosis* H37Rv lab strain at 31.25 μM [15]. Compound **1** also had β-lactam potentiation effects against *M. smegmatis;* however, they were substantially more moderate, achieving 2-4 fold reductions. Due to the precedent of activity displayed by compound **1** and other 2-AI containing molecules, this structure activity relationship (SAR) chose to keep the 2-AI portion of the molecule consistent and use alterations of the alkyl tail portion in attempts to improve activity. Additionally, recent literature has indicated that increasing the length of alkyl chain within a pyrazinamide derivative increases potency against *M. tuberculosis* [16]. With this precedent and the activity exhibited by compound **1**, a SAR was performed to probe what role, if any, alkyl chains play in anti-biofilm and potentiation activity**.** Interestingly, we find that these activities are somewhat decoupled, as the most potent anti-biofilm compound and the most potent β-lactam potentiator are distinct.

Materials and Methods

Broth Microdilution Method for MIC Determination of *M. smegmatis*

Cultures (48 h) were subcultured to 5 x 10^5 CFU/mL in 7H9 broth supplemented with ADC enrichment. Aliquots (1 mL) were placed in culture tubes, and compound was added from 100 mM stock samples in DMSO, such that the compound concentration equaled the highest concentration tested (200 μM). Samples were then aliquoted (200 μL) into the first row of wells of a 96-well plate, with all remaining wells being filled with 100 μL of initial bacterial subculture. Row one wells were mixed five times before 100 μL was transferred to the following row (row two). Row two was then mixed five times, and 100 μL was transferred to row three. This process provided a serial dilution of the compound and was continued until the final row had been mixed. Plates were covered with Glad Press n' Seal and were incubated under stationary conditions at 37°C for 48 hours. After 48 hours, the wells were stained with alamarBlue (Thermo- Fisher) and were incubated under stationary conditions at 37°C for 8 hours. After 8 hours, the wells with bacterial growth were visualized as pink and the wells with no viable bacteria were visualized as blue. MIC values were recorded as the lowest concentration at which no bacterial growth was observed.

Broth Microdilution Method for Antibiotic Resensitization of *M. smegmatis*

Cultures (48 h) were subcultured to 5 x 10^5 CFU/mL in 7H9 broth supplemented with ADC enrichment. Aliquots (4 mL) were placed in culture tubes, and compound was added from 100 mM stock samples in DMSO, such that the compound concentration was 30% of the MIC of the compound against the particular bacterial strain. One milliliter of the resulting solution was aliquoted into a separate culture tube and was dosed with antibiotic so that the resulting concentration was the highest desirable concentration to be tested. Bacteria treated with antibiotic alone were used as a control. Row one of a 96-well plate was filled with 200 µL of the antibiotic/ compound solution, and the remaining rows were filled with 100 µL per well of the remaining 4 mL bacterial subculture. Row one wells were mixed five times before 100 µL was transferred to the following row (row two). Row two was then mixed five times, and 100 µL was transferred to row three. This process was repeated until the second to last row had been reached. The last row would have only compound and serve as a negative control. The antibiotic only treated bacteria was plated by aliquoting 200 µL of the treated bacteria into row one and filling the remaining rows with untreated bacteria from the original subculture. The rows were mixed in the same way as described above. Plates were covered with Glad Press n' Seal and were incubated under stationary conditions at 37 °C for 48 hours. After 48 hours, the wells were stained with alamarBlue (Thermo-Fisher) and were incubated under stationary conditions at 37 °C for 8 hours. After 8 hours, the wells with bacterial growth were visualized as pink and the wells with no viable bacteria were visualized as blue. MIC values were recorded as the lowest concentration at which no bacterial growth was observed, which was determined by the blue well at the lowest concentration. Fold reductions were determined by comparison of the compound treated wells with the antibiotic only control well.

General static dispersion assay protocols for *M. smegmatis*

Cultures were incubated for 48 hours and then subcultured to 0.01 in Difco M9 minimal salts media. 100 µL of the subculture was aliquoted into every well in columns 2-11 of a 96-well PVC microtiter plate. Columns 1 and 12 were left empty to serve as control wells. Plates were covered with Glad Press n' Seal and were incubated under stationary conditions at 37 °C for 48 hours. After 48 hours, the media was discarded, and the plates were washed thoroughly with water. 100 µL of fresh media containing the appropriate concentration of compound was added to all of the wells in columns 2-4 and 9-11. 100 µL of sterile media was added to all of the wells in columns 1 and 12 and columns 5-8. Plates were covered with Glad Press n' Seal and were incubated under stationary conditions at 37 °C for 24 hours. After 24 hours, media was discarded, and the plates were washed thoroughly with water. 110 µL of a 0.1% aqueous solution of crystal violet was added to every well, and the plates were left at ambient temperature for 30 minutes. After 30 minutes, the crystal violet was disposed, and the plates were washed thoroughly with water. 200 µL of 95% ethanol was added to each well, and the plates were left at ambient temperature for 10 minutes. 125 µL of the ethanol solution was transferred to a fresh polystyrene microtiter plate, and the plate was quantified by measuring the OD_{540}. The percent dispersion was calculated by comparing the OD_{540} of the treated wells with the OD_{540} of the untreated wells, which contained only media after biofilm growth. The first and last column, which had only sterile

media, were used as blanks and those values were subtracted from the OD_{540} obtained in the other columns.

Compound synthesis and characterization

4-(2-amino-1*H*-imidazol-5-yl)-*N*-propylbutanamide hydrochloride (9A)

Compound **3** (0.50 g, 1.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and propylamine (1.5 eq) were added to the solution followed by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3.5 eq). The solution was allowed to stir at room temperature under nitrogen for 24 hours. After 24 hours, the reaction was quenched with 100 mL ethyl acetate and was washed with 100 mL of water followed by three washes with 100 mL of brine. The intermediate was submitted to flash column chromatography with a 1% methanol saturated with ammonium and DCM solvent system to separate excess tail and starting material from the desired product. The intermediate was still impure after flash column chromatography so presence was confirmed by LC/MS. Due to the presence of boc groups, the intermediate could not be fully characterized by NMR. To create the final product, the intermediate was solubilized (0.29 g, 0.56 mmol) in anhydrous dichloromethane (5.6 mL) and was cooled to 0°C. TFA (5.6 mL) was charged into the flask and the reaction was stirred for 24 hours. After this time, the reaction was evaporated to dryness and toluene (3.0 mL) was added. Again, the mixture was concentrated and the process was repeated two additional times. The resulting TFA salt was dissolved in methanol (2.0 mL) and concentrated HCl was added (1.5 mL). The mixture was then evaporated to dryness. Once dry, cold ether (5.0 mL) was added to the mixture, and the mixture was sonicated. The cold ether was pipetted off leaving the target compound **9A** (0.14 g, 0.56 mmol, 99 %) as a brown oil. ^{1}H NMR (300 MHz, [D_6] CD_3OD): δ 6.52 (s, 1H), 3.14 (t, 2H, *J*=7.2 Hz), 2.52 (t, 2H, *J*=7.5 Hz), 2.27 (t, 2H, *J*=7.8 Hz), 1.89 (q, 2H, *J*=6.9 Hz), 1.53 (sex, 2H, *J*=7.2 Hz), 0.92 (t, 2H, *J*=7.2 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 175.7, 148.4, 128.1, 110.1, 42.5, 35.6, 25.6, 24.9, 23.4, 11.6 ppm; IR ν_{max} (cm^{-1}) 3255, 3141, 2963, 2935, 2876, 1674, 1628, 1550, 1458; λ_{max}= 288 nm; HRMS (ESI) calculated for $C_{10}H_{18}N_4O$ $[M+H]^+$ 211.15534, found 211.15455.

4-(2-amino-1*H*-imidazol-5-yl)-*N*-butylbutanamide hydrochloride (9B)

Using the same general procedure used for **9A,** Compound **3** (0.75 g, 1.6 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and butylamine (1.5 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc- protected intermediate as a brown oil. The corresponding intermediate (0.39 g, 0.75 mmol) was then converted to the title compound **9B** (0.11 g, 0.41 mmol, 55 %) as a brown oil. ^{1}H NMR (300 MHz, [D_6] CD_3OD): δ 6.49 (s, 1H), 3.13 (t, 2H, *J*=6.9 Hz), 2.48 (t, 2H, *J*=7.5 Hz), 2.22 (t, 2H, *J*=7.5 Hz), 1.85 (q, 2H, 7.5 Hz), 1.44 (q, 2H, *J*=1.2 Hz), 1.31 (, 0.89 (t, 3H, *J*=7.5 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 175.2, 148.4, 128.1, 109.9, 40.1, 35.9, 32.4, 25.5, 24.9, 21.1, 14.1 ppm; IR ν_{max} (cm^{-1}) 3268, 3141, 2957, 2932, 2872, 1670, 1626, 1551, 1458,1438; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{11}H_{20}N_4O$ $[M+H]^+$ 225.17099, found 225.17016.

4-(2-amino-1*H*-imidazol-5-yl)-*N*-pentylbutanamide hydrochloride (9C)

Using the same general procedure used for **9A,** Compound **3** (0.76 g, 1.6 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and amylamine (1.5 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc-protected intermediate as a brown oil. The corresponding intermediate (0.47 g, 0.87 mmol) was solubilized in anhydrous dichloromethane (8.7 mL) and was cooled to 0°C. TFA (8.7 mL) was charged into the flask and the reaction was stirred for 24 hours. After this time, the reaction was evaporated to dryness and toluene (3.0 mL) was added. Again the mixture was concentrated and the process was repeated two additional times. The resulting TFA salt was dissolved in methanol (2.0 mL) and concentrated HCl was added (1.5 mL). The mixture was then evaporated to dryness. Once dry, cold acetone (5.0 mL) was added to the mixture, and the mixture was sonicated. The cold acetone was pipetted off leaving the target compound **9C** (0.11 g, 0.39 mmol, 44 %) as a tan oil. ^{1}H NMR (300 MHz, [D_6] CD_3OD): δ 6.51 (s, 1H), 3.15 (t, 2H, *J*=6.9 Hz), 2.51 (t, 2H, *J*=7.2 Hz), 2.24 (t, 2H, *J*=7.5 Hz), 1.87 (q, 2H, *J*=7.8 Hz), 1.50 (q, 2H, 6.6 Hz), 1.32 (m, 4H), 0.91 (t, 3H, *J*=6.6 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 175.1, 148.4, 128.0, 109.9, 40.3, 35.9, 30.2, 30.0, 25.5, 24.9, 23.3, 14.4 ppm; IR ν_{max} (cm^{-1}) 3269, 3151, 2956, 2931, 1674, 1630, 1554; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{12}H_{22}N_4O$ $[M+H]^+$ 239.18664, found 239.18565.

4-(2-amino-1*H*-imidazol-5-yl)-*N*-heptylbutanamide hydrochloride (9E)

Using the same general procedure used for **9A,** Compound **3** (1.0 g, 2.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and heptylamine (1.5 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc-protected intermediate as a brown oil. The corresponding intermediate (0.65 g, 1.1 mmol) was converted using the same procedure described in the synthesis of **9A** and yielded the title compound **9E** (0.34 g, 1.1 mmol, 98 %) as a white solid. ^{1}H NMR (300 MHz, [D_6] CD_3OD): δ 6.51 (s, 1H), 3.16 (t, 2H, *J*=6.9 Hz), 2.51 (t, 2H, *J*=7.8 Hz), 2.25 (t, 2H, *J*=7.5 Hz), 1.88 (q, 2H, 7.2 Hz), 1.49 (m, 2H), 1.31 (m, 8H), 0.89 (t, 3H, *J*=6.9 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 175.3, 148.2, 127.9, 109.9, 40.4, 35.8, 32.8, 30.2, 30.0, 27.9, 25.5, 24.9, 23.6, 14.5 ppm; IR ν_{max} (cm^{-1}) 3257, 3149, 2955, 2927, 2856, 1676, 1638; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{14}H_{26}N_4O$ $[M+H]^+$ 267.21794, found 267.21675.

4-(2-amino-1*H*-imidazol-5-yl)-*N*-nonylbutanamide hydrochloride (9G)

Using the same general procedure used for **9A,** Compound **3** (0.50 g, 1.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and nonylamine (1.5 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc-protected intermediate as a brown oil. Using the same procedure described above, The boc-protected intermediate (0.36 g, 0.60 mmol) was converted to the title compound **9G** (0.074 g, 0.23 mmol, 38 %) as a white solid. ^{1}H NMR (300 MHz, [D_6] CD_3OD): δ 6.51 (s, 1H), 3.16 (t, 2H, *J*=6.6 Hz), 2.51 (t, 2H, *J*=8.1 Hz), 2.24 (t, 2H, *J*=7.2 Hz), 1.87 (q, 2H, *J*= 7.2 Hz), 1.49 (m, 2H), 1.29 (m, 12H), 0.89 (t, 3H, *J*=6.6 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 175.2,

148.2, 128.1, 109.9, 40.4, 35.9, 33.0, 30.6, 30.4, 30.4, 30.4, 28.0, 25.6, 24.9, 23.7, 14.5 ppm; IR v_{max} (cm^{-1}) 3269, 3148, 2955, 2923, 2854, 1674, 1633, 1552; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{16}H_{40}N_4O$ $[M+H]^+$ 295.24924, found 295.24808.

4-(2-Amino-1*H*-imidazol-5-yl)-*N*-(pentan-3-yl) butanamide hydrochloride (10A)

Compound **3** (0.50 g, 1.1 mmol) was solubilized in anhydrous dichloromethane (DCM) (30 mL). Triethylamine (3.5 eq) and 1-ethylpropylamine (1.1 eq) were added to the solution followed by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3.5 eq). The solution was allowed to stir at room temperature under nitrogen for 24 hr. After 24 hours, the reaction was quenched with 100 mL ethyl acetate and was washed with 100 mL of water followed by three washes with 100 mL of brine. The intermediate was submitted to flash column chromatography with a 1% methanol saturated with ammonium and DCM solvent system to separate starting material from the desired product. Due to the presence of boc groups, the intermediate could not be fully characterized by NMR. The intermediate (0.6 g, 1.1 mmol) was dissolved in anhydrous DCM (10.5 mL) and was cooled to 0°C. TFA (10.5 mL) was charged into the flask and the reaction was stirred for 24 hours. After this time, the reaction was evaporated to dryness and toluene (3 mL) was added. Again the mixture was concentrated and the process was repeated two additional times. The resulting TFA salt was dissolved in methanol (2 mL) and concentrated HCl was added (1.5 mL). The mixture was then evaporated to dryness. Once dry, cold ether (5.0 mL) was added to the mixture, and the mixture was sonicated. The cold ether was pipetted off leaving the target compound **10A** (0.29 g, 1.1 mmol, 99 %) as a brown oil. ^{1}H NMR (300 MHz, CD_3OD): δ 6.52 (s, 1H), 3.65 (m, 1H), 2.53 (t, 2H, *J*=7.5 Hz), 2.29 (t, 2H, *J*=6.9 Hz), 1.90 (quint. 2H, *J*=7.5 Hz), 1.55 (m, 2H), 1.41(m, 2H), 0.89 (t, 6H, *J*=7.2 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD) δ 175.4, 148.4, 128.0, 110.0, 53.9, 35.9, 28.3, 28.3, 25.8, 24.9, 10.9, 10.9 ppm; IR v_{max} (cm^{-1}) 3255, 3146, 2964, 2934, 1674, 1630, 1547; λ_{max} = 286 nm; HRMS (ESI) calculated for $C_{12}H_{22}N_4O$ $[M+H]^+$ 239.18664, found 239.18570.

4-(2-Amino-1*H*-imidazol-5-yl)-*N*-(heptan-4-yl) butanamide hydrochloride (10B)

According to the procedure used for **10A,** compound **3** (1.0 g, 2.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and 1-propylbutylamine (1.1 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded boc-protected intermediate as a colorless oil. Using the deprotection procedure described in the synthesis of **10A**, the corresponding intermediate (0.61 g, 1.1 mmol) gave the title compound **10B** (0.24 g, 0.78 mmol, 73 %) as a brown oil. ^{1}H NMR (300 MHz, CD_3OD): δ 6.52 (s, 1H), 3.84 (m, 1H), 2.52 (t, 2H, *J*=7.5 Hz), 2.26 (t, 2H, *J*=7.2 Hz), 1.88 (quint. 2H, *J*=7.5 Hz), 1.36 (m, 8H), 0.91 (t, 6H, *J*=6.9 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD) δ 174.9, 148.5, 128.1, 109.9, 50.0, 38.3, 38.3, 36.0, 25.7, 24.9, 20.3, 20.3, 14.3, 14.3 ppm; IR v_{max} (cm^{-1}) 3256, 3152, 2957, 2933, 1673, 1629, 1548, 1459; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{14}H_{26}N_4O$ $[M+H]^+$ 267.21794, found 267.21674.

4-(2-Amino-1*H*-imidazol-5-yl)-*N*-(nonan-5-yl) butanamide hydrochloride (10C)

According to the procedure used for **10A,** compound **3** (1.0 g, 2.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and 5-nonanamine (1.1 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc-protected intermediate as a colorless oil. Using the deprotection procedure described in the synthesis of **10A**, the corresponding intermediate (0.73 g, 1.2 mmol) gave the title compound **10C** (0.39 g, 1.2 mmol, 96 %) as a brown oil. ^{1}H NMR (300 MHz, CD_3OD): δ 6.52 (s, 1H), 3.80 (m, 1H), 2.52 (t, 2H, *J*=7.8 Hz), 2.26 (t, 2H, *J*=7.5 Hz), 1.89 (quint. 2H, *J*=7.8 Hz), 1.38 (m, 12H), 0.90 (t, 6H, *J*=6.3 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD) δ 174.9, 148.4, 128.0, 109.9, 50.4, 36.0, 35.7, 35.7, 29.4, 29.4, 25.8, 24.9, 23.5, 23.5, 14.4, 14.4 ppm; IR v_{max} (cm^{-1}) 3256, 2955, 2929, 2858, 1669, 1630, 1545, 1457; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{16}H_{30}N_4O$ $[M+H]^+$ 295.24924, found 295.24817.

4-(2-Amino-1*H*-imidazol-5-yl)-*N*-(undecan-6-yl) butanamide hydrochloride (10D)

According to the procedure used for **10A,** compound **3** (1.0 g, 2.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and 6-undecanamine (1.1 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc-protected intermediate as a colorless oil. Using the deprotection procedure described in the synthesis of **10A**, the corresponding intermediate (0.95 g, 1.5 mmol) gave the title compound **10D** (0.48 g, 1.3 mmol, 87 %) as a brown oil. ^{1}H NMR (300 MHz, CD_3OD): δ 6.52 (s, 1H), 3.81 (m, 1H), 2.53 (t, 2H, *J*=7.2 Hz), 2.27 (t, 2H, *J*=7.5 Hz), 1.89 (quint. 2H, *J*=7.2 Hz), 1.32 (m, 16H), 0.89 (t, 6H, *J*=6 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 175.1, 148.5, 128.1, 109.9, 50.7, 36.0, 36.0, 36.0, 32.8, 32.8, 26.8, 26.8, 25.9, 24.9, 23.6, 23.6, 14.4, 14.4 ppm; IR v_{max} (cm^{-1}) 3269, 2928, 1678, 1639, 1549, 1458; λ_{max}= 288 nm; HRMS (ESI) calculated for $C_{18}H_{34}N_4O$ $[M+H]^+$ 323.28054, found 323.27932.

4-(2-amino-1*H*-imidazol-5-yl)-*N*-(pentadecan-8-yl) butanamide hydrochloride (10E)

According to the procedure used for **10A,** compound **3** (1.0 g, 2.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and 8-pentadecanamine (1.1 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc-protected intermediate as a brown oil. Using the deprotection procedure described in the synthesis of **10A**, the corresponding intermediate (0.76 g, 1.1 mmol) gave the title compound **10E** (0.27 g, 0.64 mmol, 57 %) as a brown oil. ^{1}H NMR (300 MHz, CD_3OD): δ 6.50 (s, 1H), 3.80 (m, 1H), 2.52 (t, 2H, *J*=7.2 Hz), 2.26 (t, 2H, *J*=7.2 Hz), 1.88 (quint. 2H, *J*=7.2 Hz), 1.28 (m, 24H), 0.88 (t, 6H, *J*=6.6 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 174.2, 147.4, 128.3, 110.1, 50.4, 36.1, 36.1, 36.1, 33.0, 33.0, 30.5, 30.5, 30.4, 30.4, 27.2, 27.2, 25.9, 25.0, 23.7, 23.7, 14.4, 14.4 ppm; IR v_{max} (cm^{-1}) 3289, 2921, 2851, 1686, 1639, 1549, 1456; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{22}H_{42}N_4O$ $[M+H]^+$ 379.34314, found 379.34168.

4-(2-amino-1*H*-imidazol-5-yl)-*N*-(heptadecan-9-yl) butanamide hydrochloride (10F)

According to the procedure used for **10A,** compound **3** (1.0 g, 2.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and 9-heptadecanamine (1.1 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the boc-protected intermediate as a brown oil. Using the deprotection procedure described in the synthesis of **10A**, the corresponding intermediate (0.91 g, 1.3 mmol) gave the title compound **10F** (0.44 g, 1.0 mmol, 78 %) as a tan solid. ^{1}H NMR (300 MHz, CD_3OD): δ= 6.52 (s, 1H), 3.80 (m, 1H), 2.52 (t, 2H, *J*=7.2 Hz), 2.26 (t, 2H, *J*=7.5 Hz), 1.89 (quint. 2H, *J*=7.8 Hz), 1.29 (m, 28H), 0.89 (t, 6H, *J*=4.5 Hz) ppm; ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.0, 146.8, 125.8, 108.5, 47.5, 34.2, 34.2, 34.2, 31.0, 31.0, 28.7, 28.7, 28.7, 28.7, 28.4, 28.4, 25.2, 25.2, 23.9, 23.3, 21.8, 21.8, 13.7, 13.7 ppm; IR v_{max} (cm^{-1}) 3290, 3151, 2921, 2850, 1690, 1641, 1551, 1457; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{24}H_{46}N_4O$ $[M+H]^+$ 407.37444, found 407.37286.

4-(2-amino-1*H*-imidazol-5-yl)-*N*-(nonadecan-10-yl)butanamide hydrochloride (10G)

According to the procedure used for **10A,** compound **3** (1.0 g, 2.1 mmol) was solubilized in anhydrous DCM (30 mL). Triethylamine (3.5 eq) and 10-nonadecanamine (1.1 eq) were added to the solution followed by HBTU (3.5 eq). This synthesis yielded the correspond intermediate as a brown oil. Using the deprotection procedure described in the synthesis of **10A**, the corresponding intermediate (1.0 g, 1.4 mmol) gave the title compound **10G** (0.40 g, 0.86 mmol, 61 %) as a tan solid. ^{1}H NMR (300 MHz, CD_3OD): δ 6.51 (s, 1H), 3.3 (m, 1H), 2.52 (t, 2H, *J*=7.5 Hz), 2.26 (t, 2H, *J*=7.5 Hz), 1.88 (quint. 2H, *J*=7.5 Hz), 1.28 (m, 32H), 0.89 (t, 6H, *J*=6.9 Hz) ppm; ^{13}C NMR (400 MHz, DMSO-d_6): δ 180.5, 156.3, 135.7, 118.0, 57.3, 44.1, 44.0, 44.0, 40.8, 40.8, 38.5, 38.5, 38.5, 38.5, 38.4, 38.4, 38.2, 38.2, 35.0, 35.0, 33.7, 33.1, 31.6, 31.6, 23.4, 23.4 ppm; IR v_{max} (cm^{-1}) 3268, 2922, 2853, 1677, 1637, 1546, 1458; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{26}H_{50}N_4O$ $[M+H]^+$ 435.40574, found 435.40386.

4-(2-amino-1*H*-imidazol-5-yl)-*N*- (1-phenyloctyl) butanamide (11A)

Compound **3** (0.40 g, 0.85 mmol) was solubilized in anhydrous dichloromethane (DCM) (30 mL). Triethylamine (3.5 eq) and tail **S4A** (1.5 eq) were added to the solution followed by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3.5 eq). The solution was allowed to stir at room temperature under nitrogen for 18 hr. After 18 hours, the reaction was quenched with 100 mL ethyl acetate and was washed with 100 mL of water followed by three washes with 100 mL of brine. The intermediate was submitted to flash column chromatography with a 1.25% methanol saturated with ammonium and DCM solvent system to separate starting material from the desired product. Due to the presence of boc groups, the intermediate could not be fully characterized by NMR. The intermediate (0.43 g, 0.66 mmol) was dissolved in anhydrous DCM (6.5 mL) and was cooled to 0°C. TFA (6.5 mL) was charged into the flask and the reaction was stirred for 24 hours. After this time, the reaction was evaporated to dryness and toluene (3 mL) was added. Again the mixture was concentrated and the process was repeated two additional times. The resulting TFA salt was dissolved in methanol (2 mL) and concentrated HCl was added (1.5 mL). The mixture was then evaporated to dryness. Once dry, cold acetone (5.0 mL) was added to the mixture, and the mixture was sonicated. The cold acetone was pipetted off leaving the target

compound **11A** (0.11 g, 0.29 mmol, 45%) as a brown oil. ^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.29(m, 5H), 6.44 (s, 1H), 4.81 (t, 1H, *J*=7.5 Hz), 2.46 (t, 2H, *J*= 7.2 Hz), 2.27 (dd, 2H, *J*= 7.2 Hz), 1.86 (q, 2H, *J*= 7.5Hz), 1.73 (m, 2H), 1.27 (m, 10H), 0.86 (t, 3H, *J*= 7 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 174.5, 148.4, 144.5, 129.4, 129.4, 128.2, 128.0, 127.6, 127.6, 109.9, 54.8, 37.4, 35.6, 32.9, 30.3, 30.3, 27.5, 25.6, 24.9, 23.6, 14.4 ppm; IR v_{max} (cm^{-1}) 2925, 1675, 1539, 1454, 699; λ_{max}= 288 nm; HRMS (ESI) calculated for $C_{21}H_{32}N_4O$ $[M+H]^+$ 357.26489, found 357.26335.

4-(2-amino-1*H*-imidazol-5-yl)-*N*- (1-phenylnonyl) butanamide (11B)

Compound **3** (0.40 g, 0.85 mmol) was solubilized in anhydrous dichloromethane (DCM) (30 mL). Triethylamine (3.5 eq) and tail **S4B** (1.5 eq) were added to the solution followed by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3.5 eq). The solution was allowed to stir at room temperature under nitrogen for 18 hr. After 18 hours, the reaction was quenched with 100 mL ethyl acetate and was washed with 100 mL of water followed by three washes with 100 mL of brine. The intermediate was submitted to flash column chromatography with a 1.25% methanol saturated with ammonium and DCM solvent system to separate starting material from the desired product. Due to the presence of boc groups, the intermediate could not be fully characterized by NMR. The intermediate (0.33 g, 0.49 mmol) was dissolved in anhydrous DCM (5 mL) and was cooled to 0°C. TFA (5 mL) was charged into the flask and the reaction was stirred for 24 hours. After this time, the reaction was evaporated to dryness and toluene (3 mL) was added. Again the mixture was concentrated and the process was repeated two additional times. The resulting TFA salt was dissolved in methanol (2 mL) and concentrated HCl was added (1.5 mL). The mixture was then evaporated to dryness. Once dry, cold acetone (5.0 mL) was added to the mixture, and the mixture was sonicated. The cold acetone was pipetted off leaving the target compound **11B** (0.11 g, 0.29 mmol, 57%) as a brown oil. ^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.29 (m, 5H), 6.45 (s, 1H), 4.81 (t, 1H, *J*= 6.6 Hz), 2.46 (t, 2H, *J*=7.6 Hz), 2.28 (dt, 2H, *J*= 7.6 Hz), 1.85 (q, 2H, *J*= 7.7 Hz), 1.73 (m, 2H), 1.26 (m, 12H), 0.86 (t, 3H, *J*= 7.1 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 174.5, 148.4, 144.5, 129.5, 129.5, 128.2, 128.0, 127.6, 127.6, 109.9, 54.8, 37.4, 35.9, 32.9, 30.6, 30.3, 30.3, 27.5, 25.6, 24.9, 23.7, 14.4 ppm; IR v_{max} (cm^{-1}) 2925, 2854, 1675, 1638, 1539, 1494, 699; λ_{max}= 288 nm; HRMS (ESI) calculated for $C_{22}H_{34}N_4O$ $[M+H]^+$ 371.28054, found 371.27927.

4-(2-amino-1*H*-imidazol-5-yl)- *N*-(1-phenylundecan-5-yl)butanamide (12)

Compound **3** (0.25 g, 0.53 mmol) was solubilized in anhydrous dichloromethane (DCM) (30 mL). Triethylamine (3.5 eq) and tail **S9** (1.5 eq) were added to the solution followed by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3.5 eq). The solution was allowed to stir at room temperature under nitrogen for 18 hr. After 18 hours, the reaction was quenched with 100 mL ethyl acetate and was washed with 100 mL of water followed by three washes with 100 mL of brine. The intermediate was submitted to flash column chromatography with a 1.25% methanol saturated with ammonium and DCM solvent system to separate starting material from the desired product. Due to the presence of boc groups, the intermediate could not be fully characterized by NMR. The intermediate

(0.15 g, 0.21 mmol) was dissolved in anhydrous DCM (3 mL) and was cooled to 0°C. TFA (3 mL) was charged into the flask and the reaction was stirred for 24 hours. After this time, the reaction was evaporated to dryness and toluene (3 mL) was added. Again the mixture was concentrated and the process was repeated two additional times. The resulting TFA salt was dissolved in methanol (2 mL) and concentrated HCl was added (1.5 mL). The mixture was then evaporated to dryness. Once dry, cold acetone (5.0 mL) was added to the mixture, and the mixture was sonicated. The cold acetone was pipetted off leaving the target compound **12** (0.07 g, 0.18 mmol, 83%) as a brown oil. ^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.20(t, 2H, *J*= 8 Hz), 7.12 (m, 3H, *J*= 8 Hz), 6.49 (s, 1H), 3.80 (bs, 1H), 2.57 (t, 2H, *J*= 8 Hz), 2.49 (t, 2H, *J*= 8 Hz), 2.26 (t, 2H, *J*= 8 Hz), 1.86 (m, 2H), 1.61 (m, 2H), 1.42 (m, 4H), 1.27 (m, 10H), 0.87 (t, 3H, *J*= 8 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 175.0, 148.5, 143.6, 129.4, 129.4, 129.2, 129.2, 128.1, 126.6, 110.0, 50.6, 36.7, 36.0, 36.0, 35.8, 32.9, 32.3, 30.2, 27.1, 26.6, 25.9, 24.9, 23.6, 14.8 ppm; IR ν_{max} (cm^{-1}) 2926, 2856, 1637, 1543, 1454, 1201, 697; λ_{max}= 292 nm; HRMS (ESI) calculated for $C_{24}H_{38}N_4O$ $[M+H]^+$ 399.31184, found 399.31204.

4-(2-amino-1*H*-imidazol-5-yl)- *N*-(1-(4-hexylphenyl)heptyl)butanamide (13)

Compound **3** (0.465 g, 0.99 mmol) was solubilized in anhydrous dichloromethane (DCM) (30 mL). Triethylamine (3.5 eq) and tail **S13** (1.5 eq) were added to the solution followed by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (3.5 eq). The solution was allowed to stir at room temperature under nitrogen for 18 hr. After 18 hours, the reaction was quenched with 100 mL ethyl acetate and was washed with 100 mL of water followed by three washes with 100 mL of brine. The intermediate was submitted to flash column chromatography with a 1.25% methanol saturated with ammonium and DCM solvent system to separate starting material from the desired product. Due to the presence of boc groups, the intermediate could not be fully characterized by NMR. The intermediate (0.45 g, 0.62 mmol) was dissolved in anhydrous DCM (6 mL) and was cooled to 0°C. TFA (6 mL) was charged into the flask and the reaction was stirred for 24 hours. After this time, the reaction was evaporated to dryness and toluene (3 mL) was added. Again the mixture was concentrated and the process was repeated two additional times. The resulting TFA salt was dissolved in methanol (2 mL) and concentrated HCl was added (1.5 mL). The mixture was then evaporated to dryness. Once dry, cold acetone (5.0 mL) was added to the mixture, and the mixture was sonicated. The cold acetone was pipetted off leaving the target compound **13** (0.08 g, 0.18 mmol, 30%) as a brown oil. ^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.21(d, 2H, *J*= 8 Hz), 7.11 (d, 2H, *J*= 8 Hz), 6.44 (s, 1H), 4.80 (t, 1H, *J*= 6.9 Hz), 2.56 (t, 2H, *J*= 7.6 Hz), 2.48 (t, 2H, *J*= 7.6 Hz), 2.29 (m, 2H), 1.87 (m, 2H), 1.74 (m, 2H), 1.56 (m, 2H), 1.28 (m, 14H), 0.88 (m, 6H) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 174.6, 148.4, 142.8, 141.5, 129.5, 129.5, 128.1, 127.5, 127.5, 110.0, 54.7, 37.4, 36.5, 35.8, 32.9, 32.8, 32.7, 30.0, 30.0, 27.5, 25.6, 24.8, 23.6, 23.6, 14.4, 14.4 ppm; IR ν_{max} (cm^{-1}) 3250, 2925, 2855, 1676, 1542, 1457, 564; λ_{max}= 292 nm; HRMS (ESI) calculated for $C_{26}H_{42}N_4O$ $[M+H]^+$ 427.34314, found 427.34363.

1-phenylundecan-5-one (S8)

5-Phenylvaleric acid **(S5)** (2.5g, 14.02mmol) was purchased from a commercial source and was converted to compound **S6** using oxalyl chloride (5 eq.), anhydrous DCM (10mL), and catalytic DMF (0.1mL) under nitrogen. After 16 hours, the reaction was evaporated to dryness and placed under vacuum. The acid chloride **S6** (3g, 15.25 mmol) was then converted to the Weinreb amide **(S7)** using N,O dimethylhydroxylamine (1.1 eq), TEA (2.5 eq.) and anhydrous DCM (50mL). After 16 hours, 30mL of sodium bicarbonate was added to the reaction and stirred for 10 minutes. After 10 minutes, the product was extracted with DCM and flash column chromatography was performed using 1:3 ethyl acetate, petroleum ether solvent system. To obtain compound **S8**, compound **S7** was dissolved in anhydrous THF (10mL) on ice and was then treated with hexylmagnesium bromide (2M) and allowed to stir at room temperature for two hours. After two hours, ammonium chloride (10mL) was added to the reaction mixture and was allowed to stir for 10 minutes. The product was extracted using ethyl acetate and was evaporated to dryness. This yielded compound **S8** as a white solid in 49% yield. ^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.23(m, 2H, *J*=7.5 Hz), 7.15 (t, 3H, *J*=8.4 Hz), 2.81 (m, 1H), 2.61 (t, 2H, *J*=7.5 Hz), 1.57 (m, 4H), 1.33 (m, 12H), 0.90 (t, 3H, *J*= 7 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 213.6, 143.5, 129.3, 129.3, 129.2, 129.2, 126.7, 43.4, 43.2, 36.6, 32.7, 32.1, 29.9, 24.8, 24.4, 23.5, 14.4 ppm; IR v_{max} (cm^{-1}) 2928, 2852, 1711, 1454, 746, 698; λ_{max}= 284 nm; HRMS (ESI) calculated for $C_{17}H_{26}O$ $[M+H]^+$ 247.20564, found 247.20553.

1-phenylundecan-5-amine (S9)

Compound **S8** (0.75g, 3mmol) was dissolved in anhydrous methanol (30mL) and ammonium acetate (10eq) was added to the reaction. The reaction mixture stirred for 90 minutes before sodium cyanoborohydride (5eq) was added. The reaction stirred under nitrogen for 64 hours. After 64 hours, concentrated HCl was added to the reaction mixture until the pH was 2. The reaction mixture was then evaporated to dryness. Water (500mL) was added and solid KOH was added until the pH reached 10. The product was extracted with DCM and was evaporated to dryness. This yielded the desired product **S9** as a colorless oil in 99% yield. ^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.23(m, 2H, *J*=7.5 Hz), 7.15 (t, 3H, *J*=8.4 Hz), 2.81 (m, 1H), 2.61 (t, 2H, *J*=7.5 Hz), 1.57 (m, 4H), 1.33 (m, 12H), 0.90 (t, 3H, *J*= 7 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 143.6, 129.4, 129.4, 129.2, 129.2, 126.7, 52.3, 36.7, 36.6, 36.4, 32.9, 32.7, 30.5, 26.6, 26.2, 23.7, 14.4 ppm; IR v_{max} (cm^{-1}) 2925, 2850, 1451, 732; λ_{max}= 284 nm; HRMS (ESI) calculated for $C_{17}H_{29}N$ $[M+H]^+$ 248.23728, found 248.23724.

1-(4-hexyl-henyl)heptan-1-one (S12)

4-Hexylbenzoyl chloride **(S10)** (2.5g, 11.12mmol) was purchased from a commercial source and was converted to compound **S11** using N,O dimethylhydroxylamine (1.1 eq), TEA (2.5 eq.) and anhydrous DCM (50mL). After 16 hours, 30mL of sodium bicarbonate was added to the reaction and stirred for 10 minutes. After 10 minutes, the product was extracted with DCM and flash column chromatography was performed using 1:3 ethyl acetate, petroleum ether solvent system. This yielded compound **S11** as a colorless oil in 98% yield. To obtain compound **S12**, compound **S11** was dissolved in anhydrous THF (10mL) on ice and was

then treated with hexylmagnesium bromide (2M) and allowed to stir at room temperature for two hours. After two hours, ammonium chloride (10mL) was added to the reaction mixture and was allowed to stir for 10 minutes. The product was extracted using ethyl acetate and was evaporated to dryness. This yielded compound **S12** as a colorless oil in 99% yield. ^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.87(d, 2H, *J*= 8 Hz), 7.27 (d, 2H, *J*=8 Hz), 2.96 (t, 2H, *J*= 7.4 Hz), 2.65 (t, 2H, *J*= 7.4 Hz), 1.65 (m, 4H), 1.33 (m, 12H), 0.89 (t, 6H, *J*= 5.6 Hz) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 202.5, 150.1, 136.0, 129.7, 129.7, 129.4, 129.4, 39.4, 36.9, 32.9, 32.8, 32.3, 30.1, 30.0, 25.7, 23.6, 23.6, 14.4, 14.4 ppm; IR v_{max} (cm^{-1}) 2927, 2856, 1683, 1607, 1465, 1180, 978; λ_{max}= 286 nm; HRMS (ESI) calculated for $C_{19}H_{30}O$ $[M+H]^+$ 275.23694, found 275.23717.

1-(4-hexylphenyl) heptan-1-amine (S13)

Compound **S12** (1g, 3.64mmol) was dissolved in anhydrous methanol (120mL) and ammonium acetate (10eq) was added to the reaction. The reaction mixture stirred for 90 minutes before sodium cyanoborohydride (5eq) was added. The reaction stirred under nitrogen for 64 hours. After 64 hours, concentrated HCl was added to the reaction mixture until the pH was 2. The reaction mixture was then evaporated to dryness. Water (500mL) was added and solid KOH was added until the pH reached 10. The product was extracted with DCM and was evaporated to dryness. This yielded the desired product **S13** as a colorless oil in 99% yield.^{1}H NMR (400 MHz, [D_6] CD_3OD): δ 7.16(m, 4H), 3.81 (t, 1H, *J*= 6.7 Hz), 2.56 (m, 2H), 1.64 (m, 4H), 1.25 (m, 14H), 0.85 (m, 6H) ppm; ^{13}C NMR (400 MHz, CD_3OD): δ 143.3, 142.3, 129.7, 129.7, 127.7, 127.7, 57.0, 39.1, 36.6, 32.9, 32.9, 32.7, 30.2, 30.0, 27.4, 23.7, 23.6, 14.4, 14.4 ppm; IR v_{max} (cm^{-1}) 2924, 2854, 1458, 822, 558; λ_{max}= 284 nm; HRMS (ESI) calculated for $C_{19}H_{33}N$ $[M+H]^+$ 276.26858, found 276.26807.

Results and Discussion

Analogue Synthesis

For this study, we elected to explore the SAR of the alkyl tail region of compound **1**, looking at linear chains as well as branched symmetric and asymmetric chains. Compounds were synthesized utilizing the strategy outlined in Scheme 1, using the protected 2-AI **3** as the common coupling partner.

The synthesis of **3** was initiated with the desymmerization of glutaric anhydride **5** to produce the gamma-keto ester **6**. This intermediate was treated with oxalyl chloride to form the acid chloride, which was further reacted with diazomethane followed by the quenching with hydrobromic acid (HBr) to afford the desired α-bromo ketone **4**. The 2-AI ring was then installed via Boc guanidine cyclization to yield the 2-AI **7.** Finally, treatment with Boc anhydride followed by hydrogenation to remove the benzyl group yielded 2-AI **3** (Scheme 2).

With **3** in hand, all final 2-AI derivatives were synthesized by activating the carboxylic acid with HBTU, coupling with the appropriate amine, removing the Boc-groups with TFA, and performing counter ion exchange to generate the HCl salt of the desired product **(9A-K, 10A-G, 11A-B, 12, 13)**(Scheme 3)**.** All the single carbon chain amines (as well as a select number of the branched amines) were commercially available. The branched amines that were not commercially available were synthesized from the corresponding ketones through a reductive amination using ammonium acetate and sodium cyanoborohydride in anhydrous methanol (Scheme S.1 and S.2). The asymmetric ketones necessary to access amines for the synthesis of **12** and **13** were generated through the synthesis of the Weinreb amide followed by a Grignard addition to produce the desired ketone. The previously described reductive amination procedure was used to deliver the desired amines (Scheme S.3 and S.4). Despite the addition of a chiral center, enantioselectivity was not controlled or resolved during synthesis. Ultimately, this SAR study was to determine whether there was an increase or decrease in activity with the absence of symmetry in the tail portion of the molecule. If activity is observed, further studies to enhance activity by controlling enantioselectivity will be performed.

Screening for biofilm dispersion

Initial screening focused on the ability of the compounds to induce dispersion of pre-formed *M. smegmatis* biofilms as assessed using a crystal violet reporter assay (Table S.1) [17]. Previously, we found compound **1** to have an EC_{50} of 52 μM. Upon evaluation of the branched derivative library, it was revealed that lengthening the tail one carbon **(10E)** increased activity, with this compound exhibiting an EC_{50} of 19.9 ± 5.3 μM (Figure S.1). Shortening the tail by one carbon **(10D)**, reduced activity to an EC_{50} of 86.3 ± 8.3 μM (Figure S.2). Adding or removing two carbons to the tail resulted in a further decrease in activity with compounds **10C** (two carbons shorter) and **10F** (two carbons longer) exhibiting EC_{50} values of >200 μM and 150 ± 11 μM respectively. The single linear tails **(9A-K)**, however, possessed limited dispersion activity. Compound **9H** displayed the best dispersion activity with an EC_{50} of 89.9 ± 15 μM**.** None of the other branched derivatives induced dispersion at 200 μM (highest concentration tested). Next, we evaluated the asymmetric derivatives that contained the most active branched tail lengths. Compound **11A,** which has the same length carbon tail (seven total carbons) as the most active compound **10E,** exhibited an EC_{50} value of 109 ± 13 μM. The addition of one carbon in compound **11B** resulted in a decrease in EC_{50} value to 57.8 ± 4.6 μM, though this EC_{50} was still not comparable to lead compound **1** or **10E** (Figure S.3). With that, the synthesis efforts shifted to incorporating a phenalkyl chain to one side of the branch while keeping the other branch consistent with a linear chain of six carbons. Compounds **12** and **13** returned EC_{50} values of 66.9 ± 3.0 and 85.1 ± 13 μM respectively, which were again less active than compounds **1** or **10E.** The incorporation of the aryl substituents did not improve upon the activity of **10E** (Complete results can be found in Table S.1).

Screening for potentiation activity

We previously reported that compound **1** exhibits synergy with β-lactam antibiotics against planktonic *M. tuberculosis* [18]. Based upon this observation, these newly synthesized derivatives were screened for the ability to potentiate several cephalosporin and penicillin antibiotics against planktonic *M. smegmatis*. In order to evaluate the potentiation activity of these compounds against *M. smegmatis*, the MIC of antibiotics and the compounds alone were first determined. Following this determination, the MIC of each antibiotic was measured in the presence of each compound at 30% its MIC, which is the typical concentration we employ for potentiation assays [18, 19]. Interestingly, none of the symmetric branched tail compounds produced any notable reductions at this concentration. When compound **1** was tested at 50% its MIC, results were modest producing at most a 16-fold reduction of ceftazidime's MIC. For the linear tail compounds at 30% the MIC, activity increased slightly as the length of the tail increased, but remained modest with the largest fold reduction observed being a four-fold reduction with cefotaxime and compound **9K**. Interestingly, when the asymmetric tails were analyzed, compound **11A** showed no notable activity at 30% its MIC for five of the six antibiotics, but effected a 32-fold reduction in the cefotaxime MIC. Compound **11B,** which was a more potent biofilm inhibitor than **11A**, showed only an eight fold reduction in the MIC of cefotaxime and again no notable reduction in the MICs of the other antibiotics tested (Table 1). Additionally, compounds **12** and **13** were screened and again, it was seen that the less active biofilm disruptor, compound **13**, was more active than the more potent antibiofilm derivative (**12)**, but activity was limited to two and four-fold reductions except again in the case of cefotaxime, where the reduction was 32-fold (Complete results can be found in Tables S.2 and S.3). The selectivity of this series of compounds for cefotaxime potentiation over other β-lactam antibiotics mirrors that observed with a 2-aminobenzimidazole series previously reported, which possess a pattern of selectivity distinct from direct β-lactamase inhibitors, such as clavulanic acid, and does not directly inhibit β-lactamase activity [18].

Conclusion

In conclusion, the previously reported potentiation and biofilm dispersion activity of compound **1** against *M. smegmatis* and *M. tuberculosis* led us to investigate the SAR of the alkyl tail region of this compound. A library consisting of linear and branched symmetric and asymmetric tails were synthesized and screened for their ability to disperse biofilms and potentiate a subset of β-lactam antibiotics against *M. smegmatis*. This screen revealed that compound **10E** improved upon the previously reported EC_{50} value of compound **1**, but did not improve potentiation capabilities. Screening of the linear tails revealed little to no dispersion or potentiation capabilities. The asymmetric tails containing alkyl chains with lengths comparable to the most active branched compounds and phenyl rings added in different orientations proved to be better than the linear compounds at biofilm dispersion, but did not have EC_{50} values comparable to compound **1** or **10E**. Despite the disappointing antibiofilm activity, we found that the asymmetric tails possessed improved potentiation capabilities, with compounds **11A** and **13** reducing the MIC of cefotaxime from 64 to 2 μg/mL. These results show that biofilm dispersion capabilities may be decoupled from antibiotic potentiation capabilities for this series of compounds.

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Conflict of Interest

Christian Melander is a co-founder of Agile Sciences, a biotechnology company focused on commercial applications of nitrogen-rich small molecules, and currently sits on its board-of-directors.

Figure legends

Figure 1- Structure of previously reported compound **1.**

Figure S.1- Determination of EC_{50} for compound **10E.**

Figure S.2- Determination of EC_{50} for compound **10D.**

Figure S.3- Determination of EC_{50} for compound **11B.**

Scheme 1- Retrosynthetic approach to the synthesis of the proposed 2-AI library.

Scheme 2- Synthesis of the 2-AI region of derivative library. a)BnOH, TEA, DMAP, DCM (96%); b)i) $(COCl)_2$, DMF, DCM; ii) CH_2N_2; iii) HBr (70%); c)Boc-guanidine, DMF (50%); d) $(Boc)_2O$, TEA, DMAP (90%); e) 10 wt% Pd, H_2, THF (99%).

Scheme 3- Coupling amine tails to produce desired reverse amide compounds. a) H_2N-R, HBTU, TEA, DCM (40-70%); b) TFA, DCM; c) HCl, MeOH (50-99%).

Scheme S.1- Synthesis of the branched tails from the respective carbonyl derivatives. a) i) $NH_4CH_3CO_2$, Anhydrous CH_3OH, 90 minutes; ii) $NaBH_3CN$, 60 hours.

Scheme S.2- Synthesis of the asymmetric tails for compounds **11A-B** from the respective carbonyl derivatives. a) i) $NH_4CH_3CO_2$, Anhydrous CH_3OH, 90 minutes; ii) $NaBH_3CN$, 60 hours.

Scheme S.3- Synthesis of asymmetric tail for compound **12** from the commercially available carboxylic acid. a) Oxalyl chloride, Anhydrous DCM, catalytic DMF; b) N,O dimethyl hydroxylamine, TEA, Anhydrous DCM; c) Hexylmagnesium bromide, THF; d) i) $NH_4CH_3CO_2$, Anhydrous CH_3OH, 90 minutes; ii) $NaBH_3CN$, 60 hours.

Scheme S.4- Synthesis of asymmetric tail for compound **13** from the commercially available acid chloride. a) N,O dimethyl hydroxylamine, TEA, Anhydrous DCM; b) Hexylmagnesium bromide, THF; c) i) $NH_4CH_3CO_2$, Anhydrous CH_3OH, 90 minutes; ii) $NaBH_3CN$, 60 hours.

Table 1- Biofilm dispersion and repotentiation activity of the most active derivatives (Fold reductions in parenthesis).

Table S.1- EC_{50} values for derivative library obtained from biofilm dispersion assay with *M. smegmatis*.

Table S.2- Potentiation of common penicillin β-lactam antibiotics against *M. smegmatis* using 2-AI derivative library.

Table S.3- Potentiation of common cephalosporin β-lactam antibiotics against *M. smegmatis* using 2-AI derivative library.

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Table 1

Compound	EC_{50} (μM)	MIC (μM)	Concentration Tested (μM)	Penicillin MIC (μg/mL)	Cefotaxime MIC (μg/mL)	Ceftazidime MIC (μg/mL)
-	-	-	-	256	64	512
1	52	40	10	128 (2)	64 (0)	512 (0)
	-	-	20	64 (4)	8 (8)	32 (16)
10E	19.9 ± 5.3	50	12.5	256(0)	64 (0)	512 (0)
11A	109 ± 13	100	30	128 (2)	2 (32)	128 (4)
11B	57.8±4.6	50	15	128 (2)	8 (8)	128 (4)
12	66.9±3.0	50	15	64 (4)	8 (8)	512 (0)
13	85.1±13	50	15	64(4)	2(32)	512 (0)