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Structural Studies on 4,5-Disubstituted 2-Aminoimidazole-Based Biofilm Modulators that Suppress Bacterial Resistance to β-Lactams

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A library of 4,5-disubstituted 2-aminoimidazole triazole amide (2-AITA) conjugates has been successfully assembled. Upon biological screening, this class of small molecules was discovered as enhanced biofilm regulators through non-microbicidal mechanisms against methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Acinetobacter baumannii* (MDRAB), with active concentrations in the low micromolar range. The library was also subjected to synergism and resensitization studies with β-lactam antibiotics against MRSA. Lead compounds were identified that suppress the antibiotic resistance of MRSA by working synergistically with oxacillin, a β-lactam antibiotic resistant to penicillinase. A further structure–activity relationship (SAR) study on the parent 2-AITA compound delivered a 2-aminoimidazole diamide (2-AIDA) conjugate with significantly increased synergistic activity with oxacillin against MRSA, decreasing the MIC value of the β-lactam antibiotic by 64-fold. Increased anti-biofilm activity did not necessarily lead to increased suppression of antibiotic resistance, which indicates that biofilm inhibition and resensitization are most likely occurring via distinct mechanisms.

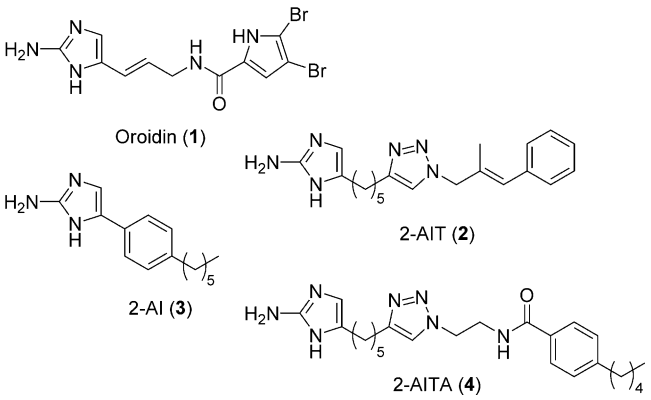
Introduction

Infectious disease is the second leading cause of mortality throughout the world, accounting for more than 25% of the annual global deaths, and remains a serious threat to our society.[1] This scenario has been exacerbated in the past decades by the emergence of wide-spread, drug-resistant pathogens.[2] Bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Acinetobacter baumannii* (MDRAB) are now common nosocomial pathogens and mediate infections that are extremely difficult or even impossible to treat.[3] MRSA accounts for 80% of the hospital-acquired *S. aureus* infections and is now a greater cause of deaths annually in comparison with complications due to HIV,[4,5] while MDRAB is related to many cases of serious infections among wounded soldiers in the Middle East.[6]

Bacterial biofilms, defined as sessile communities of microorganisms that coexist as highly differentiated associates in an extracellular matrix of exo-polysaccharides,[7] play a significant role in the development of antibiotic resistance by bacteria.[8] Within a biofilm state, bacteria are up to 1000-fold more resistant to conventional antibiotics in comparison with their planktonic counterparts.[9] Biofilms have been recognized over the past decade as a global threat.[10] Approximately 80% of the microbial mass on earth is in a biofilm state, and the US National Institutes of Health (NIH) estimates that over 80% of bacterial infections in humans are related to biofilms.[11]

A number of natural products, such as the marine alkaloid oroidin (**1**), are known to possess moderate anti-biofilm activity.[12] Given the ubiquitous nature of biofilms in antimicrobial therapy, efforts have been directed toward the development of small molecules that modulate biofilm development.[13] A class of 2-aminoimidazole triazole conjugates, exemplified here by 2-AIT (**2**), has been discovered to inhibit and disperse biofilms through non-microbicidal mechanisms across bacterial order, class and phylum.[14] Further structure–activity relationship (SAR) studies on these 2-AIT analogues have identified molecules with improved anti-biofilm activity.[15]

Given the significant pressure that antibiotic-resistant bacteria have posed on the medical community and contrasted with



the scarce development of novel antibiotics,[16] methods to deal with the threat of multidrug-resistant bacteria are sorely needed. Recently, Klitgaard and co-workers reported that thioridazine, an antipsychotic drug, was able to reverse oxacillin resistance in MRSA without affecting bacteria growth.[17] In an-

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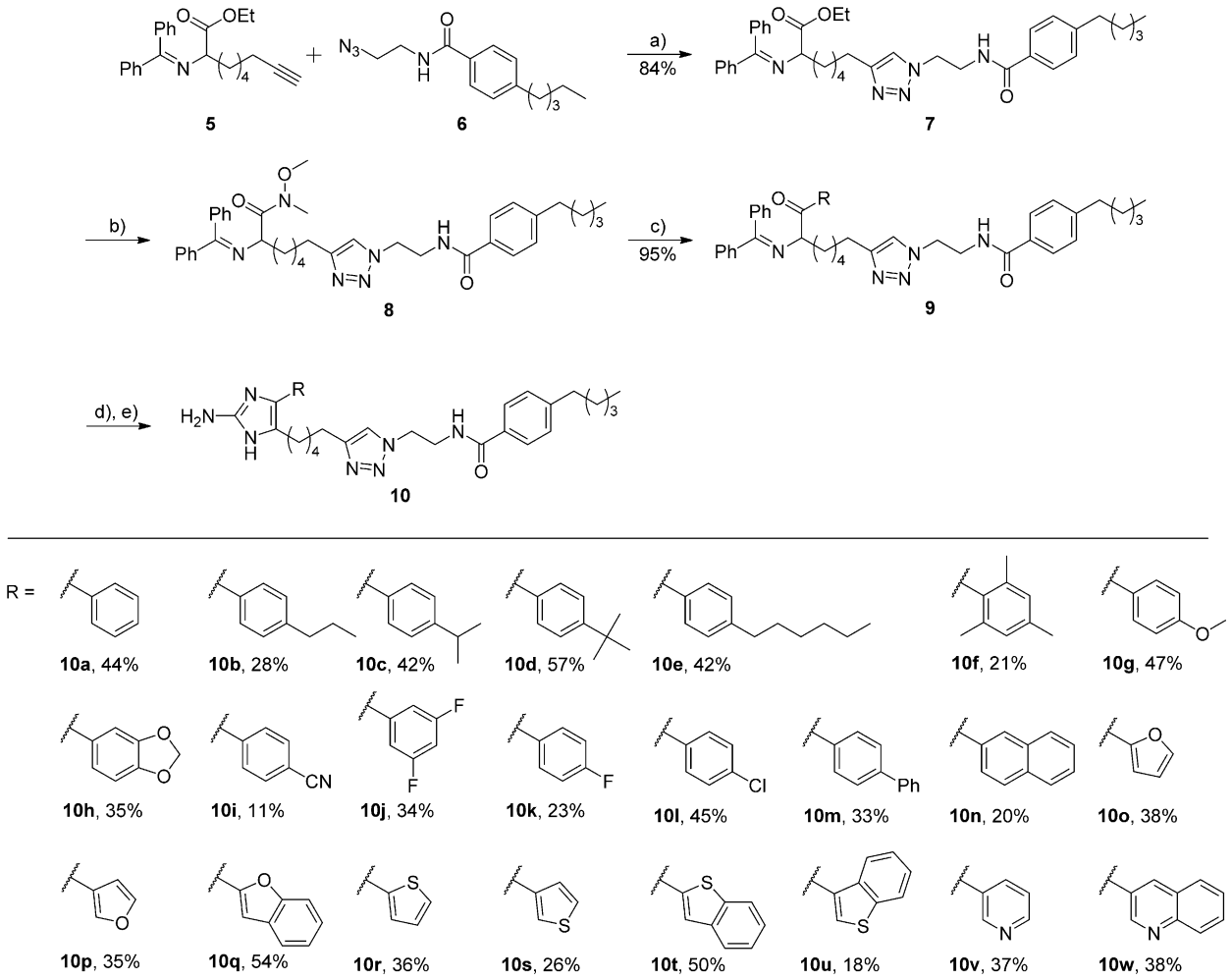
other study, we found that 2-AI (**3**) was able to suppress carbapenem resistance in New Delhi metallo-β-lactamase-1 (NDM-1)-producing *Klebsiella pneumoniae*.[18]

In an effort to combat antibiotic resistance and reveal a correlation between antibiotic resistance and biofilm formation, we screened our in-house 2-aminoimidazole (2-AI) library seeking dual inhibitors of biofilm formation and β-lactam resistance in MRSA. From these studies, the 2-aminoimidazole triazole amide conjugate **4** (2-AITA) was identified as the most active molecule to resensitize a representative MRSA strain (ATCC BAA-44) toward oxacillin. In order to maximize the biological activity, we were interested in studying the impact of installing a 4,5-disubstitution pattern on 2-AITA (**4**). It has been previously demonstrated that a 4,5-disubstitution pattern enhances biological activity, and 2-AI analogues with aromatic substitutions were most active.[19,20] Herein, we report the assembly of 4,5-disubstituted 2-AITA derivatives with a variety of aromatic substitutions, including heterocycles, based on the use of Weinreb amide intermediates and organometallic additions.[20] This class of 4,5-disubstituted 2-AITA analogues possess improved antimicrobial activity, which is consistent with our previous findings on polysubstituted 2AIs.[19,21] Furthermore, these compounds work synergistically with oxacillin against MRSA (ATCC# BAA-44). Compounds with heterocyclic substitutions were found to be less toxic to bacteria and continue to regulate biofilm formation through non-microbicidal mechanisms. The necessity of the triazole was also investigated, and a 2-aminoimidazole diamide conjugate (2-AIDA) was identified with augmented resensitization activity against MRSA toward oxacillin.

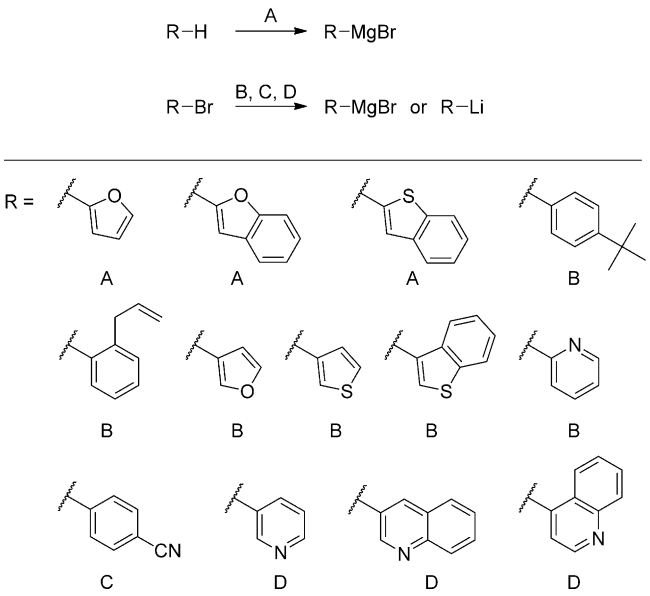
Results and Discussion

Synthesis of the 4,5-disubstituted 2-AITA library is outlined in Scheme 1. Protected α-amino-alkynyl ethylester **5** and azido benzamide **6** were prepared as previously described.[20,22] The copper-catalyzed [3+2] cycloaddition (click reaction) was performed between **5** and **6** to deliver desired triazole **7** in 84% yield. Triazole **7** was then treated with *N*,*O*-dimethylhydroxyamine hydrochloride and isopropylmagnesium chloride to generate target Weinreb amide **8** in 95% yield. With amide **8** in hand, a variety of commercially available Grignard reagents



Scheme 1. Synthetic route to 4,5-disubstituted 2AITAs. *Reagents and conditions:* a) sodium ascorbate, $CuSO_4$, *t*BuOH/H_2O/CH_2Cl_2 (2:2:1), RT, 12 h (84%); b) *O*,*N*-dimethylhydroxyamine·HCl, *i*PrMgCl, THF, −20→0 °C, 3 h (95%); c) RMgBr, THF, −20→0 °C, 4 h; d) 2 N HCl, EtOH, RT, 12 h; e) NH_2CN, EtOH/H_2O (1:1), pH 4.3, 95 °C, 3 h. Yields given for compounds **10** are overall yields from **8** (three steps).

were reacted with **8** to yield corresponding ketones **9**. Magnesium/lithium reagents of heterocycles and some phenyl derivatives were also generated in situ from the corresponding aromatics or aryl halides (Scheme 2). Deprotection of ketones **9**



Scheme 2. Preparation of aryl Grignard/lithium reagents. *Reagents and conditions*: Method A: 1) *n*BuLi, THF, −78–0 °C, 2 h; 2) $MgBr_2{\cdot}Et_2O$, THF, 0 °C-RT, 1 h; Method B: Mg turnings, LiCl, DIBAL (I_2), THF, RT, 4 h; Method C: *i*PrMgCl–LiCl, −15 °C-RT, 6 h; Method D: *n*BuLi, −78 °C, Et_2O, 4 h.

with 2 N hydrochloric acid in ethanol and cyclization with cyanamide was performed in tandem to deliver the final 4,5-disubstituted 2-AITA derivatives (**10**). We found that the aminoketones resulting from addition of 2-allylphenyl, 2-pyridinyl, and 4-quinolinyl organometallic reagents were unstable to the deprotection/cyclization sequence and so were not studied further. After purification (CH_2Cl_2/MeOH/NH_3), all 2-AITA derivatives were converted to their hydrochloride salts for biological screening.

The pilot library was first tested for its anti-biofilm activity at 100 μM against MRSA and MDRAB (ATCC BAA-1605) using a crystal violet reporter assay.[23] Compounds that produced >95% biofilm inhibition activity were then subjected to a dose–response study, and the IC_{50} value for each compound was determined. This data is summarized in Table 1. When the library was screened for biofilm inhibition against MDRAB, most compounds showed a precipitous drop over a narrow concentration range in their dose–response curves that typically indicates that biofilm inhibition is acting through a strict microbicidal mechanism. However, compounds **10e**, **10q** and **10t** were able to modulate biofilm development without effecting bacterial growth (Table 1), with IC_{50} values of 25.8 μM, 69.4 μM, and 50.4 μM, respectively. In comparison, parent 2-AITA **4** inhibited MDRAB biofilm formation <5% at 100 μM. A number of compounds were identified that inhibit the development of MRSA biofilm development, yet a majority of these compounds also affected bacterial growth. In comparison with

Table 1. Inhibition of MRSA and MDRAB biofilm formation by compounds **4** and **10a–w**.

Compd	IC_{50} [μM][a]	Compd	IC_{50} [μM][a]	Compd	IC_{50} [μM][a]
MRSA					
10a	4.1±0.3	**10i**	8.7±1.6	**10q**	6.0±2.0
10b	2.1±1.0	**10j**	4.4±0.6	**10r**	10.9±1.9
10c	2.9±0.7	**10k**	3.9±0.3	**10s**	5.7±0.5
10d	3.7±2.3	**10l**	3.6±1.0	**10t**	4.8±0.7
10e	2.8±0.5	**10m**	2.0±0.2	**10u**	2.6±0.9
10f	3.5±0.8	**10n**	2.3±0.6	**10v**	19.2±1.8
10g	3.6±0.8	**10o**	13.9±2.7	**10w**	4.2±1.0[b]
10h	3.7±0.8[b]	**10p**	7.2±1.8[b]	**4**	15.2±1.7[b]
MDRAB					
10e	25.8±3.4[b]	**10q**	69.4±6.8[b]	**10t**	50.4±13.4[b]

[a] Values represent the mean ±SD of at least three independent experiments. [b] Compound has no affect on bacteria growth.

parent compound **4** (IC_{50} = 15.2 μM), compounds **10h**, **10p** and **10w** exhibited increased activity to modulate MRSA biofilms through a non-microbicidal mechanism (IC_{50} values of 3.7, 7.2, and 4.2 μM, respectively).

Next, we assessed the ability of the pilot library to reverse antibiotic resistance of MRSA toward β-lactams. In this assay, the minimum inhibitory concentration (MIC) of each 4,5-disubstituted 2-AITA was first determined, and 25% of the MIC concentration of each compound was subsequently employed in the resensitization activity. It is our experience from previous studies that 2-AI derivatives typically exhibit weak microbicidal activity when tested at 25% of their MIC values, thus allowing us to attribute the suppression of antibiotic resistance mainly to non-microbicidal effects of the compounds. MRSA was pretreated with each compound for 30 min, and the MIC values for oxacillin and penicillin G were established (in the presence of 25% MIC of each 2-AI compound; Table 2). We previously established that parent compound **4** decreases the MIC value of oxacillin against the BAA-44 strain of MRSA by fourfold.[19] Compounds **10e**, **10m** and **10p** showed comparative resensitization activity and lowered the MIC value of oxacillin against MRSA by fourfold. The library was also screened for the ability

Table 2. Resensitization of MRSA to oxacillin and penicillin G.

Compd (MIC/ test concn)[a]	MIC [μg mL^{-1}]		Compd (MIC/ test concn)[a]	MIC [μg mL^{-1}]	
	oxacillin	penicillin		oxacillin	penicillin
10a (4/1)	16	32	**10m** (4/1)	8	16
10b (2/0.5)	32	32	**10n** (4/1)	16	16
10c (2/0.5)	16	32	**10o** (16/4)	16	32
10d (2/0.5)	16	32	**10p** (16/4)	8	16
10e (8/2)	8	16	**10q** (8/2)	16	16
10f (4/1)	16	32	**10r** (16/4)	16	32
10g (4/1)	32	32	**10s** (8/2)	32	32
10h (8/2)	16	32	**10t** (16/4)	16	16
10i (16/4)	16	32	**10u** (8/2)	16	16
10j (4/1)	32	32	**10v** (32/8)	32	32
10k (4/1)	32	32	**10w** (8/2)	32	32
10l (2/0.5)	32	32	no compd	32	32

[a] MIC values and test concentrations are given in μg mL^{-1}.

to resensitize MDRAB to the effects of imipenem, a broad-spectrum β-lactam antibiotic. None of the compounds elicited any appreciable resensitization activity against MDRAB, recording less than a twofold decrease in the MIC value of imipenem.

As resensitization activity was not significantly improved by imparting the 4,5-disubstitution pattern on 2-AITA (**4**), we elected to pursue additional SAR studies to better understand the structural requirements necessary to augment activity. The parent compound 2-AITA (**4**) consists of three functional regions: the 2-AI head, the triazole linker, and benzamide tail (Figure 1). Since the tail region has been previously modified,[22] and modifications to the head region are described

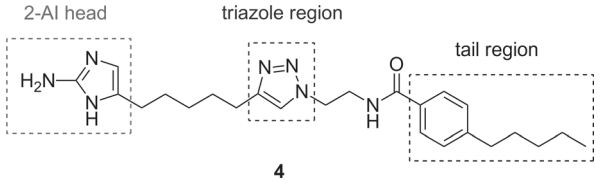


Figure 1. Overview of the structure–activity relationship (SAR) study of parent compound 2-AITA (**4**).

above, the last region left for modification is the triazole. Our initial thought was to replace the triazole with an amide moiety based upon our earlier findings that the triazole and amide functionalities are interchangeable in the context of 2-AI biological activity and that replacement of one with the other can result in augmentation of activity.[24]

The approach employed to access derivatives of 2-AITA (**4**) with the triazole moiety either removed or replaced with an amide is outlined in Scheme 3. Bromohexanoic acid **11** was treated with sodium azide to give azidohexanoic acid **12** in 84% yield. Acid **12** was reacted with oxalyl chloride and diazomethane followed by quenching the resulting α-diazoketone with hydrogen bromide to generate the α-bromoketone intermediate in 90% yield. Cyclization with *tert*-butyloxycarbonyl (Boc)-protected guanidine (51% yield) and subsequent exhaustive Boc protection (83% yield) delivered desired precursor azido tri-Boc-protected 2-AI **13**. Another precursor, tri-Boc-protected 2-AI benzoate **16**, was generated through the treatment of dicarboxylic acid **14** with acetic anhydride and benzyl alcohol to give the target benzyl-protected dicarboxylic acid (**15**) in 44% yield. Acid **15** was then converted to the corresponding α-bromoketone followed by cyclization with Boc-guanidine and exhaustive Boc protection to deliver **16**. Amide **17** was accessed by reduction of **6** with hydrogen in the presence of palladium on carbon, while **19** was generated from coupling of **18** with β-alanine in 45% yield.

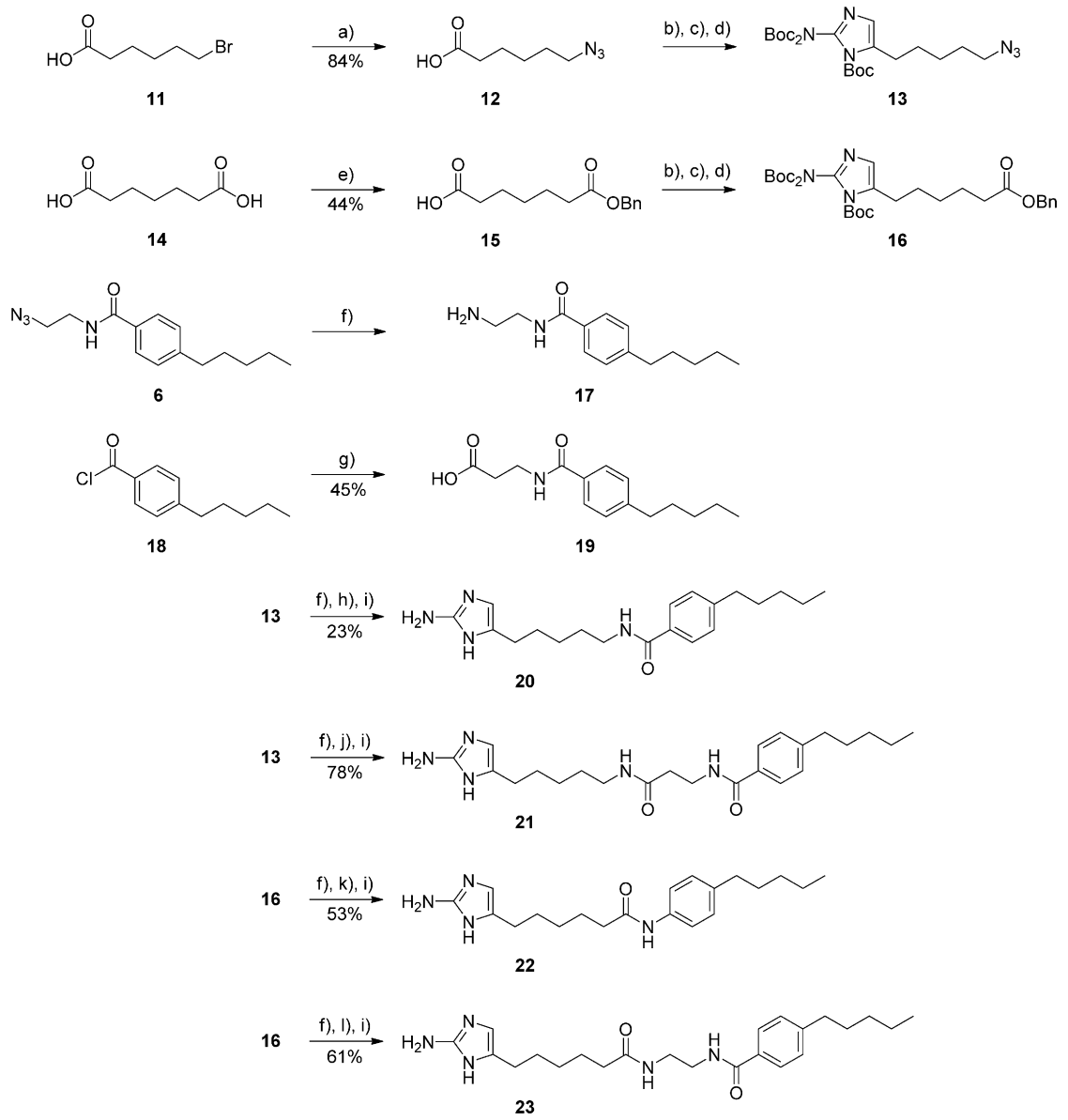
With all intermediates in hand, azide **13** was reduced to the primary amine by palladium-catalyzed hydrogenation and coupled with **18** or **19** in the presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxybenzotriazole (HOBt) and *N*,*N*-diisopropylethylamine (DIEA). Finally, Boc deprotection with 2 N hydrochloric acid in ethanol delivered target 2-AI derivatives **20** and **21** in 23% and 78% yield, respectively. Compounds **22** and **23** were accessed by first debenzylating **16** with hydrogen and palladium on carbon to generate the corresponding carboxylic acids, which were then coupled with either 4-pentylaniline or **17** followed by Boc deprotection to give desired products **22** and **23** in 53% and 61% yield, respectively.

Compounds **20**–**23** were screened for anti-biofilm and resensitization activity, and the results are summarized in Table 3. Gratifyingly, this class of 2-AI derivatives was also able to inhibit MRSA and MDRAB biofilm formation via non-microbicidal mechanisms. More importantly, derivative **23** was identified to possess significantly enhanced resensitization activity and suppressed β-lactam resistance of MRSA by lowering the MIC value of oxacillin from 32 $\mu g\,mL^{-1}$ to 0.5 $\mu g\,mL^{-1}$ (64-fold) and the MIC value of penicillin G from 32 $\mu g\,mL^{-1}$ to 2 $\mu g\,mL^{-1}$ (16-fold) when the bacteria was grown in the presence of 16 $\mu g\,mL^{-1}$ (35.6 μM) of compound **23**. Growth curve analysis indicated that all four compounds altered early (0–8 h) MRSA growth, with **20** having the weakest effect; however, bacterial growth was equivalent for all treated and untreated samples by 24 h. Parent compound **4** and the most active derivative (**23**) have similar effects on bacteria growth inhibition (see Supporting Information).

Given the activities of the lead compounds, a checkerboard assay was employed to determine the synergistic activity of all 2-AI derivatives in combination with oxacillin.[25] The ΣFIC value is used to evaluate whether two combined agents are working synergistically, and when the ΣFIC value is less than or equal to 0.5, the combination is considered to be synergistic. As shown in Table 4, most compounds work synergistically with oxacillin, with derivative **23** determined to be the most promising compound with a ΣFIC value of 0.25.

Conclusions

We have synthesized a number of 4,5-disubstituted 2-AITA derivatives and evaluated their anti-biofilm and antibiotic resensitization activity against MRSA and MDRAB. For most of these compounds, this substitution pattern increased the microbicidal and anti-biofilm activities. Compounds **10h**, **10p**, and **10w** were identified as lead compounds against MRSA, while compounds **10e**, **10q**, **10t** were most active against MDRAB; all six compounds acted via a non-microbicidal mechanism. Compounds with heterocycles fused with phenyl substitutions generally displayed enhanced anti-biofilm activity and less toxicity. Unfortunately, no significant improvement in suppressing antibiotic resistance of MRSA toward β-lactams was observed in comparison with parent compound 2-AITA (**4**). A further SAR study indicated that replacement of the triazole moiety in compound **4** with an amide enhanced its ability to suppress antibiotic resistance of MRSA toward β-lactams without compromising the anti-biofilm activity. Compound **23** was identified as the most potent molecule in this study and decreased the MIC value of oxacillin against MRSA by 64-fold. The fact that augmentation of the anti-biofilm activity does not necessarily lead to increased suppression of antibiotic resistance indicates that biofilm inhibition and resensitization are most likely occurring via distinct mechanisms. Further mechanistic



Scheme 3. Synthesis of 2-AI amide/diamide derivatives. *Reagents and conditions*: a) NaN_3, DMF, RT, 12 h (84%); b) 1. oxalyl chloride, CH_2Cl_2, DMF (cat), 0 °C→RT, 1 h; 2. CH_2N_2, Et_2O/CH_2Cl_2 (1:1), 0 °C, 1 h; 3. HBr, 0 °C, 30 min (99%); c) Boc-guanidine, DMF, RT, 3 d (51%); d) Boc_2O, DMAP, THF, RT, 12 h (87%); e) 1. Ac_2O, xylenes, reflux, 2 h; 2. BnOH, 60 °C, 12 h (44%); f) H_2, Pd/C, 1 atm, THF, RT, 12 h; g) β-analine, Et_3N, CH_2Cl_2, 0 °C→RT, 12 h (45%); h) **18**, Et_3N, CH_2Cl_2, 0 °C→RT, 12 h (23%); i) TFA/CH_2Cl_2 (1:4), RT, 12 h; j) **19**, EDC·HCl, HOBt, DIEA, DMF, RT, 12 h (82%); k) 4-pentylaniline, EDC·HCl, HOBt, DIEA, DMF, RT, 12 h (63%); l) **17**, EDC·HCl, HOBt, DIEA, DMF, RT, 12 h (74%).

Table 3. Biological activity of 2-AI amide/diamides derivatives.

Compd (MIC/test concn)[a]	MIC [μg mL^{-1}]		IC_{50} [μM]	
	oxacillin	penicillin	MRSA	MDRAB
20 (16/4)	4	16	14.6 ± 1.8[b]	136.6 ± 2.2
21 (32/8)	4	16	35.0 ± 3.8	150.1 ± 2.5
22 (8/2)	16	16	6.4 ± 2.8	127.9 ± 15.2
23 (64/16)	0.5	2	6.5 ± 3.1	N/A
no compd	32	32	–	–

[a] MIC values and test concentrations against MRSA BAA-44 are given in μg mL^{-1}. [b] Values represent the mean ± SD of at least three independent experiments.

studies and evaluation of these classes of compounds in animal models of infections are currently underway and will be reported in due course.

Experimental Section

Chemistry

General: All reagents were purchased from commercially available sources and used without further purification. Chromatography was performed using 60 Å mesh standard grade silica gel from Sorbtech (Atlanta, GA, USA). Infrared (IR) spectra were obtained on a FT/IR-4100 spectrophotometer; $\tilde{\nu}$ are reported in cm^{-1}. UV/Vis

Table 4. Synergistic activity of all compounds with oxacillin against MRSA.

Compd	ΣFIC[a]	Compd	ΣFIC[a]	Compd	ΣFIC[a]
10a	0.62	**10k**	1	**10t**	0.38
10b	0.62	**10l**	0.62	**10u**	0.5
10c	0.5	**10m**	0.38	**10v**	0.75
10d	0.5	**10n**	0.38	**10w**	0.5
10e	0.5	**10o**	0.56	**20**	0.5
10f	0.62	**10p**	0.38	**21**	0.5
10g	1	**10q**	0.31	**22**	0.56
10h	0.53	**10r**	0.75	**23**	0.25
10i	0.56	**10s**	0.53	**4**	0.5
10j	0.38	–	–	–	–

[a] $\Sigma FIC = FIC_{Compd} + FIC_{Antibiotic}$; $FIC_{Compd} = [MIC_{Compd}$ in combination$]/[MIC_{Compd}$ alone$]$; $FIC_{Antibiotic} = [MIC_{Antibiotic}$ in combination$]/[MIC_{Antibiotic}$ alone$]$. The combination is considered synergistic if $\Sigma FIC \leq 0.5$, indifferent if $0.5 < \Sigma FIC < 2$, and antagonistic if $\Sigma FIC \geq 2$.

absorbance was recorded on a Genesys 10 scanning UV/Vis spectrophotometer; λ_{max} are reported in nm. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded at 25 °C on Varian Mercury spectrometers. Deuterated solvents were obtained from Cambridge Isotope Labs and used as received. Chemical shifts (δ) are given in ppm relative to tetramethylsilane (TMS) or the respective residual solvent peak. Coupling constants (J) are in given in Hertz (Hz). Abbreviations used are s = singlet, bs = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, td = triplet of doublets, tt = triplet of triplets, bt = broad triplet, q = quartet, m = multiplet, bm = broad multiplet. Mass spectrometry (MS) was performed by the North Carolina State University Department of Chemistry Mass Spectrometry Facility.

Full synthetic protocols for the preparation of Grignard/lithium reagents (S2) and the synthesis of 4,5-disubstituted-2-AITA derivatives (S3), along with NMR data for compounds **7, 8, 10a–w** (S20) and conjugates **12–23** (S45) are available in the Supporting Information.

Preparation of Grignard/lithium reagents

Method A: The appropriate heterocycle was dissolved in anhydrous THF in a flame-dried 50 mL round-bottomed flask, and the solution was cooled to −78 °C. *n*BuLi (1.6 M) was added, and the mixture was stirred for 2 h while warming to 0 °C. The resulting solution was mixed with a slurry of $MgBr_2{\cdot}Et_2O$ in anhydrous THF at 0 °C and stirred at RT for 1 h until all solids were dissolved.

Benzo[*b*]thiophen-2-ylmagnesium bromide: Benzo[*b*]thiophene (0.805 g, 6 mmol) and *n*BuLi (1.6 M, 3.75 mL, 6 mmol) in dry THF (10 mL) was reacted with $MgBr_2{\cdot}Et_2O$ (1.549 g, 6 mmol) in dry THF (10 mL) according to method A to deliver the desired Grignard reagent.

Benzofuran-2-ylmagnesium bromide: Benzofuran (0.236 g, 2 mmol) and *n*BuLi (1.6 M, 1.25 mL, 6 mmol) in dry THF (3 mL) was reacted with $MgBr_2{\cdot}Et_2O$ (0.516 g, 2 mmol) in dry THF (2 mL) according to method A to deliver the desired Grignard reagent.

Method B: A flame-dried vial (23×85 mm) was charged with Mg turnings and LiCl followed by diisobutylaluminum hydride (DIBAL) in anhydrous THF. The resulting suspension was stirred at RT for 5 min. The resulting mixture was reacted with the appropriate aryl bromide in dry THF and stirred at RT until all Mg had disappeared.

Furan-3-ylmagnesium bromide: 3-Bromofuran (0.294 g, 2 mmol) in dry THF (2 mL) was reacted with Mg turnings (0.054 g, 2.2 mmol), LiCl (0.085 g, 2 mmol) and DIBAL (1.0 M, 0.02 mL, 0.02 mmol) in dry THF (4 mL) for 3 h according to method B to deliver the desired Grignard reagent.

Method C: **(4-Cyanophenyl)magnesium bromide**: A flame-dried vial (23×85 mm) was charged with 4-bromobenzonitrile (0.724 g, 4 mmol), cooled to −15 °C, and *i*PrMgCl·LiCl (4.5 mL, 0.93 M) was added. The reaction was stirred at −15 °C→0 °C for 3 h, giving the desired Grignard reagent in 97% yield (0.86 M, 4.5 mL), as determined by titration with salicylaldehyde phenylhydrazone.[26]

Method D: The appropriate aryl bromide in a flame-dried 50 mL round-bottomed flask was dissolved in anhydrous Et_2O and cooled to −78 °C. The reaction was treated dropwise with *n*BuLi (1.6 M) and then stirred at −78 °C→−60 °C for 3 h.

Quinolin-3-ylmagnesium bromide: 3-Bromoquinoline (0.813 g, 3.9 mmol) and *n*BuLi (1.6 M, 2.9 mL, 4.6 mmol) were reacted in dry Et_2O (12 mL) according to method D to deliver the desired lithium reagent.

Synthesis of 4,5-disubstituted 2-AITA derivatives 10a–w

Ethyl 2-(diphenylmethyleneamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1*H*-1,2,3-triazol-4-yl)heptanoate (7): Compound **5** (2.167 g, 6 mmol) was dissolved in CH_2Cl_2/*t*BuOH/H_2O (1:2:2, 50 mL) in a 150 mL round-bottomed flask. Compound **6** (1.874 g, 7.2 mmol) was added to the solution at RT. With vigorous stirring, $CuSO_4$ (0.144 g, 0.9 mmol) and sodium ascorbate (0.475 g, 2.4 mmol) were added, and the resulting solution was stirred at RT for 12 h. The mixture was extracted with CH_2Cl_2 (3×15 mL). The combined organic extracts were washed with brine (20 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting residue was purified by column chromatography (hexane/EtOAc, 9:1→1:4) to afford target compound **7** as a yellow oil (3.135 g, 84%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.03 (s, 1H), 7.76 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 7.2 Hz, 2H), 7.41 (m, 3H), 7.35 (m, 2H), 7.28 (m, 2H), 7.15 (m, 4H), 4.52 (m, 2H), 4.14 (m, 2H), 4.04 (t, J = 6.8 Hz, 1H), 3.88 (m, 2H), 2.58 (t, J = 7.6 Hz, 4H), 1.90 (m, 2H), 1.57 (m, 4H), 1.29 (m, 6H), 1.23 (t, J = 7.6 Hz, 3H), 1.22 (m, 2H), 0.87 ppm (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.2, 170.1, 167.8, 147.8, 146.7, 139.2, 136.2, 131.1, 130.1, 128.5, 128.4, 128.3, 128.2, 127.8, 127.5, 127.1, 121.7, 65.1, 60.6, 49.0, 39.8, 35.5, 33.3, 31.1, 30.6, 29.0, 28.7, 25.5, 25.2, 22.2, 14.0, 13.8 ppm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{38}H_{47}N_5O_3$: 622.3751, found 622.3759.

***N*-(2-(4-(6-(Diphenylmethyleneamino)-7-(methoxy(methyl)amino)-7-oxoheptyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide (8)**: A flame-dried 50 mL round-bottomed flask was charged with **7** (1.819 g, 2.9 mmol) and anhydrous THF (10 mL). The solution was stirred under nitrogen at −20 °C until **7** was fully dissolved. The solution was then treated with *N*,*O*-dimethylhydroxylamine hydrochloride (1.141 g, 11.7 mmol), and *i*PrMgCl (14.8 mL, 23.4 mmol, 1.58 M) was then added dropwise, while the reaction temperature was maintained below −5 °C. The resulting solution was stirred at 0 °C for 3 h. The reaction was quenched with saturated aq NH_4Cl (10 mL) and then extracted with EtOAc (3×10 mL). The combined organic extracts were washed with brine (30 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting residue was purified by column chromatography (100% EtOAc) to give desired product **8** as a colorless oil (1.779 g, 95%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (m, 3H), 7.61 (d, J = 7.6 Hz, 2H), 7.43 (m, 3H), 7.32 (m, 2H), 7.27 (m, 2H), 7.16 (m, 4H), 4.53 (t, J = 6.0 Hz, 2H), 4.31 (t, J = 6.4 Hz, 1H), 3.88 (m, 2H), 3.22 (s, 3H), 3.12 (s, 3H),

2.59 (t, $J=7.6$ Hz, 4H), 1.58 (m, 4H), 1.30 (m, 10H), 0.87 ppm (t, $J=7.2$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=169.8$, 168.2, 148.2, 147.2, 139.6, 137.1, 131.5, 130.4, 128.9, 128.8, 128.7, 128.7, 128.2, 128.0, 127.8, 127.5, 122.0, 62.9, 61.1, 49.4, 40.2, 36.0, 33.8, 31.6, 31.1, 29.4, 29.2, 29.1, 26.1, 25.7, 22.7, 14.3 ppm; IR (neat): $\tilde{\nu}=3324$, 3135, 3059, 3027, 2930, 2856, 2360, 2340, 1957, 1817, 1771, 1652, 1615, 1571, 1541, 1503, 1444, 1383, 1291, 1181, 1143, 1050, 990, 855, 781, 734, 700 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 205, 238 nm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{38}H_{48}N_6O_3$: 637.3861, found 637.3845.

General procedure for tandem synthesis of 10: A flame-dried vial (23×85 mm) was charged with compound **8** and anhydrous THF (1 mL). The solution was stirred under nitrogen at −20 °C, and the appropriate Grignard/lithium reagent was added dropwise. The resulting mixture was allowed to warm to 0 °C and stirred for 4 h. The reaction was quenched with saturated aq NH_4Cl (2 mL), and then extracted with EtOAc (3×2 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was dissolved in a minimum amount of CH_2Cl_2 and purified by column chromatography (hexane/EtOAc, 1:1). The desired α-diphenylmethyleneamino ketone still contained a minor impurity that could only be observed in ^{13}C NMR; the crude material was carried forward. Ketone **9** was redissolved in EtOH, and the solution was treated with 2 N HCl and stirred at RT for 12 h. The pH of the resulting solution was then adjusted to 4.3 with 0.1 M aq NaOH. Cyanamide was added, and the resulting mixture was heated at 95 °C for 3 h. The EtOH was removed in vacuo, and the residue was purified by column chromatography (CH_2Cl_2/MeOH (sat. NH_3), 4:1) to afford target 4,5-disubstituted 2-AITA **10** in its free base form. To form the HCl salt, a solution of free base in MeOH (1 mL) was treated with concd HCl. Removal of the solvent in vacuo delivered corresponding **10**·HCl.

***N*-(2-(4-(5-(2-Amino-4-(4-hexylphenyl)-1*H*-imidazol-5-yl)pentyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide (10e)**: Compound **8** (0.098 g, 0.15 mmol) was treated with (4-hexylphenyl) magnesium bromide (2.2 mL, 1.1 mmol, 0.5 M), and the resulting crude ketone **9e** was reacted with 2 N aq HCl (1.2 mL) in EtOH (1 mL) followed by cyclization with cyanamide (0.157 g, 3.7 mmol) according to the general procedure. Purification by column chromatography afforded target compound **10e** as a pale yellow solid (0.038 g, 42% over three steps): ^{1}H NMR (400 MHz, CD_3OD): $\delta=8.51$ (s, 1H), 7.68 (d, $J=8.0$ Hz, 2H), 7.34 (d, $J=8.4$ Hz, 2H), 7.29 (d, $J=8.0$ Hz, 2H), 7.21 (d, $J=8.0$ Hz, 2H), 4.83 (t, $J=5.6$ Hz, 2H), 3.94 (t, $J=5.2$ Hz, 2H), 2.84 (t, $J=7.6$ Hz, 2H), 2.61 (m, 6H), 1.59 (m, 8H), 1.31 (m, 12H), 0.87 ppm (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): $\delta=170.4$, 148.7, 148.1, 145.9, 144.8, 132.3, 130.3, 129.7, 128.6, 128.2, 127.0, 123.9, 123.6, 54.0, 40.6, 36.8, 36.7, 33.0, 32.6, 32.2, 30.1, 29.7, 29.3, 29.0, 25.0, 24.2, 23.8, 23.6, 14.6, 14.5 ppm; IR (neat): $\tilde{\nu}=3319$, 2929, 2851, 1683, 1653, 1541, 1507, 1457, 1397, 1138, 1124, 1099, 596 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 202, 220 nm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{36}H_{51}N_7O$: 598.4228, found 598.4212.

***N*-(2-(4-(5-(2-Amino-4-(benzofuran-2-yl)-1*H*-imidazol-5-yl)pentyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide (10q)**: Compound **8** (0.078 g, 0.12 mmol) was treated with prepared benzofuran-2-yl-MgBr (0.442 g, 2 mmol), and the resulting crude ketone **9q** was reacted with 2 N aq HCl (1.6 mL) in EtOH (1 mL) followed by cyclization with cyanamide (0.085 g, 2 mmol) according to the general procedure. Purification by column chromatography afforded target compound **10q** as a pale yellow solid (0.036 g, 54% over three steps): ^{1}H NMR (400 MHz, CD_3OD): $\delta=7.73$ (s, 1H), 7.66 (d, $J=8.4$ Hz, 2H), 7.57 (dd, $J=0.8$, 7.6 Hz, 1H), 7.46 (d, $J=8.4$ Hz, 1H), 7.23 (m, 2H), 7.18 (d, $J=8.0$ Hz, 2H), 6.98 (s, 1H), 4.60 (t, $J=6.0$ Hz, 2H), 3.82 (t, $J=5.6$ Hz, 2H), 2.75 (t, $J=7.6$ Hz, 2H), 2.65 (t, $J=7.6$ Hz, 2H), 2.53 (t, $J=8.0$ Hz, 2H), 1.64 (m, 4H), 1.51 (m, 2H), 1.36 (m, 2H), 1.24 (m, 4H), 0.83 ppm (t, $J=7.2$ Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta=170.4$, 155.8, 148.9, 148.6, 148.5, 146.7, 132.6, 129.7, 128.7, 128.5, 127.1, 126.2, 124.8, 124.0, 122.3, 115.3, 112.0, 104.0, 50.5, 41.1, 36.7, 32.6, 32.2, 30.0, 29.6, 29.3, 26.0, 25.3, 23.6, 14.5 ppm; IR (neat): $\tilde{\nu}=3396$, 2933, 2856, 1685, 1653, 1542, 1124, 1092, 618 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 205, 229, 301 nm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{32}H_{39}N_7O_2$: 554.3238, found 554.3220.

Synthesis of 2-AI amide/diamide conjugates 20–23

6-Azidohexanoic acid (12): A flame-dried 50 mL round-bottomed flask was charged with 6-bromohexanoic acid **11** (3.901 g, 20 mmol) and anhydrous DMF (10 mL). NaN_3 (5.201 g, 80 mmol) was then added, and the reaction was stirred at RT for 12 h. The resulting solution was diluted with CH_2Cl_2/H_2O (1:1, 30 mL), and the organic layer was separated and extracted with H_2O (2×15 mL). The combined aqueous portions was then back extracted with CH_2Cl_2 (3×15 mL). The combined organic extracts were washed with brine (20 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. Resulting product **12** was carried forward without further purification (2.652 g, 84%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=3.28$ (t, $J=7.2$ Hz, 2H), 2.38 (t, $J=7.6$ Hz, 2H), 1.67 (m, 4H), 1.43 ppm (m, 2H).

7-Azido-1-bromoheptan-2-one: Compound **12** (2.652 g, 16.9 mmol) was dissolved in dry CH_2Cl_2 (10 mL) in a flame-dried 100 mL round-bottomed flask. A catalytic amount of DMF (5 drops) was added, and the solution was cooled to 0 °C. Oxalyl chloride was added dropwise [*note*: vigorous effervescence occurs], and the resulting solution was stirred for 15 min at 0 °C and then 1 h at RT. The solvent was removed in vacuo, and the crude acyl chloride was redissolved in anhydrous CH_2Cl_2 (20 mL) and added dropwise at 0 °C to a solution of CH_2N_2 in Et_2O (0.451 M, 150 mL), prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald; 67.6 mmol) and KOH (15 g, 0.267 mol) in H_2O (25 mL), EtOH (35 mL) and Et_2O (150 mL). The reaction was stirred at 0 °C for 1 h, treated with concd HBr (48%; 7 mL) at 0 °C, and then stirred for another 30 min at 0 °C. Saturated aq $NaHCO_3$ (70 mL) was added to the reaction at 0 °C, and the mixture was extracted with Et_2O (3×30 mL). The combined organic extracts were washed with saturated aq $NaHCO_3$ (20 mL) and brine (20 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by column chromatography (hexane/EtOAc, 9:1→1:1) gave the desired α-bromoketone as a yellow oil (3.535 g, 90%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=3.87$ (s, 2H), 3.27 (t, $J=6.8$ Hz, 2H), 2.67 (t, $J=7.2$ Hz, 2H), 1.62 (m, 4H), 1.38 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=202.0$, 51.3, 39.7, 34.4, 28.8, 26.3, 23.4 ppm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_7H_{12}BrN_3O$: 234.0236, found 234.0223.

***tert*-Butyl 2-amino-5-(5-azidopentyl)-1*H*-imidazole-1-carboxylate**: A 100 mL round-bottomed flask was charged with α-bromoketone (3.535 g, 15.2 mmol) and DMF (20 mL). Boc-guanidine (5.714 g, 35.9 mmol) was then added, and the reaction was stirred at RT for 3 d. The resulting solution was mixed with H_2O (20 mL) and EtOAc (40 mL), and the mixture was extracted with H_2O (3×20 mL). The combined aqueous portions were extracted with EtOAc (1×20 mL), and the combined organic extracts were washed with brine (20 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting residue was purified by column chromatography (hexane/EtOAc, 4:1→1:1) to afford the target Boc-2-AI as a pale yellow solid (2.282 g, 51%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=6.44$ (s,

2H), 6.40 (s, 1H), 3.16 (t, $J=7.2$ Hz, 2H), 2.26 (t, $J=7.6$ Hz, 2H), 1.48 (m, 13H), 1.31 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=150.7$, 149.4, 138.5, 106.0, 84.2, 51.2, 28.5, 27.8, 27.7, 26.3, 26.2 ppm; IR (neat): $\tilde{\nu}=3427$, 3402, 3121, 2981, 2934, 2089, 1734, 1642, 1369, 1268, 1253, 1201, 1157, 1121, 884, 772 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 259 nm; HRMS (FAB) *m/z* $[M+H]^+$ calcd for $C_{13}H_{22}N_6O_2$: 295.1876, found 295.1885.

***tert*-Butyl 5-(5-azidopentyl)-2-(bis(*tert*-butoxycarbonyl)amino)-1*H*-imidazole-1-carboxylate (13)**: Boc-2-AI (1.751 g, 6 mmol) was dissolved in dry THF (20 mL) in a 100 mL round-bottomed flask, and the solution was treated with DMAP (0.073 g, 0.6 mmol) and Boc_2O (3.893 g, 17.9 mmol) and then stirred at RT for 12 h. The solvent was removed in vacuo. Purification by column chromatography (hexane/EtOAc, 9:1→1:1) gave desired product **13** as a pale yellow oil (2.446 g, 83%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=6.94$ (s, 1H), 3.09 (t, $J=6.8$ Hz, 2H), 2.37 (t, $J=7.6$ Hz, 2H), 1.41 (m, 13H), 1.25 ppm (m, 20H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=149.2$, 146.2, 139.7, 137.4, 113.8, 85.3, 83.0, 51.1, 28.4, 28.0, 27.8, 27.6, 27.6, 25.8 ppm; IR (neat): $\tilde{\nu}=2979$, 2936, 2863, 2096, 1807, 1752, 1585, 1537, 1458, 1395, 1370, 1276, 1254, 1150, 1119, 1100, 1019, 850, 772 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 244 nm; HRMS (FAB) *m/z* $[M+H]^+$ calcd for $C_{23}H_{38}N_6O_6$: 495.2926, found 495.2905.

7-(Benzyloxy)-7-oxoheptanoic acid (15): A 1 L round-bottomed flask was charged with heptanedioic acid **14** (6.403 g, 40 mmol) and Ac_2O (16.334 g, 160 mmol) in xylenes (500 mL). The reaction was stirred at 140 °C for 2 h. The solvent was then evaporated in vacuo, and the resulting crude cyclic anhydride was redissolved in BnOH (5 mL, 5.191 g, 48 mmol) and stirred at 60 °C for 12 h. Et_2O (30 mL) was added, and the reaction was extracted with saturated aq $NaHCO_3$ (3×20 mL). The combined aqueous extracts were acidified to pH 2 with 2 N aq HCl. The acidic solution was then extracted with Et_2O (3×20 mL), and the combined organic extracts were washed with brine (60 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting residue was purified by column chromatography (hexane/EtOAc, 4:1→1:1) to afford target compound **15** as a colorless oil (4.438 g, 44%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=$ 7.36 (m, 5H), 5.12 (s, 2H), 2.37 (t, $J=8.0$ Hz, 2H), 2.34 (t, $J=7.6$ Hz, 2H), 1.67 (m, 4H), 1.37 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=179.9$, 173.6, 136.2, 128.8, 128.4, 66.4, 34.2, 34.0, 28.6, 24.7, 24.4 ppm; HRMS (FAB) *m/z* $[M+H]^+$ calcd for $C_{14}H_{18}O_4$: 251.1277, found 251.1283.

Benzyl 8-bromo-7-oxooctanoate: Compound **15** (4.438 g, 17.7 mmol) was reacted with oxalyl chloride (6.757 g, 53.2 mmol) and diazald (15.205 g, 71 mmol) according to the same procedure described for the synthesis of 7-azido-1-bromoheptan-2-one. Purification by column chromatography (hexane/EtOAc, 9:1→1:1) afforded the target α-bromoketone (5.749 g, 99%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.36$ (m, 5H), 5.11 (s, 2H), 3.87 (s, 2H), 2.64 (t, $J=7.6$ Hz, 2H), 2.36 (t, $J=7.6$ Hz, 2H), 1.61 (m, 4H), 1.33 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=202.2$, 173.6, 136.2, 128.8, 128.4, 66.4, 39.7, 34.5, 34.2, 28.6, 24.8, 23.6 ppm; HRMS (FAB) *m/z* $[M+H]^+$ calcd for $C_{15}H_{19}BrO_3$: 327.0590, found 327.0579.

***tert*-Butyl 2-amino-5-(6-(benzyloxy)-6-oxohexyl)-1*H*-imidazole-1-carboxylate**: Benzyl 8-bromo-7-oxooctanoate (5.749 g, 17.6 mmol) was reacted with Boc-guanidine (8.416 g, 52.9 mmol) according to the same procedure described for the synthesis of *tert*-butyl 2-amino-5-(5-azidopentyl)-1*H*-imidazole-1-carboxylate. Purification by column chromatography (hexane/EtOAc, 4:1→1:1) afforded the target Boc-2-AI as a yellow oil (2.459 g, 36%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.35$ (m, 5H), 6.49 (s, 1H), 5.57 (s, 2H), 5.11 (s, 2H), 2.36 (m, 4H), 1.67 (m, 2H), 1.58 (m, 11H), 1.37 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=173.8$, 150.0, 149.7, 139.3, 136.3, 128.7, 128.4, 106.7, 84.8, 66.3, 34.5, 29.0, 28.2, 28.2, 25.0 ppm; IR (neat): $\tilde{\nu}=3454$, 2935, 2860, 1734, 1643, 1456, 1370, 1253, 1160, 1123, 847, 773, 698 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 259 nm; HRMS (FAB) *m/z* $[M+H]^+$ calcd for $C_{21}H_{29}N_3O_4$: 388.2231, found 388.2218.

***tert*-Butyl 5-(6-(benzyloxy)-6-oxohexyl)-2-(bis(*tert*-butoxycarbonyl)amino)-1*H*-imidazole-1-carboxylate (16)**: *tert*-Butyl 2-amino-5-(6-(benzyloxy)-6-oxohexyl)-1*H*-imida-zole-1-carboxylate (2.214 g, 5.7 mmol) was treated with DMAP (0.07 g, 0.6 mmol) and Boc_2O (3.739 g, 17.2 mmol) according to the same procedure described for the synthesis of **13**. Purification by column chromatography (hexane/EtOAc, 4:1→1:1) afforded target product **16** as a pale yellow oil (2.918 g, 87%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.30$ (m, 5H), 7.04 (s, 1H), 5.06 (s, 2H), 2.46 (t, $J=7.2$ Hz, 2H), 2.30 (t, $J=$ 7.6 Hz, 2H), 1.63 (m, 4H), 1.53 (s, 9H), 1.37 (s, 18H), 1.32 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=173.5$, 149.5, 146.6, 140.2, 137.6, 136.2, 128.6, 128.2, 114.0, 85.6, 83.3, 66.1, 34.3, 28.5, 28.4, 27.9, 27.7, 24.8 ppm; IR (neat): $\tilde{\nu}=2979$, 2936, 2863, 1807, 1749, 1586, 1537, 1457, 1394, 1370, 1276, 1254, 1151, 1119, 1100, 1019, 850, 751, 69 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 244 nm; HRMS (FAB) *m/z* $[M+H]^+$ calcd for $C_{31}H_{45}N_3O_8$: 588.3279, found 588.3265.

***N*-(2-Aminoethyl)-4-pentylbenzamide (17)**: A vial (23×85 mm) was charged with *N*-(2-azidoethyl)-4-pentylbenzamide **6** (0.2 g, 0.77 mmol) and anhydrous THF (6 mL), and the solution was treated with Pd/C (0.081 g, 0.08 mmol). The resulting solution was first flushed with H_2 for 5 min, and then stirred under 1 atm H_2 for 12 h. The mixture was filtered through Celite, and the solvent was evaporated in vacuo to deliver the desired crude primary amine, which was used without further purification.

3-(4-Pentylbenzamido)propanoic acid (19): A 50 mL round-bottomed flask was charged with 4-pentylbenzoyl chloride **6** (1.054 g, 5 mmol) and anhydrous CH_2Cl_2 (6 mL). The mixture was cooled to 0 °C and treated with Et_3N (1.012 g, 10 mmol) and β-analine (0.534 g, 6 mmol) sequentially. The reaction was stirred for 12 h while warming from 0 °C to RT. The resulting solution was treated with 1 N aq HCl (3 mL) and then extracted with CH_2Cl_2 (3×4 mL). The combined organic extracts were washed with brine (12 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting residue was purified by column chromatography (hexane/EtOAc, 2:1→1:2) to afford target compound **19** as a white solid (0.64 g, 45%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=10.32$ (bs, 1H), 7.65 (d, $J=8.0$ Hz, 2H), 7.20 (d, $J=8.4$ Hz, 2H), 7.13 (t, $J=6.0$ Hz, 1H), 3.70 (m, 2H), 2.67 (t, $J=6.0$ Hz, 2H), 2.60 (t, $J=8.0$ Hz, 2H), 1.58 (m, 2H), 1.30 (m, 4H), 0.87 ppm (t, $J=6.8$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=177.0$, 168.4, 147.4, 131.3, 128.8, 127.2, 35.9, 35.5, 34.0, 31.6, 31.0, 22.6, 14.2 ppm; IR (neat): $\tilde{\nu}=3342$, 2952, 2925, 2852, 1698, 1636, 1539, 1509, 1454, 1431, 1294, 1225, 1076, 925, 853, 648, 626 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 235 nm; HRMS (FAB) *m/z* $[M+H]^+$ calcd for $C_{15}H_{21}NO_3$: 264.1594, found 264.1586.

***N*-(5-(2-Amino-1*H*-imidazol-5-yl)pentyl)-4-pentylbenzamide (20)**: A vial (23×85 mm) was charged with **13** (0.2 g, 0.4 mmol) and anhydrous THF (4 mL), and the solution was treated with Pd/C (0.043 g, 0.04 mmol). The resulting solution was first flushed with H_2 for 5 min, and then stirred under 1 atm H_2 for 12 h. The mixture was filtered through Celite, and the solvent was evaporated in vacuo. The crude primary amine was redissolved in anhydrous CH_2Cl_2 (2 mL) and cooled to 0 °C. Et_3N (0.047 g, 0.46 mmol) and 4-pentylbenzoyl chloride **18** (0.054 g, 0.25 mmol) were added, and the solution was stirred for 12 h while warming from 0 °C to RT. Then, the reaction was treated with trifluoroacetic acid (TFA; 0.5 mL, 6.53 mmol) and stirred at RT for 12 h. The solvent was removed in

vacuo, and the resulting residue was purified by column chromatography (CH_2Cl_2/MeOH (sat. NH_3), 20:1→4:1) to afford desired product **20** as a pale yellow solid (0.018 g, 23% over three steps): ^{1}H NMR (400 MHz, CD_3OD): δ = 7.72 (d, J = 8.0 Hz, 2H), 7.25 (d, J = 8.0 Hz, 2H), 6.48 (s, 1H), 3.37 (t, J = 7.2 Hz, 2H), 2.64 (t, J = 8.0 Hz, 2H), 2.50 (t, J = 8.0 Hz, 2H), 1.63 (m, 6H), 1.42 (m, 2H), 1.32 (m, 4H), 0.89 ppm (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 170.4, 148.3, 133.2, 129.7, 129.1, 128.4, 109.8, 40.8, 36.8, 32.7, 32.3, 30.3, 29.0, 27.4, 25.5, 23.7, 14.5 ppm; IR (neat): $\tilde{\nu}$ = 3382, 2923, 2850, 1632, 1403, 1070, 1019 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 205, 229 nm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{20}H_{30}N_4O$: 343.2492, found 343.2483.

***N*-(3-(5-(2-Amino-1*H*-imidazol-5-yl)pentylamino)-3-oxopropyl)-4-pentylbenzamide (21)**: A vial (23×85 mm) was charged with **13** (0.2 g, 0.4 mmol) and anhydrous THF (4 mL), and the solution was treated with Pd/C (0.043 g, 0.04 mmol). The resulting solution was first flushed with H_2 for 5 min, and then stirred under 1 atm H_2 for 12 h. The mixture was filtered through Celite, and the solvent was evaporated in vacuo. The crude primary amine was added to a solution of **19** (0.053 g, 0.2 mmol) in DMF (2 mL) at RT. The solution was treated with EDC (0.077 g, 0.4 mmol), HOBt (0.068 g, 0.4 mmol) and DIEA (0.105 g, 0.8 mmol), and then stirred at RT for 12 h. The reaction was diluted with H_2O (3 mL) and EtOAc (3 mL), and the aqueous phase was extracted with EtOAc (3×3 mL). The combined organic extracts were washed with H_2O (3 mL) and brine (3 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The crude product was redissolved in CH_2Cl_2 (2 mL), treated with TFA (0.5 mL), and stirred at RT for 12 h. The solvent was evaporated in vacuo, and the residue was purified by column chromatography (CH_2Cl_2/MeOH (sat. NH_3), 20:1→4:1) to afford desired product **21** as a pale yellow solid (0.065 g, 95% over three steps): ^{1}H NMR (400 MHz, CD_3OD): δ = 7.77 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 6.46 (s, 1H), 3.70 (t, J = 6.8 Hz, 2H), 3.23 (t, J = 6.8 Hz, 2H), 2.67 (t, J = 6.4 Hz, 2H), 2.62 (t, J = 7.6 Hz, 2H), 2.39 (t, J = 7.6 Hz, 2H), 1.54 (m, 6H), 1.31 (m, 6H), 0.87 ppm (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.8, 170.4, 148.6, 148.4, 132.4, 129.7, 128.8, 128.6, 109.6, 41.0, 37.7, 36.8, 36.6, 32.6, 32.2, 29.6, 28.9, 27.3, 25.3, 23.6, 14.5 ppm; IR (neat): $\tilde{\nu}$ = 3288, 2931, 2857, 1680, 1636, 1542, 1504, 1439, 1307, 761 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 205, 226 nm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{23}H_{35}N_5O_2$: 414.2864, found 414.2855.

6-(2-Amino-1*H*-imidazol-5-yl)-*N*-(4-pentylphenyl)hexanamide (22): A vial (23×85 mm) was charged with **16** (0.104 g, 0.18 mmol) and Pd/C (0.024 g, 0.02 mmol) in anhydrous THF (2 mL). The resulting solution was first flushed with H_2 for 5 min, and then stirred under 1 atm H_2 for 12 h. The mixture was filtered through Celite, and the solvent was evaporated in vacuo. The crude carboxylic acid was reacted with 4-pentylaniline (0.048 g, 0.29 mmol), EDC (0.056 g, 0.29 mmol), HOBt (0.050 g, 0.29 mmol) and DIEA (0.076 g, 0.59 mmol) in DMF (2 mL), and the mixture was stirred at RT according to procedure described for **21**. Deprotection of the crude product with TFA (0.5 mL, 6.53 mmol) in CH_2Cl_2 (2 mL) and purification by column chromatography (CH_2Cl_2/MeOH (sat. NH_3), 20:1→4:1) afforded desired product **22** as a yellow solid (0.012 g, 26% over three steps): ^{1}H NMR (400 MHz, CD_3OD): δ = 7.77 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 6.46 (s, 1H), 3.70 (t, J = 6.8 Hz, 2H), 3.23 (t, J = 6.8 Hz, 2H), 2.67 (t, J = 6.4 Hz, 2H), 2.62 (t, J = 7.6 Hz, 2H), 2.39 (t, J = 7.6 Hz, 2H), 1.54 (m, 6H), 1.31 (m, 6H), 0.87 ppm (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.8, 170.4, 148.6, 148.4, 132.4, 129.7, 128.8, 128.6, 109.6, 41.0, 37.7, 36.8, 36.6, 32.6, 32.2, 29.6, 28.9, 27.3, 25.3, 23.6, 14.5 ppm; IR (neat): $\tilde{\nu}$ = 3308, 3156, 2959, 2928, 2856, 1679, 1598, 1536, 1414, 1384, 1180, 1146, 1073, 692 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 205, 241 nm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{20}H_{30}N_4O$: 343.2492, found 343.2481.

***N*-(2-(6-(2-amino-1*H*-imidazol-5-yl)hexanamido)ethyl)-4-pentylbenzamide (23)**: A vial (23×85 mm) was charged with **16** (0.095 g, 0.16 mmol) and Pd/C (0.024 g, 0.02 mmol) in anhydrous THF (2 mL). The resulting solution was first flushed with H_2 for 5 min, and then stirred under 1 atm H_2 for 12 h. The mixture was filtered through Celite, and the solvent was evaporated in vacuo. The crude carboxylic acid was reacted with **17** (0.063 g, 0.27 mmol), EDC·HCl (0.052 g, 0.27 mmol), HOBt (0.046 g, 0.27 mmol) and DIEA (0.070 g, 0.54 mmol) in DMF (2 mL), and the mixture was stirred at RT as described for the synthesis of **22**. Deprotection of the crude product with TFA (0.5 mL, 6.53 mmol) in CH_2Cl_2 (2 mL) and purification by column chromatography (CH_2Cl_2/MeOH (sat. NH_3), 20:1→4:1) afforded the desired product **23** as a white solid (0.040 g, 61% over three steps): ^{1}H NMR (400 MHz, CD_3OD): δ = 7.76 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 6.46 (s, 1H), 3.55 (m, 4H), 2.64 (t, J = 8.0 Hz, 2H), 2.41 (t, J = 7.6 Hz, 2H), 2.32 (t, J = 7.6 Hz, 2H), 1.61 (m, 6H), 1.32 (m, 6H), 0.88 ppm (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 177.7, 170.6, 148.6, 148.5, 132.7, 129.7, 128.9, 128.6, 109.7, 40.8, 40.6, 36.8, 36.4, 32.6, 32.2, 29.4, 28.9, 26.6, 25.2, 23.6, 14.5 ppm; IR (neat): $\tilde{\nu}$ = 3251, 3148, 2925, 2854, 1679, 1633, 1540, 1503, 1403, 1303 cm^{-1}; UV/Vis (CH_2Cl_2): λ_{max} (ε) = 229 nm; HRMS (FAB) m/z $[M+H]^+$ calcd for $C_{23}H_{35}N_5O_2$: 414.2864, found 414.2855.

Biological screening

General: MRSA (ATCC # BAA-44) and MDRAB (ATCC # BAA-1605) were obtained from the American Type Culture Collection (ATCC). Imipenem monohydrate was purchased from US Pharmacopeial Convention (USP; # 1337809), penicillin G and oxacillin sodium salt were purchased from Sigma–Alrich (# P3032) and TCI (# O0353), respectively. Mueller–Hinton broth (MHB) was made using materials from Becton, Dickinson and Co. (BBL beef extract: # 21230; Difco soluble starch: # 217820) and MP Biomedicals (casein hydrolysate: # 101290) using the following procedure: Deionized water (1 L) was mixed with beef extract (32 g) and soluble starch (1.5 g). Casein hydrolysate (17.5 g) was then added, and the pH was adjusted to 7.4 at RT with 10 N NaOH. The resulting solution was autoclaved at 120 °C for 15 min.

Dose–response curves of lead compounds in MRSA (S14) and MDRAB (S16) biofilm inhibition assays, along with growth curve analysis of lead compounds against both MRSA and MDRAB (S18) are available in the Supporting Information.

Inhibition assay protocols: Inhibition assays were performed by taking overnight cultures of MRSA or MDRAB and subculturing at an OD_{600} value of 0.01 into tryptic soy broth with a 0.5% glucose supplement (TSBG) for MRSA or Luria–Bertani (LB) media for MDRAB. Stock solutions of predetermined concentrations of test compound were prepared in the resulting bacterial suspension, and aliquots (100 μL) of stock solution were plated in a 96-well format. Plates were covered, sealed with GLAD Press'n Seal, and incubated under stationary conditions at 37 °C for 24 h. The media was then discarded, and the plates were washed thoroughly with water. Each well was stained with 0.1% solution of crystal violet (110 μL) at RT for 30 min. After thoroughly washing with water again, the remaining stain was dissolved in 95% EtOH (200 μL), and an aliquot of the solution (125 μL) was transferred to corresponding wells of a polystyrene microtiter dish. Biofilm inhibition was quantified by measuring the OD_{540} value of each well. Blank wells were employed as background controls.

Broth microdilution method for antibiotic resensitization: MHB was inoculated (5×10^5 CFU mL^{-1}) with either MRSA or MDRAB. The resulting bacterial suspension was aliquoted (4 mL) into culture tubes, and test compound (from DMSO stock solution) was added to give the final concentration to be tested. Bacteria not treated with the test compound served as a control. After sitting for 30 min at RT, 1 mL of each sample was transferred to a new culture tube, and the appropriate antibiotic (oxacillin and penicillin G sodium salt for MRSA/imipenem for MDRAB) was added from a 128 mg mL^{-1} water stock solution to give a concentration of 128 μg mL^{-1}. Rows 2–12 of a 96-well microtiter plate were filled with 100 μL/well from the remaining 3 mL bacterial subcultures, allowing the concentration of compound to be kept uniform throughout the antibiotic dilution procedure. After standing for 10 min, the samples containing antibiotic were aliquoted (200 μL) into the corresponding first-row wells of the microtiter plate. Row 1 wells were mixed 6–8 times, and then 100 μL were transferred to row 2. Row 2 wells were mixed 6–8 times, and then 100 μL were transferred from row 2 to row 3. This procedure was repeated to serially dilute the rest of the rows of the microtiter plate, with the exception of the final row, to which no antibiotic was added. The plate was sealed with GLAD Press'n Seal and incubated under stationary conditions at 37 °C. After 16 h, minimum inhibitory concentration (MIC) values were recorded as the lowest concentration of antibiotic at which no visible growth of bacteria was observed.

Checkerboard assay: MHB was inoculated with MRSA (5×10^5 CFU mL^{-1}), and 100 μL were distributed into each well of a 96-well plate except well 1A. Inoculated MHB (200 μL) containing test compound (at 2× the highest concentration being tested) was added to well 1A, and 100 μL of the same sample were placed in each of wells 2A–12A. Column A wells were mixed 6–8 times, and then 100 μL were withdrawn and transferred to column B. Column B wells were mixed 6–8 times, followed by a 100 μL transfer to column C. This procedure was repeated to serially dilute the rest of the columns of the plate up to column G (column H was not mixed to allow the MIC of antibiotic alone to be determined). Inoculated media (100 μL) containing antibiotic at two-times the highest concentration being tested was placed in wells A1–H1 (row 1) and serially diluted in the same manner to row 11 (row 12 was not mixed to allow the MIC of compound alone to be determined). The plates were incubated for 16 h at 37 °C. The MIC values of both compound and antibiotic in the combination were recorded, as well as the MIC values of compound alone (row 12) and antibiotic alone (column H). The ΣFIC values were calculated as follows: $\Sigma FIC = FIC_{Compd} + FIC_{Antibiotic}$, where FIC_{Compd} is the MIC of the compound in the combination/MIC of the compound alone, and $FIC_{Antibiotic}$ is the MIC of the antibiotic in the combination/MIC of the antibiotic. The combination is considered synergistic when the ΣFIC value is ≤ 0.5, indifferent when the ΣFIC value is >0.5 but <2, and antagonistic when the ΣFIC is ≥ 2.

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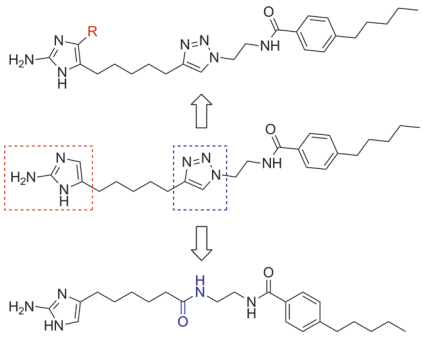
Modulating resistance: 4,5-Disubstituted 2-aminoimidazole triazole amide (2-AITA) conjugates were discovered as MRSA and MDRAB biofilm modulators, which also suppress MRSA resistance to oxacillin. Further structure–activity relationship studies identified a 2-aminoimidazole diamide (2-AIDA) conjugate with significantly increased resensitization activity without compromising the anti-biofilm activity.



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Structural Studies on 4,5-Disubstituted 2-Aminoimidazole-Based Biofilm Modulators that Suppress Bacterial Resistance to β-Lactams

