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Synthesis and biological evaluation of 2-aminoimidazole/carbamate hybrid anti-biofilm and anti-microbial agents

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ABSTRACT

The successful marriage of structural features from our 2-aminoimidazole and menthyl carbamate classes of anti-biofilm agents has resulted in the development of a novel hybrid scaffold of biofilm modulators. The compounds were evaluated against a panel of four bacterial strains for anti-biofilm and anti-microbial activity.

Biofilms represent a particularly hardy phenotype of bacterial growth.[1] Owing to their encasement in a robust extracellular matrix of biomolecules, bacteria in these surface-adhered communities are uniquely resilient, often displaying resistance toward conventional antibiotics, antiseptics, and host defense mechanisms.[2] Indeed, more than 80% of all bacterial infections are the direct result of biofilms comprising medically relevant pathogens.[3] Biofilms have been implicated in persistent infections of medical implants,[4] and are responsible for the mortality and morbidity of cystic fibrosis (CF) patients.[5]

Despite the involvement of bacterial biofilms in a host of medical maladies, the development of small, drug-like compound classes that influence their formation and maintenance has lagged significantly.[6] Currently, relatively few scaffolds are known to possess anti-biofilm activity, and these include homoserine lactones,[7] brominated furanones,[8] and ursine triterpenes.[9] Additionally, computer aided drug design protocols[10] and high throughput screening methods[11] have also led to the discovery of a few novel scaffolds that possess anti-biofilm activity. Despite these advances, potent biofilm modulators are still sorely underdeveloped.

Our group has developed an array of novel molecular scaffolds that both inhibit and disperse bacterial biofilms across order, class, and phylum via a non-microbicidal mechanism.[12] Our inspiration for the design of these molecules was to extract and systematically optimize structural motifs embedded within the marine natural product bromoageliferin (**1**, Fig. 1).[13] The 2-aminoimidazole (2-AI, highlighted in red) heterocycle has proven crucial for the observed biological activity of these compounds. These studies have culminated in the discovery of our current lead compound **5** that is active against both Gram-positive and Gram-negative bacteria.

Very recently we disclosed a new, structurally unrelated class of molecules based on a menthyl carbamate scaffold (e.g., **7**, Fig. 2) that possess potent non-microbicidal biofilm inhibition activity against various *Staphylococcal* strains.[14] We arrived at **7** through the systematic optimization of the SCRC3P79 (*Cytophaga* sp.)

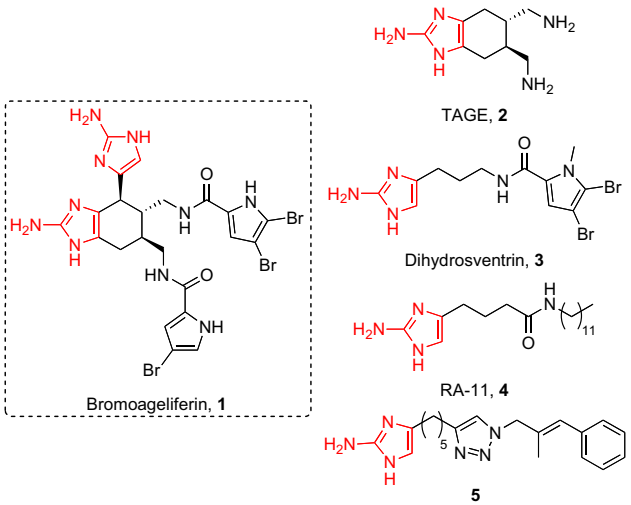

Figure 1. 2-Aminoimidazole anti-biofilm agents based on Bromoageliferin.

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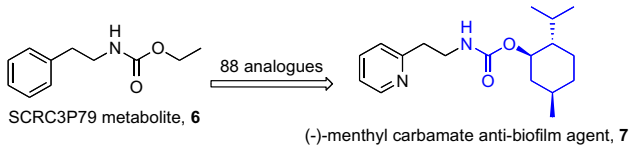


Figure 2. (−)-Menthyl carbamate anti-biofilm agent.

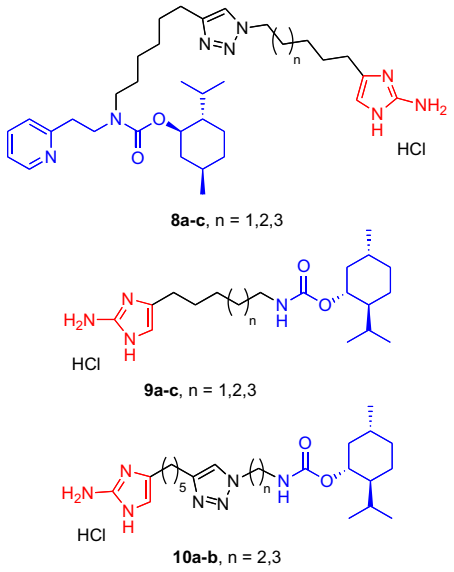


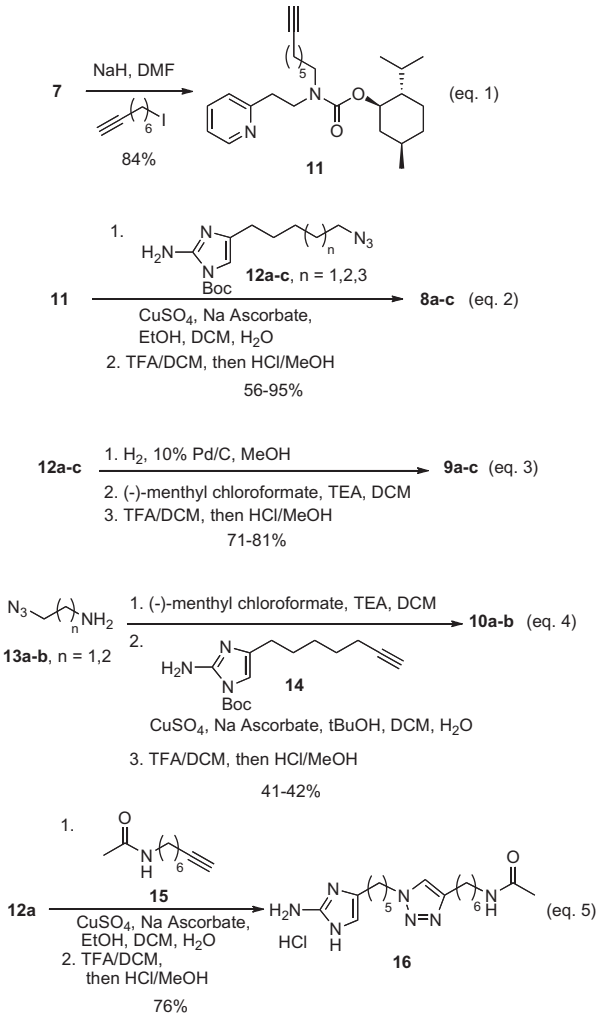
Figure 3. Hybrid 2-AI/menthyl carbamate anti-biofilm targets.

bacterial metabolite **6**.[13] Unlike our 2-aminoimidazole scaffold, the menthyl carbamate series lacked the ability to disperse pre-formed biofilms and demonstrated poor anti-biofilm properties against non-*Staphylococcal* strains. Nonetheless, these shortcomings were somewhat offset by the trivial synthesis of compounds such as **7**.

We next sought to investigate hybrid scaffolds that included structural motifs from both classes of molecules. Namely, we proposed a series of targets that blended the 2-AI head group from our bromoageliferin analogues (**2–5**) with the menthyl carbamate (highlighted in blue) moiety in our most recent class of biofilm inhibitors (e.g., **7**). Presented herein is an account of the successful marriage of these two classes of biofilm inhibitors and the evaluation of these hybrid structures as anti-biofilm and anti-microbial agents.

Initially we targeted three separate scaffolds that combined structural elements of the 2-AI and menthyl carbamate motifs (Fig. 3). Compounds **8a–c** represent a series of three molecules that comprise the entire menthyl carbamate lead compound **7** directly tethered to the 2-aminoimidazole head group with an intervening triazole similar to our current lead compound **5**. Compounds **9a–c** represent menthyl carbamate analogues of 2-AI amide derivatives dihydrosventrin (**3**) and RA-11 (**4**). Finally, **10a–b** were designed to closely resemble our lead compound **5** by replacing the aryl olefin in **5** with a menthyl carbamate linkage.

Scheme 1 details the preparation of compounds **8a–10b** (see Supplementary data for full details). Compound **7**[14] was alkylated with 8-iodo-1-octyne, providing carbamate derivative **11** (Eq. 1). Azides **12a–c**[12] were each independently reacted with alkyne **11** in the presence of copper sulfate and sodium ascorbate,[15] followed by Boc deprotection and HCl exchange to return the target hybrid structures **8a–c** (Eq. 2). Alternatively, 2-AI carbamate analogues **9a–c** were prepared from 2-AI azides **12a–c** via an efficient three step sequence including azide reduction, carbamate formation, and Boc deprotection/HCl exchange (Eq. 3).[12]



Scheme 1. Preparation of 2-AI/menthyl carbamate hybrids.

Table 1
Biofilm inhibition (IC_{50} values) against various bacterial strains

Compound	MRSA[a]	PA14[a]	*A. baumannii* (ATCC# 19606)[a]
8a	—	>200	19.2 ± 2.0
8b	—	>200	18.4 ± 0.95
8c	—	>200	16.7 ± 1.5
9a	—	18.0 ± 5.1	19.2 ± 2.0
9b	—	18.0 ± 5.3	—
9c	—	—	16.7 ± 1.2
10a	29.9 ± 5.8	58.7 ± 1.5	94.9 ± 0.2
10b	20.5 ± 4.9	40.3 ± 5.2	—
16	>200	>200	>200

[a] IC_{50} values are in μM.

The 2-AI triazole analogues **10a–b** were generated by initially reacting amino azides **13a–b** with (−)-menthyl chloroformate in the presence of triethylamine. The azido carbamates were then coupled with known 2-AI alkyne **14**[12] via the Cu(I) mediated click reaction.[15] Antibiofilm agents **10a–b** were isolated after Boc removal and HCl exchange (Eq. 4).

Additionally, we elected to prepare the acetamido analogue of 2-AI/menthyl carbamate hybrid **8a** as a control compound featuring the 2-AI head group capped with an acetamido group in lieu of the (−)-menthyl carbamate moiety (Eq. 5). The 2-AI azide **12a** was reacted with acetamido-alkyne **15** under copper mediated click conditions[15] to generate the corresponding 2-AI 1,4-disubsti-

tuted 1,2,3-triazole, which was then deprotected to provide acetamido analogue **16** after HCl salt exchange.

After their synthesis, compounds **8a–10b** and **16** were initially screened for their ability to inhibit biofilms of the medically relevant bacteria methicillin resistant *Staphylococcus aureus* (MRSA), *S. aureus* (ATCC# 29213), *Pseudomonas aeruginosa* (PA14), and *Acinetobacter baumannii* (ATCC# 19606). We subjected each compound to a dose–response study in an attempt to determine the IC_{50} value for biofilm inhibition (i.e., the concentration necessary to inhibit 50% of biofilm formation) as judged by a crystal violet reporter assay.[16] The data for these experiments is collected in Table 1. During the course of these experiments, most of the compounds displayed a precipitous drop in biofilm inhibition activity over a narrow concentration range. For example, the compounds would exhibit >90% inhibition at 10 μM concentrations, but a dismal <10% inhibition at 5 μM. Such behavior is commonly diagnostic of an underlying microbicidal mechanism for biofilm inhibition. Such dose–response behavior precludes the ability to calculate a reliable IC_{50} value, and compounds exhibiting this behavior are indicated with a dash (−) in Table 1. It should be noted that all of the compounds exhibited this type of behavior for *S. aureus* (ATCC# 29213) (data not depicted in Table 1).

Some compounds, however, returned dose–response data suitable for the determination of IC_{50} values. Against MRSA, compounds **10a** and **10b** returned IC_{50} values of 29.9 and 20.5 μM, respectively. Against PA14, compounds **8a–c** exhibited no anti-biofilm properties, while **9a** and **9b** each returned an IC_{50} value of 18 μM. Additionally, **10a** and **10b** gave IC_{50} values of 58.8 and 40.3 μM, respectively against PA14. For *A. baumannii*, IC_{50} values of 19.2, 18.4, 16.7, 19.2, 16.7, and 94.9 were obtained for compounds **8a–c**, **9a**, **9c**, and **10a**, respectively. Control acetamido compound **16** exhibited no anti-biofilm activity for any of the four bacterial strains at the highest concentration tested (200 μM), thus confirming the necessity of the carbamate moiety for anti-biofilm activity.

We next conducted growth curves at the IC_{50} concentration for each of the compounds in Table 1 (where appropriate) to assess the viability of planktonic bacteria. Growth curve analysis against MRSA revealed that compound **10a** and **10b** reduced bacterial growth at their respective IC_{50} value, thus some of their anti-biofilm activity was due to inhibition of bacterial growth. The same was true for compounds **8a**, **8b**, **9a**, **9c**, and **10a** against *A. baumannii* (ATCC# 19606). Importantly, however, compounds **9a**, **9b**, and **10a–b** inhibited PA14 biofilm formation via non-microbicidal activity. Additionally, compound **8c** was found to inhibit *A. baumannii* (ATCC# 19606) biofilms in a non-toxic fashion.

Given that most of the 2-AI/carbamate hybrids exhibited anti-biofilm activity by toxic means, we elected to evaluate their potential as anti-microbial agents. To determine the extent of microbicidal activity, we measured the minimum inhibitory concentration (MIC) of each compound against MRSA, *S. aureus* (ATCC# 29213), PA14, and *A. baumannii* (ATCC# 19606). Additionally, we evaluated the compounds against multi-drug resistant *A. baumannii* (MDRAB, ATCC# BAA-1605). The data for this study is summarized in Table 2.

Compounds **8a–c**, as well as **9b** and **9c** proved to be especially active against the *Staphylococcal* strains, each exhibiting MIC values of 8 μg/mL or lower. Compounds **9a**, **10a**, and **10b** exhibited MIC values of 32, 64, and 16 μg/mL respectively against MRSA, and 32, 16, and 16 μg/mL against *S. aureus* (ATCC# 29213). In keeping with the biofilm inhibition data collected in Table 1, all of the compounds exhibited relatively high MIC values for PA14. Compounds **8a–c** and **10a** were found to be non-toxic at the highest concentration tested (128 μg/mL) while compounds **9a–c** and **10b** exhibited MIC values of 32, 64, 64, and 64 μg/mL, respectively.

Against the two *A. baumannii* strains, compound **8a** returned an MIC value of 16 μg/mL. Compound **8b** exhibited an MIC value of 32 μg/mL against the ATCC# 19606 strain, but was completely inactive against MDRAB (>128 μg/mL). Analogue **8c** exhibited no toxicity against either *A. baumannii* strain. In the **9a–c** series, a structure activity trend emerged whereby lengthening the intervening methylene tether in between the 2-AI head and (−)-menthyl carbamate tail resulted in increased potency. Against both *A. baumannii* strains MIC values of 32, 16, and 8 μg/mL were found for **9a**, **9b**, and **9c**, respectively. Finally, compound **10a** returned MIC values of 64 and 128 μg/mL against ATCC# 19606 and MDRAB, respectively, while **10b** gave an MIC of 64 μg/mL against both *A. baumannii* strains. In the case of control compound **16**, we did not observe any anti-microbial behavior up to the highest concentration tested (128 μg/mL). This result serves to highlight the importance of both the 2-AI head and the (−)-menthyl carbamate functionality in eliciting the observed anti-microbial properties.

Table 2
MIC values against various bacterial strains

Compound	MRSA[a]	*S. aureus* (ATCC# 29213)[a]	PA14[a]	*A. baumannii* (ATCC# 19606)[a]	MDRAB[a]
8a	8	4	>128	16	16
8b	4	4	>128	32	>128
8c	4	4	>128	>128	>128
9a	32	32	32	32	32
9b	8	8	64	16	16
9c	8	8	64	8	8
10a	64	16	>128	64	128
10b	16	16	64	64	64
16	>128	>128	>128	>128	>128

[a] MIC values are in μg/mL.

Given their observed anti-biofilm activity via both toxic and non-toxic means, we were eager to evaluate whether or not several of the 2-AI/menthyl carbamate hybrids could disperse pre-formed bacterial biofilms. This study was of particular interest given the dichotomy between to two parent scaffolds. While our 2-AI leads (e.g., Scheme 1) effectively disperse pre-formed biofilms across order, class, and phylum, our menthyl carbamate lead **7** failed to exhibit biofilm dispersal capability.

In order to assess the potential for biofilm dispersal, we chose compounds **8a** and **9c**, based on the fact that they exhibited the lowest MIC values against the *S. aureus* and *A. baumannii* strains. Additionally, we elected to screen **8c** for non-toxic dispersal activity given that it inhibited the formation of *A. baumannii* (ATCC# 19606) films in a non-microbicidal fashion. Pre-formed biofilms from MRSA, *S. aureus* (ATCC # 29213), and *A. baumannii* (ATCC# 19606) were treated with lead compounds **8a**, **8c**, and **9c**. Dose–response curves were generated to determine the EC_{50} values for biofilm dispersal (i.e., concentration required to disperse 50% of a pre-formed biofilm, see Table 3).

While compound **8a** failed to disperse pre-formed *Staphylococcal* biofilms, it exhibited an EC_{50} value of 37.5 μM against *A. baumannii* (ATCC# 19606). Compound **8c** gave EC_{50} values of 38.1 and 32.5 μM against MRSA and *S. aureus* (ATCC# 29213) respectively; however, these concentrations are microbicidal to planktonic bacteria (MIC = 4 μg/mL against both strains). Conversely,

Table 3
Biofilm dispersal (EC_{50} values) against various bacterial strains

Compound	MRSA[a]	*S. aureus* (ATCC# 29213)[a]	*A. baumannii* (ATCC# 19606)[a]
8a	>200	>200	37.5 ± 2.3
8c	38.1 ± 5.7	32.5 ± 4.6	20.6 ± 1.7
9c	53.5 ± 5.1	38.8 ± 4.5	68.3 ± 2.7

[a] EC_{50} values are in μM.

Table 4
Blood lysis (HD_{50}) assay of compounds **8a**–**10b** and **16**

Compound	HD_{50}[a]
8a	93.8 ± 3.5
8b	46.0 ± 4.9
8c	42.7 ± 1.7
9a	463.7 ± 0.7
9b	228.8 ± 29.9
9c	473.7 ± 15.0
10a	474.0 ± 7.2
10b	317.6 ± 14.6
16	>800

[a] HD_{50} values are in μM.

compound **8c** also showed *non-toxic* (MIC >128 μg/mL, vide supra) dispersal activity against *A. baumannii* (ATCC# 19606) with an EC_{50} value of 20.6 μM (13.1 μg/mL). Finally, compound **9c** had EC_{50} values of 53.5, 38.8, and 68.3 μM against MRSA, *S. aureus* (ATCC# 29213), and *A. baumannii* (ATCC# 19606), respectively. In addition to the data collected in Table 3, we also screened the entire library against pre-formed *P. aeruginosa* (PA14) biofilms owing to their relatively low toxicity in the MIC studies against that strain. Unfortunately, none of the compounds in this study effectively dispersed pre-formed PA14 biofilms.

In a final set of experiments, all of the compounds in the study were preliminarily assessed for mammalian cytotoxicity using a red blood cell hemolysis assay using defibrinated sheep blood.[17] HD_{50} values (i.e., the concentration at which 50% hemolysis is observed) are collected in Table 4. No hemolysis is observed at the MIC concentrations for the lead antibiotics depicted in Table 2.

In conclusion, we have introduced a new class of hybrid 2-aminoimidazole/menthyl carbamate anti-biofilm agents that exhibit the ability to inhibit biofilm formation and to disperse mature, pre-formed biofilms. While most of the 2-AI/carbamate hybrids elicited their anti-biofilm activity via underlying microbicidal means, this study also resulted in a panel of non-toxic inhibitors for the *P. aeruginosa* strain PA14 (i.e., compounds **9a**, **9b**, **10a**, and **10b**). Additionally, compound **8c** both inhibited and dispersed biofilms of *Acinetobacter baumannii* (ATCC# 19606) in a non-toxic fashion.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.057.

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