Synthesis and bacterial biofilm inhibition studies of ethyl *N*-(2-phenethyl) carbamate derivatives†

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An 88 member library based upon the marine bacterial metabolite ethyl *N*-(2-phenethyl) carbamate was evaluated for bacterial biofilm inhibition against a panel of medically relevant strains. These studies culminated in the discovery of a new class of molecules capable of inhibiting the formation of *S. aureus* biofilms with low micromolar IC_{50} values.

Introduction

Bacterial biofilms are defined as surface-adhered communities of bacteria encased in an extracellular matrix of biomolecules.[1] This particular phenotype of bacterial growth is incredibly resilient to conventional antibiotics, antiseptics, and host defences.[2] In fact, biofilms of medically relevant bacteria constitute more than 80% of all bacterial infections.[3] Additionally, biofilms have been implicated in the persistence of infections of indwelling medical devices,[4] and are responsible for the mortality and morbidity of all cystic fibrosis (CF) patients.[5]

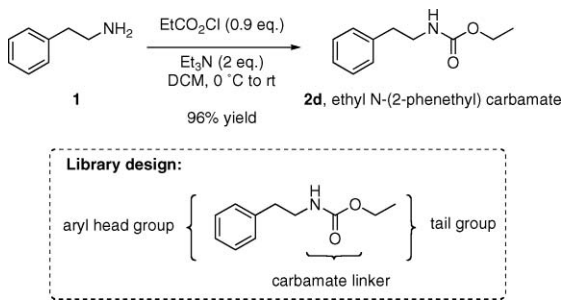
Despite the preponderance of severe medical conditions that are influenced by bacterial biofilms, there exists a relative dearth of small, drug-like molecular scaffolds that affect their formation and maintenance.[6] Examples of various compound classes known to possess *anti*-biofilm activity include homoserine lactone derivatives,[7] brominated furanones,[8] and ursine triterpenes.[9] Additionally, high throughput screening approaches[10] and computer aided drug design methods[11] have also resulted in the discovery of a few novel scaffolds that possess *anti*-biofilm activity.

Our group has had marked success in the development of novel molecular scaffolds that can both inhibit and disperse bacterial biofilms across order, class, and phylum. Our unifying strategy towards the development of these molecules has been through the systematic design and optimization of structural motifs embedded within the core structure of the marine natural product bromoageliferin.[12]

With the aim of introducing a new class of molecules possessing potent *anti*-biofilm activity, we sought to evaluate a library of analogues based upon the bacterial metabolite ethyl *N*-(2-phenethyl) carbamate (**2d**), isolated from the marine bacteria SCRC3P79 (*Cytophaga* sp.).[13] A. Yamada *et al.* reported that **2d** exhibited moderate antibiofilm activity against the marine α-proteobacteria *Rhodospirillum salexigens*. Yamada performed preliminary analogue synthesis by varying the aromatic appendage with substituted benzene rings and the ethyl appendage with a handful of aliphatic subunits. None of the analogues demonstrated improved activity in comparison to **2d**.[13] Based on these results and our success with 2-AI derivatives, we raised the question as to whether or not this metabolite (**2d**) would display *anti*-biofilm properties against more medically relevant bacteria. Furthermore, if this was the case and compound **2d** was active against medically relevant bacteria, would the synthesis and screening of a more structurally diverse library of **2d** analogues provide potent *anti*-biofilm compounds? We were particularly eager to investigate a library of analogues based on **2d**, due to their relative structural simplicity and ease of synthesis and purification as compared to our 2-aminoimidazole-based modulators. Presented herein is an account of the results of this pilot study.

Results and discussion

Ethyl *N*-(2-phenethyl) carbamate **2d** was synthesized from commercially available materials by routine acylation methodology (ethyl chloroformate/TEA in DCM) (Scheme 1). Compound **2d** was isolated in 96% yield without recourse to chromatographic purification.



Scheme 1 Preparation of metabolite **2d** and library design.

Similar to Yamada *et al.*, we found that **2d** displayed mediocre antibiofilm activity against *R. salexigens*, giving a 59.7% inhibition at a 200 μM concentration as judged by a crystal violet reporter assay.[14] Interestingly, a 200 μM concentration of **2d** also displayed activities against various medically relevant bacterial strains, inhibiting 63.1%, 68.1%, 80.2%, 52.0% and 40.8% of biofilm formation for *S. epidermidis*, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), multi-drug resistant *Acinetobacter baumannii* (MDRAB), and *E. coli* respectively (Table 1).

After successfully obtaining antibiofilm activity for **2d** against medically relevant bacteria, a three part structure–activity analysis was designed (see Scheme 1), which entailed a systematic modulation of the metabolite's aromatic head region, carbamate linkage, and tail group.

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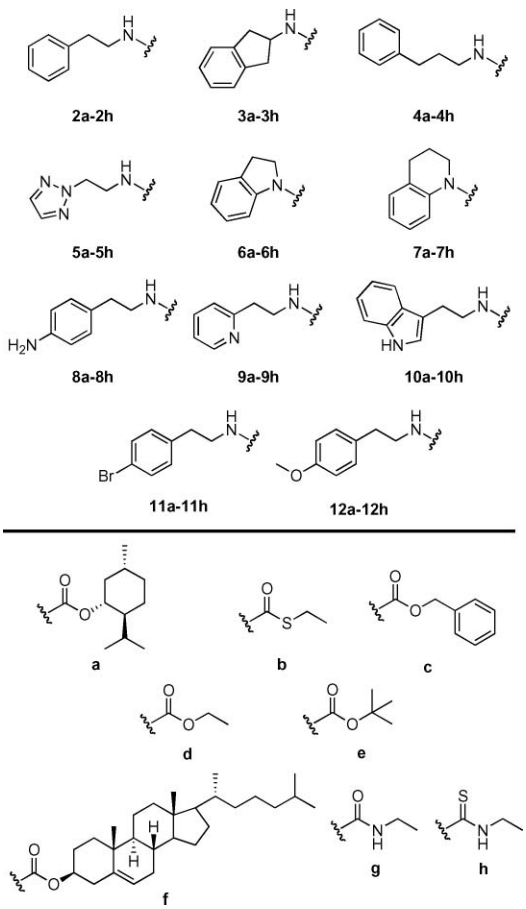
Table 1 Biofilm inhibition activity of **2d** against various bacteria

Strain	% Inhibition (200 μM 2d)
S. epidermidis	63.1
MRSA	68.1
VRE	80.2
R. salexigens	59.7
MDRAB	52.0
E. coli	40.8

The natural product analogues were synthesized using the same method used to prepare **2d**. Specifically, the respective amine was reacted with 0.9 equivalents of the requisite chloroformate, isocyanate, dicarbonate, or isothiocyanate in the presence of 2.0 equivalents of triethylamine in dichloromethane (see ESI for details†). Each of the listed amines was reacted independently with each acylating reagent to produce an 88 member pilot library in yields ranging from 76–98%. Various aromatic head groups were used, incorporating the indole, triazole, indane, tetrahydroquinoline, indoline, and pyridine, as well as *para*-amino, *para*-methoxy, and *para*-bromo substituted phenyl rings. The carbamate heteroatomic core was varied through the substitution with a thiocarbamate, urea, and thiourea linkages. Tail modifications were made through the incorporation of the (–)-menthyl, benzyl, *t*-butyl and cholesteryl groups (Scheme 2).



Scheme 2 Analogue library.

Once **2a–12h** had been synthesized, they were screened for their ability to inhibit biofilm formation of *S. epidermidis*, MRSA, VRE, *R. salexigens*, MDRAB and *E. coli* at a 200 μM concentration. None of the compounds displayed notable antibiofilm activity against *S. epidermidis*, VRE, *R. salexigens*, or MDRAB. Nevertheless, compounds **4c**, **8a**, **9a**, and **10a** displayed greater than 90% inhibition of MRSA biofilms at 200 μM concentration. Furthermore, compounds **5a**, **9a**, and **10c** exhibited greater than 80% inhibition of *E. coli* biofilms at 200 μM concentration.

Table 2 Biofilm inhibition (IC_{50}) against MRSA and *E. coli*

Compound	MRSA IC_{50}/μM	*E. coli* IC_{50}/μM
5a	—	28.3
8a	49.8	—
9a	4.87	34.6
10a	4.70	—
10c	—	66.4

Dose-response curves were generated for the lead compounds for the inhibition of MRSA and *E. coli* biofilms (Table 2). Non-bactericidal antibiofilm activity was verified through colony count analysis of the planktonic viability in the presence and in the absence of each compound at their IC_{50} value (*i.e.* the concentration that inhibits 50% of biofilm formation, see ESI for details†). Against MRSA, IC_{50} values were determined to be 49.8 μM, 4.87 μM and 4.70 μM for **8a**, **9a**, and **10a** respectively. Against *E. coli*, IC_{50} values were determined to be 28.3 μM, 34.6 μM and 66.4 μM for **5a**, **9a**, and **10c** respectively.

Given the potency of our lead compounds toward inhibiting MRSA biofilms, we next explored their activity against various other *S. aureus* strains. Specifically, we screened **8a**, **9a**, and **10a** against three additional *S. aureus* strains (ATCC #'s 29213, 29740, and 25923). IC_{50} values were determined for each compound against each of the *S. aureus* strains; in some cases, the compounds were found to be more potent than they were against MRSA.

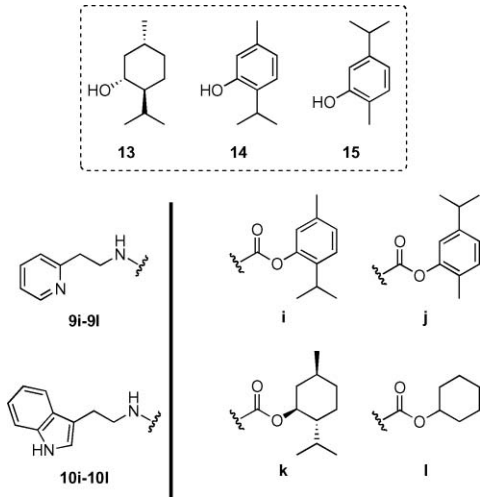
IC_{50} values for compound **8a** were found to be 21.2 μM, 24.3 μM and 71.9 μM against 29213, 29740 and 25923 respectively. For **9a** they were found to be 124 μM, 82.2 μM and 19.7 μM for 29213, 29740 and 25923 respectively. Lastly, for compound **10a**, which was found to be the most active compound overall, IC_{50} values were determined to be 4.70 μM, 2.84 μM and 37.4 μM for 29213, 29740 and 25923 respectively (Table 3). Again, planktonic viability in the presence of the test compounds was verified through colony count analysis.

Interestingly, our most potent inhibitors of the *S. aureus* strains including MRSA contained (–)-menthyl carbamates. Indeed, (–)-menthol and its derivatives have long been shown to have various antimicrobial and antiplasmid effects on bacteria.[15] Along with (–)-menthol (**13**), the related natural products thymol (**14**) and carvacrol (**15**) (Scheme 3, dashed box) are also known to possess antimicrobial activity.[16] In light of this observation, we prepared the thymyl and carvacryl carbamate analogues of **9a** and **10a**. We chose these two compounds for analogue design because they had the lowest IC_{50} values against MRSA and both worked well against 29213, 29740, and 25923 (see Tables 2 and 3). Additionally, we prepared the stereochemical antipodes of **9a** and **10a** by employing (+)-menthyl carbamate. Finally, we prepared the cyclohexyl carbamate derivatives of **9a** and **10a** as a control (Scheme 3).

With compounds **9i–10l** in hand, they were then screened for biofilm inhibition activity along with (–)-menthol and

Table 3 Biofilm inhibition (IC_{50}) against other *S. aureus* strains

Compound	29213 IC_{50}/μM	29740 IC_{50}/μM	25923 IC_{50}/μM
8a	21.2	24.3	71.9
9a	124	82.2	19.7
10a	4.70	2.84	37.4



Scheme 3 Analogues of compounds **9a** and **10a**.

(–)-menthol methyl ether against MRSA and 29213. Interestingly, none of the analogues depicted in Scheme 3 displayed any notable biofilm inhibition activity against either MRSA or 29213 at a 200 μM concentration, with the exception of **9k** and **10k**. These compounds were found to have identical antibiofilm properties as their enantiomers, **9a** and **10a**. Importantly, both (–)-menthol and (–)-menthol methyl ether were found to be completely inactive.

Lastly, **2d**, **8a**, **9a**, and **10a** were preliminarily screened for cytotoxicity. This was assessed using a red blood cell hemolysis assay using difibrinated sheep blood. In each case, the carbamates were found to show no red blood cell lysis up to the highest concentration tested (1.2 mM, see ESI for details†).

In summary, by targeting analogues of the bacterial metabolite **2d**, we have discovered a novel class of biofilm inhibitors based upon a menthyl carbamate scaffold. The culmination of this study resulted in two potent compounds (**9a** and **10a**) that display low micromolar IC_{50} values for the inhibition of various *S. aureus* biofilms including those from the medically relevant MRSA. This scaffold represents a unique new class of compounds for combating bacterial biofilms. Although they currently lack the ability to disperse preformed biofilms, this disadvantage is offset by their trivial preparation and inherent tunability. It is also noteworthy to mention that high concentrations of antibiofilm agents that have low IC_{50} values were needed to completely inhibit biofilm formation. This may in part be due to the inherit equilibrium of the biofilm development cycle in that planktonic cells will always continue to form films as long as they are viable. We have recently demonstrated that employing a combination therapy of antibiofilm agents with antibiotics is more effective at completely alleviating the biofilm source since the planktonic cells are constantly being eliminated from the equilibrium.[17] Current efforts in our labs are focused on further tuning this new scaffold as well as marrying this novel menthyl carbamate motif with our 2-aminoimidazole compounds. The results of these studies will be reported in due course.

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