ACS Medicinal Chemistry Letters

Letter

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1,2,4-Triazolidine-3-thiones as Narrow Spectrum Antibiotics against Multidrug-Resistant *Acinetobacter baumannii*

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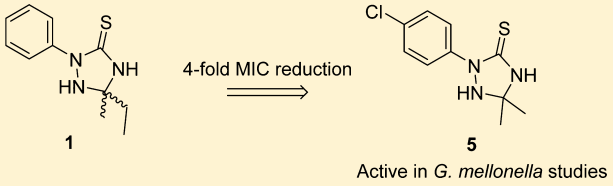
Supporting Information

ABSTRACT: With only two new classes of antibiotics developed in the last 40 years, novel antibiotics are desperately needed to combat the growing problem of multidrug-resistant and extensively drug resistant bacteria, particularly Gram-negative bacteria. Described in this letter is the synthesis and antibiotic activity of 1,2,4-triazolidine-3-thiones as narrow spectrum antibiotics. Optimization of the 1,2,4-triazolidine-3-thione scaffold identified a small molecule with potent antibiotic activity against multiple strains of multidrug-resistant and extensively drug-resistant *Acinetobacter baumannii*. This small molecule also shows single dose, *in vivo* activity in a *Galleria mellonella* infection model with *A. baumannii* and represents a promising start in the development of a class of drugs that can target this bacterial pathogen.



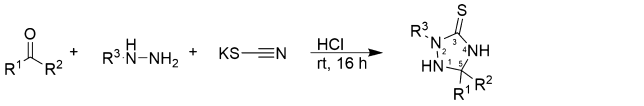
KEYWORDS: *antibiotic resistance, antibiotics, Acinetobacter baumannii*

Antibiotic resistance has become one of the forefront issues in global health because of the emergence of multidrug-resistant (MDR) bacteria. According to the Centers for Disease Control and Prevention (CDC), an estimated two million people each year acquire MDR bacterial infections, of which 23,000 are fatal.[1] Even more alarming is the dearth of antibiotic options emerging to treat these infections. Only two antibiotics belonging to novel structural classes have been brought into the clinic in the past 40 years, daptomycin and linezolid.[2] Of great concern is that both of these antibiotics are only effective against Gram-positive bacteria, which leaves treatment options for MDR Gram-negative bacteria as an urgent unmet medical need. Prominent Gram-negative pathogens include *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, which along with the Gram-positive species *Enterococcus faecium* and *Staphylococcus aureus* make up the bacterial species that are often referred to as "ESKAPE" pathogens.[3] Currently, the most effective option to treat MDR Gram-negative bacteria remains the polymyxin colistin, which has significant side effects including nephrotoxicity.[4] These side effects, coupled with colistin's effectiveness, have made it a last resort antibiotic against MDR Gram-negative bacteria. Worryingly, colistin-resistant strains of bacteria are being isolated with greater frequency, and recently, a colistin-resistance gene was found on a plasmid that could easily move between bacterial species.[5] Therefore, the threat of superbugs, bacteria becoming extensively drug resistant (XDR) and pandrug-resistant (PR), is a real possibility. As a result, new antibiotics with activity against Gram-negative pathogens are sorely needed.

The development of narrow spectrum antibiotics has some advantages over the traditional approach of developing broad-spectrum antibiotics, namely, the possibility of slower resistance generation and a reduction in detrimental effects on the gut microbiome including a decreased risk of antibiotic-associated colitis.[6] As more rapid and sensitive diagnostic tests are developed, the use of narrow-spectrum antibiotics is becoming more feasible, and the EMA and the FDA have recently included antibacterial compounds active against a narrow spectrum of MDR pathogens in a list of treatments that qualify for a shorter route to registration.[7] Of the Gram-negative ESKAPE pathogens, *A. baumannii* has recently come under the microscope because of its high mortality rates in the ICU (>50%), prevalence in wound infections in U.S. servicemen that have been injured in the conflicts in Iraq and Afghanistan,[8] as well as its ability to survive in hospital environments and the isolation of PR strains.[9]

In 2009 compound **1** (Scheme 1) was reported to have antifungal activity against *Candida albicans*.[10] Recently, genomic

Scheme 1. Synthesis of 1,2,4-Triazolidine-3-thiones[12,13]



1 R^3= Ph, R^1=Me, R^2=Et
2a-o R^3= Ph
3a-c, 4a-o R^1=R^2=Et
5 R^3=4-chlorophenyl, R^1=R^2=Me

Received: July 29, 2016
Accepted: October 31, 2016
Published: November 12, 2016

DOI: 10.1021/acsmedchemlett.6b00296
ACS Med. Chem. Lett. 2017, 8, 27−31

sequencing has revealed that *A. baumannii* harbors genes that share homology to a long chain fatty acid elongation pathway in fungi that was the supposed target of compound **1**. Testing of 1,2,4-triazolidine-3-thiones, for antibiotic activity against *A. baumannii* identified compound **1** as a promising antibacterial lead.[11] Using this compound as a structural template, we have synthesized analogues of the 1,2,4-triazolidine-3-thione core structure with variations introduced at the *N*-1, *N*-2, and *C*-5 positions as well as alkylations of the sulfur atom at *C*-3 in an attempt to identify new, more biologically active compounds. Herein, we report the synthesis of analogues of 1,2,4-triazolidine-3-thiones as well as their antibiotic activity against MDR-*A. baumannii*. Moreover, we report a compound that has minimum inhibitory concentrations (MICs) of up to 4-fold lower against multiple strains of MDR-*A. baumannii* compared to compound **1** and, unlike parent compound **1**, shows activity in a *Galleria mellonella* model of infection.

The 1,2,4-triazolidine-3-thione scaffold is readily accessible through a three-component reaction between ketones or aldehydes, hydrazines, and potassium thiocyanate in hydrochloric acid for 16 h in the dark (Scheme 1). The resulting triazolidines typically precipitate during the reaction, allowing for simple filtration followed by recrystallization from methanol to deliver purified material.[12,13] Moreover, this synthetic procedure easily allows for modifications to be made at the *N*-2 and *C*-5 positions on the ring, thus allowing rapid assessment of the structure−activity relationship of 1,2,4-triazolidine-3-thiones.

Initially, we evaluated the effect that substitution at carbon 5 had upon activity by varying the identity of the aliphatic substituents. A series of 14 compounds that were mono- or di-substituted with alkyl or aryl groups were synthesized and screened for antibiotic activity against AB5075 under standard CLSI broth microdilution conditions[14] (Table 1). *A. baumannii* strain AB5075 is an XDR primary clinical isolate and was chosen to evaluate the 1,2,4-triazolidine-3-thiones because of its virulence and XDR properties.[15]

Compound **1** exhibited an MIC of 8 μg/mL against AB5075, and both the dimethyl (compound **2a**) and diethyl (compound **2e**) derivatives exhibited identical activity to the parent compound. All other modifications at the *C*-5 position yielded either no change, or an increase in MIC compared to the parent compound (Table 1).

As we did not observe an increase in antibiotic activity through modification of the 5-position we next shifted our focus to the substitution at the *N*-2 position of the ring. The diethyl substituted derivatives were chosen instead of the parent methyl ethyl substituted derivatives to avoid racemic mixtures. Benzyl, cyclohexyl, and 2-pyridinyl substitutions of the *N*-2 position completely abolished biological activity (supporting information), which led to the conclusion that the phenyl ring at the *N*-2 position was required for activity (compound **3**, Scheme 1).

Given the significant impact that changing the 2-phenyl substituent to a 2-benzyl had upon activity, we then investigated whether substitution of the phenyl ring itself had any effect on the MIC (Table 3). The 4-chloro derivative **4a** exhibited increased activity compared to the parent compound, with an MIC of 2 μg/mL. Encouraged by this result, additional analogues were synthesized with various halogen substitutions in different positions on the phenyl ring. Unfortunately, only a decrease in activity was observed compared to the 4-chloro compound. Other substitutions on the phenyl ring were also made, but only halogen substitutions were tolerated (Table 2).

Table 1. MIC Values for *C*-5 Modifications against AB5075, Where R^3 = Phenyl

compd	R^1	R^2	MIC[a]
1	methyl	ethyl	8
2a	methyl	methyl	8
2b	methyl	isopropyl	8
2c	methyl	phenyl	>128
2d	methyl	H	>128
2e	ethyl	ethyl	8
2f	ethyl	isopropyl	32
2g	ethyl	H	>128
2h	*n*-propyl	methyl	16
2i	*n*-propyl	ethyl	32
2j	*n*-propyl	*n*-propyl	32
2k	*n*-propyl	H	64
2l	phenyl	H	64
2m	cyclopentyl		8
2n	cyclohexyl		8

[a]All concentrations are in μg/mL.

Table 2. MIC Values for Various Phenyl Ring Substitutions against AB5075, Where R^1 = R^2 = Ethyl

compd	R^3	MIC[a]
4a	(4-chlorophenyl)	2
4b	(4-bromophenyl)	4
4c	(4-iodophenyl)	8
4d	(4-fluorophenyl)	4
4e	(2-fluorophenyl)	32
4f	(3-fluorophenyl)	8
4g	(2-chlorophenyl)	128
4h	(3-chlorophenyl)	8
4i	(3-chloro-4-fluoro)	32
4j	(3,5-difluorophenyl)	>128
4k	(3,4-dichlorophenyl)	>128
4l	(4-isopropylphenyl)	>128
4m	(4-nitrophenyl)	>128
4n	(4-cyanophenyl)	>128
4o	(4-trifluoromethylphenyl)	>128

[a]All concentrations are in μg/mL.

With a more active derivative **4a** identified, we next tested the lead compound and parent compound against multiple strains of MDR-*A. baumannii* (representative examples shown in Table 3). The dimethyl analogue of compound **4a**, compound **5**[16−18] (Scheme 1), was also prepared to investigate whether the lipophilicity of the compounds could be tuned without affecting the activity of the compounds (note: our

Table 3. MIC Results for Compounds 1, 4a, and 5 against Multiple MDR Strains of *A. baumannii*[a]

compd	AB-4878	AB-3785	AB-3806	AB-4957	BAA-1605	AB-3638
1	8	4	4	8	8	4
4a	4	4	4	4	4	4
5	2	1	1	2	2	1

[a]The resistance profiles of these strains have been previously published.[19] All concentrations are in μg/mL.

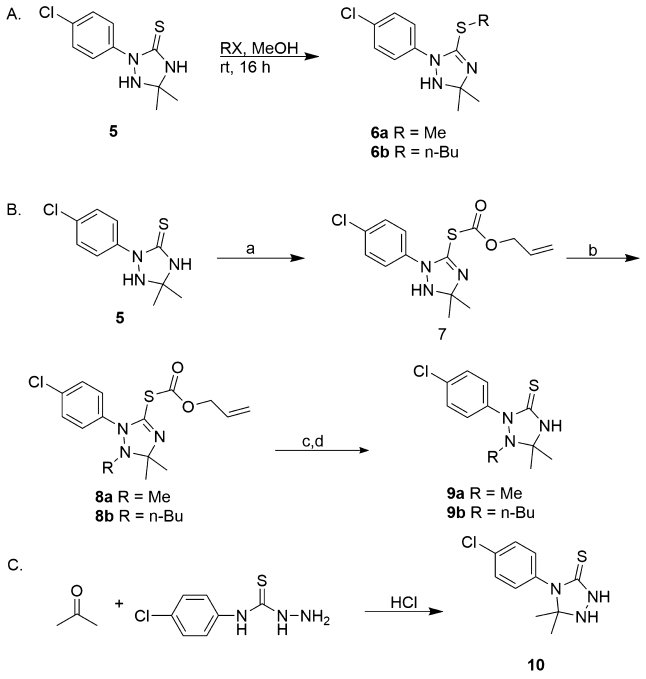
DOI: 10.1021/acsmedchemlett.6b00296
ACS Med. Chem. Lett. 2017, 8, 27−31

spectral data for compound **5** did not match previously reported data; however, the crystal structure of our synthetic sample was determined to unambiguously establish identity). Compound **4a** has a predicted log D value of 3.85, while compound **5** has a predicted log D value of 2.58 at pH 7.

All three compounds exhibited activity against multiple strains of *A. baumannii*, while compound **5** was more active against several strains of *A. baumannii* than either compound **4a** or the parent compound **1** (Table 3). Additional MIC results against more strains of *A. baumannii* can be found in the Supporting Information.

After the identification of compound **5** as the most active analogue against several *A. baumannii* strains, we next opted to investigate further structure−activity relationships of compound **5**, beginning with the effect of alkylating the sulfur atom at the *C*-3 position. Briefly, compound **5** was reacted with the corresponding alkyl halide in methanol at room temperature overnight to yield the thioethers **6a−b** (Scheme 2). The methyl

Scheme 2. Synthetic Routes To Allow for the Alkylation of the Thiol Moiety (A), *N*-1 Position (B), and Move of 4-Chloro Substituent (C) of Compound 5[a]



[a]Reagents and conditions: (a) Alloc-Cl, DMAP, Et_3N, THF rt, 16 h; (b) RX, NaH, DMF 0° C to rt, 4 h; (c) $NaBH_4$, $Pd(PPh_3)_4$, EtOH 0° C to rt, 1 h; (d) 12 N HCl (pH 2.5-3), rt, 4 h.

and benzyl analogues were prepared, and the observed MIC for both compounds was greater than 128 μg/mL, showing the necessity of the unsubstituted sulfur atom for biological activity. To further probe the SAR of the 1,2,4-triazolidine-3-thione core, effect of alkylation of the *N*-1 position of compound **5** was also investigated (Scheme 2).

In order to alkylate the *N-1* position, the thiol was protected with an alloc protecting group. The *N-1* position was then alkylated using sodium hydride and the corresponding alkyl halide. The alloc group was removed using sodium borohydride and tetrakis(triphenylphosphine)palladium(0) in ethanol at 0 °C, and the solution was then acidified with 12 N HCl at room temperature.[20] The MICs of compounds **9a−b** were all greater than128 μg/mL, demonstrating the need for the free N−H at the *N-1* position on the 1,2,4-triazolidine-3-thione ring for biological activity.

The final structural modification that was investigated was to move the 4-chlorophenyl substituent from the *N*-2 position to the *N*-4 position of compound **5** (Scheme 2C). The reverse 1,2,4-triazolidine-3-thione scaffold is accessed through the reaction of *N*-(4-chlorophenyl) hydrazinecarbothioamide with acetone in hydrochloric acid for 16 h in the dark (Scheme 2C). As with compounds **1−4o**, the resulting triazolidine precipitated during the reaction, allowing for simple filtration followed by recrystallization from methanol to deliver purified material.[12,13] The observed MIC of compound **10** was greater than 128 μg/mL, which is a greater than 64-fold increase in MIC compared to compound **5**, indicating that the original substitution pattern of the 1,2,4-triazolidine-3-thiones is the more active motif.

We next investigated whether compounds **5** was acting in a bactericidal or bacteriostatic manner by constructing a time kill curve. An antibiotic is defined as bactericidal when it kills ≥99.9% of bacteria at a concentration no greater than four times the MIC.[21] As seen in the time-kill curve below (Figure 1), compound **5** is bactericidal, effecting a greater than six log reduction in colony forming units per milliliter (CFU/mL) at double the MIC and greater.

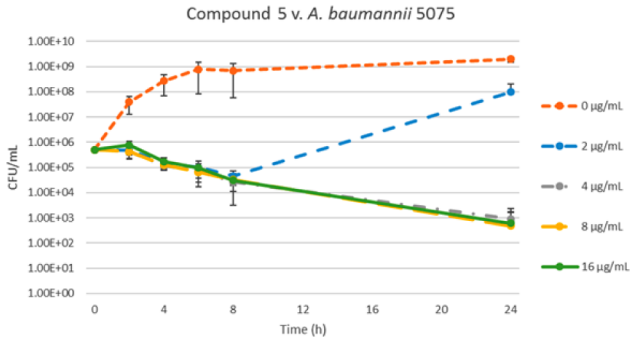


Figure 1. Time-kill curve for compound **5** against AB5075.

In *C. albicans* and *A. baumannii*, compound **1** distorts the ratio of unsaturated to saturated fatty acids.[10,11] It was also established that fatty acid supplementation of media abrogated the bactericidal effects of compound **1** against *A. baumannii*.[11] In order to test whether compound **5** exhibited similar behavior, an MIC assay under fatty acid supplementation was performed. As with compound **1**, when the media was supplemented with 0.02% linoleic acid, the activity of compound **5** against AB5075 was completely abolished with an observed MIC of >128 μg/mL.

In order to probe the antibiotic spectrum of compound **5**, MICs against *Escherichia coli*, *P. aeruginosa*, *K. pneumoniae*, and methicillin resistant *S. aureus* (MRSA) were recorded. An MIC of ≥128 μg/mL was observed for all bacterial species other than *A. baumannii*, establishing that as with the original lead **1**, compound **5** is a narrow spectrum antibiotic. The synergistic potential of compound **5** with other antibiotics was also investigated utilizing checkerboard assays.[22] Compound **5** did not show synergy with Meropenem or colistin against AB5075, or with colistin against the colistin-resistant AB3941.

Given the potential hydrolytic instability of **5**, we performed a time-dependent stability study. Compound **5** was dosed in

DOI: 10.1021/acsmedchemlett.6b00296
ACS Med. Chem. Lett. 2017, 8, 27−31

cation adjusted Mueller−Hinton Broth (CAMHB) and incubated at 37 °C. The mixture was then analyzed by taking liquid chromatography. We determined that compound **5** has a half-life of about 2.5 h in these conditions and is almost fully degraded after 16 h (Supporting Information). After observing the decomposition of the compound, compound **5** was dosed in CAMHB at 4 μg/mL and incubated for 1, 2, 4, and 8 h. The activity of each of the incubated solutions was then tested against *A. baumannii* 5075, and compound **5** was found to have no activity at its MIC after incubation in MHB for any of the time points. This indicates that compound **5**, and not any degradation products, is most likely the active chemical species.

The toxicity of compound **5** to eukaryotic cell membranes was also investigated by measuring its hemolytic activity against mechanically defibrinated sheep blood as previously reported.[23] Compound **5** did not lyse the red blood cells at any concentration tested (up to 512 μg/mL, Supporting Information).

Finally, we compared the activity of compound **1** and **5** in a *G. mellonella* infection model with AB5075.[15] Compound **1** was inactive against *AB*5075 in this model (data not shown, Supporting Information). However, compound **5** exhibited modest *in vivo* activity, with 50 mg/kg of compound **5** saving 22% of worms after 6 days, and 100 mg/kg saving 32% of worms after 6 days. In comparison, 100% of untreated, infected worms were dead after 6 days (Figure 2). The comparison is

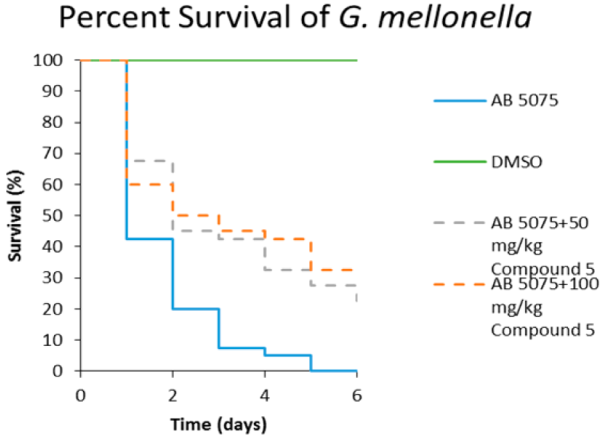


Figure 2. Percent survival of *G. mellonella* after AB5075 infection and compound **5** treatment.

more stark at 3 days, where almost 90% of the worms are dead in the untreated group, but in contrast, 42.5% and 45% survival are seen with the 50 mg/kg and 100 mg/kg treatments, respectively (Figure 2).

In conclusion, following the identification of the 1,2,4-triazolidine-3-thiones as possessing antibiotic activity against *A. baumannii* we synthesized a diverse array of analogues in attempts to identify a compound with increased activity. After making modifications at the *C*-5 and *N*-2 positions on the 1,2,4-triazolidine-3-thione core structure to no effect, substitutions were made to the phenyl ring at position 2 to yield compound **4a**, which displayed a 4-fold improvement in MIC against *A. baumannii* compared to compound **1**. Compound **5** was also synthesized and tested against multiple strains of *A. baumannii* along with compound **1** and compound **4a** in order to ascertain whether the lipophilicity could be tuned without affecting the MIC. Compound **5** displayed an up to 4-fold improvement against multiple strains of *A. baumannii* compared with compound **1**, as well as returning a lower MIC than compound **4a** in most cases. Compound **5** was found to be bactericidal, and an *in vivo* study showed positive survival results when compound **5** was used against AB5075 in a *G. mellonella* model of infection. While these results were modest, it is important to note that just a single dose was provided, and most antibiotics are dosed multiple times a day. Furthermore, AB5075 is a more virulent strain than most other *A. baumannii* isolates,[14] which provides a higher bar when evaluating activity. Future studies are now underway to develop structural modifications and conduct biological evaluations against *A. baumannii* using compound **5** as our current lead in an attempt to further augment *in vitro* activity and improve *in vivo* results.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00296.

Compound characterization for all novel compounds, ^{1}H and ^{13}C NMR spectra, MIC and full in vivo testing data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

The authors from N.C. State would like to thank the university for support. The authors from Wound Infection Department at WRAIR would like to acknowledge the constant support of the Military Infectious Diseases Research Program.

Notes

The material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Dr. Zurawski would like to thank the Multidrug-resistant Organism Repository and Surveillance Network (MRSN) for providing the isolates used in this study.

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