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Amide Isosteres of Oroidin: Assessment of Antibiofilm Activity and *C. elegans* Toxicity

Justin J. Richards,[†] Samuel Reyes,[†] Sean D. Stowe,[‡] Ashley T. Tucker,[‡] T. Eric Ballard,[†,§] Laura D. Mathies,[∥] John Cavanagh,[‡] and Christian Melander*[,†]

[†]*Department of Chemistry,* [∥]*Department of Genetics, and* [‡]*Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, North Carolina 27695.* [§]*Current address: Department of Chemistry, University of Virginia, Charlottesville, VA 22904.*

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Abstract: The synthesis and antibiofilm activities of sulfonamide, urea, and thiourea oroidin analogues are described. The most active derivative was able to selectively inhibit *P. aeruginosa* biofilm development and is also shown to be nontoxic upward of 1 mM to the development of *C. elegans* in comparison to other similar isosteric analogues and the natural product oroidin.

Bacterial biofilm formation is often described as a developmental process initiated when free floating (planktonic) bacteria adhere to a surface suitable for growth and initiate the formation of a microcolony.[1] Occupation of a biofilm growth state confers to the bacteria a unique set of phenotypic traits that include resistance to microbicides and antibiotics that would often lead to eradication.[2−4] In a medical setting, biofilms pose a serious threat to individuals who suffer from a myriad of diseases. Recent estimates have attributed biofilm-associated infections as being responsible for upward of 75% of microbial infections in the human body.[5] This problem is further exacerbated by the increased spread of antibiotic resistance. Additionally, biofilms are known to infect patients with in-dwelling medical devices (IMDs[a]) such as catheters and heart stents.[6−8] Remediation of biofilm infected IMDs is traditionally accomplished by device removal because of the lack of antibiotic efficacy.

As the medical community works toward new approaches aimed at combating the deleterious effects of biofilms, one area that has garnered significant attention is the identification of nonmicrobicidal modulators of biofilm growth and maintenance (Figure 1).[9−13] By not directly killing bacteria, it is postulated that development of resistance to these molecules would be mitigated or significantly impaired. Implementation of remediation therapies that focus on the co-dosing of an antibiofilm modulator with an antibiotic also provides an attractive avenue for treatment. One of the few naturally occurring scaffolds shown to possess nonmicrobicidal antibiofilm properties are compounds derived from the oroidin class of natural products. Oroidin **5** has been reported to be a moderate inhibitor of *Pseudomonas aeruginosa* PAO1/-PA14 biofilm growth (PAO1 IC_{50} = 190 μM, PA14 IC_{50} = 166 μM).[14]

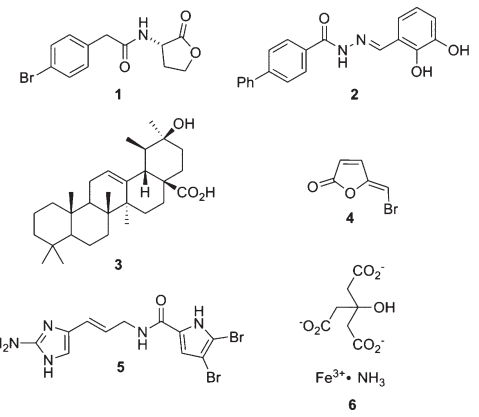

Figure 1. Nonmicrobicidal biofilm modulators.

We have recently focused on the development of methodologies to access oroidin analogues for antibiofilm screening.[15−17] Of these approaches, development of conditions for a generic reductive acylation reaction has allowed access to previously unattainable oroidin derivatives through the use of acid chlorides, anhydrides, succinimide esters, and trichloromethylketone pyrroles.[18] In a continued effort, we sought to further apply this approach in generating isosteric analogues possessing sulfonamide, urea, and thiourea functionalities to further probe the structure−activity relationships (SAR) of the oroidin family in the context of antibiofilm activity and preliminary toxicity.

The Gram-negative γ-proteobacteria *Pseudomonas aeruginosa* PA14 and *Acinetobacter baumannii* were selected as the biofilm-forming bacteria employed in the study. *Pseudomonas aeruginosa* is one of the most well studied organisms with respect to biofilm behavior.[19] It is the second most commonly isolated pathogen in cases of nosocomial acquired pneumonia.[20] Additionally, the inability to treat cystic fibrosis patients with chronic *P. aeruginosa* infections has been directly correlated with the emergence and pathogenicity of *P. aeruginosa* biofilms.[21] *Acinetobacter baumannii* has been identified in recent hospital outbreaks throughout healthcare systems in both Europe and the U.S.[22,23] The speed and prevalence with which multidrug resistance (MDR) is occurring in this bacterium has posed as a significant impediment to *A. baumannii* remediation therapies. In addition to examining the antibiofilm properties of these new derivatives, it was also a major goal of this study to further delineate the toxicity associated with oroidin-derived compounds. This was accomplished by investigating how the most active member of the newly formed library and previously synthesized isosteric analogues affected larvae development in the eukaryote *Caenorhabditis elegans* in comparison to the natural product oroidin.

A total of 19 analogues (9 prepared from sulfonyl chlorides, 8 prepared from isocyanates, 2 prepared from isothiocyanates) were synthesized by slightly varying the reaction conditions previously reported (Schemes 1 and 2).[18] While no base was used under the initial acylation conditions, it was found that

*To whom correspondence should be addressed. Phone: 919-513-2960. Fax: 919-515-5369. E-mail: christian_melander@ncsu.edu.

[a] Abbreviations: IMD, in-dwelling medical device; IC_{50}, biofilm inhibitory concentration of 50%; SAR, structure−activity relationship; MDR, multidrug resistance; Pd/C, palladium on carbon; THF, tetrahydrofuran; rt, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid; SE, standard error; 2-AI, 2-aminoimidazole; NIEHS, National Institute of Environmental Health and Safety; DHS, dihydrooroidin.

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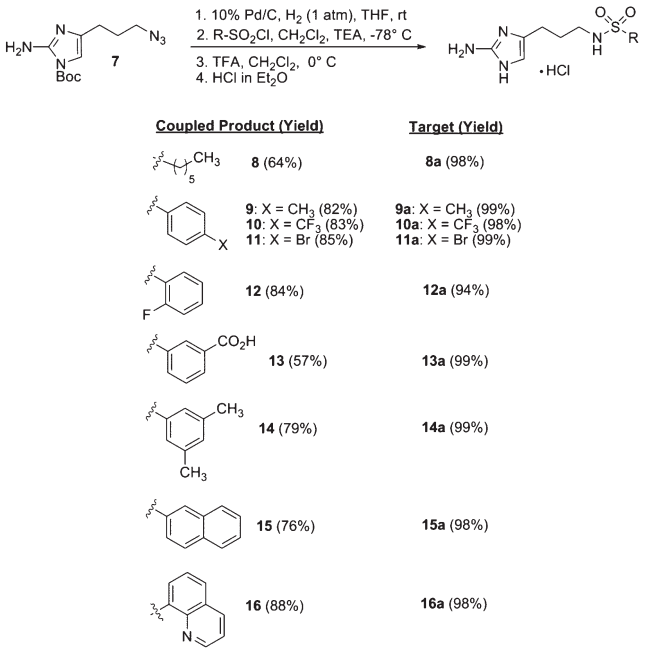
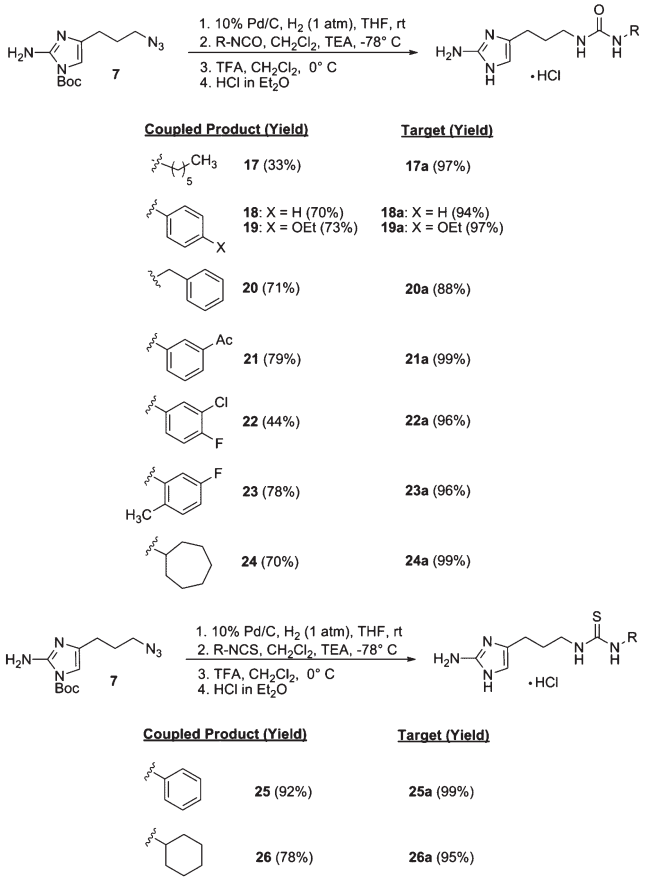
Scheme 1. Synthesis of Sulfonamide Analogues

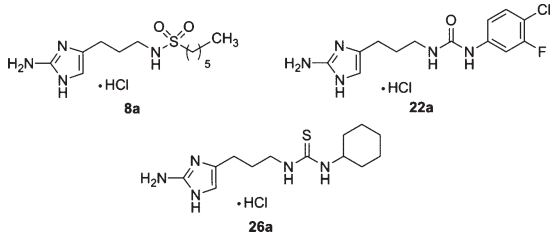


Scheme 2. Synthesis of Urea and Thiourea Analogues



addition of a single equivalent of triethylamine prior to addition of the acylating reagent and keeping the solution at −78 °C throughout the course of the reaction led to optimum yields. In general, the generation of sulfonamide derivatives proceeded smoothly (57−88%) while the corresponding yields for the formation of the ureas and thioureas were less consistent (33−92%). The commercial availability of a wide array of these acylating reagents also made it possible to probe the effect that larger ring systems (**15**, **16**, and **24**) had on antibiofilm activity. Coupled products generated through the application of the methodology were then exposed to TFA-mediated deprotection in dichloromethane. The resulting TFA salts were exchanged for the corresponding hydrochloride salts which were then used for biological assessment.

Table 1. Antibiofilm Activity of Select Analogues



analogue	PA14 $IC_{50} \pm$ SE (μM)	PA14 dispersion at 100 μM $\pm$ SE (%)
8a	10.1 ± 1.96	42 ± 4
22a	25.7 ± 2.19	55 ± 3
26a	22.6 ± 3.24	25 ± 5

All newly synthesized derivatives were first subjected to static biofilm inhibition assays performed at 100 μM utilizing a crystal violet reporter assay (Supporting Information).[15,24] Compounds whose inhibition values exceeded 80% in the initial screen were subsequently assayed for IC_{50} (biofilm inhibition) values. The most active compound in each class of molecules (sulfonamide, urea, thiourea) was then analyzed for biofilm dispersion activity. Surprisingly, none of the newly developed sulfonamides or ureas/thioureas were able to effectively inhibit the formation of *A. baumannii* biofilms greater than 30% at 100 μM. This is in contrast to previous studies where the biological activity of most analogues could be conserved over γ-proteobacteria.[25,26] However, many of the derivatives were determined to be potent inhibitors of *P. aeruginosa* PA14 biofilm development with 11 out of the 19 analogues exhibiting PA14 IC_{50} under 50 μM. In general, the sulfonamides were the most active (IC_{50} = 10−46 μM), followed closely by the two thioureas (IC_{50} = 22−26 μM) and last the ureas (IC_{50} = 25−50 μM). The most active analogues from each class are summarized in Table 1. Nonmicrobicidal behavior of **8a**, **22a**, and **26a** against PA14 was validated by performing growth curve experiments at the calculated IC_{50}. No change in bacterial cell density was observed for all compounds throughout a 24 h time period (Supporting Information).

In parallel with previous observations of other oroidin derivatives, there was a decrease in the activities observed in the dispersion experiments.[14,15,18,27] Analogues **8a**, **22a**, and **26a** were only modest dispersal agents against preformed PA14 biofilms at 100 μM (42%, 55%, and 25%, respectively). Despite the lack of biofilm dispersal activity, the inhibition results further strengthened the argument that inhibition activity of the oroidin scaffold against PA14 can be modulated to deliver molecules even more potent than those found with an amide bond in a similar arrangement to the natural products.[15,25] Also, activity can be tuned to display selectivity against biofilm forming proteobacteria which reside in the same class through the incorporation of sulfonamide or urea/thiourea functionalities.

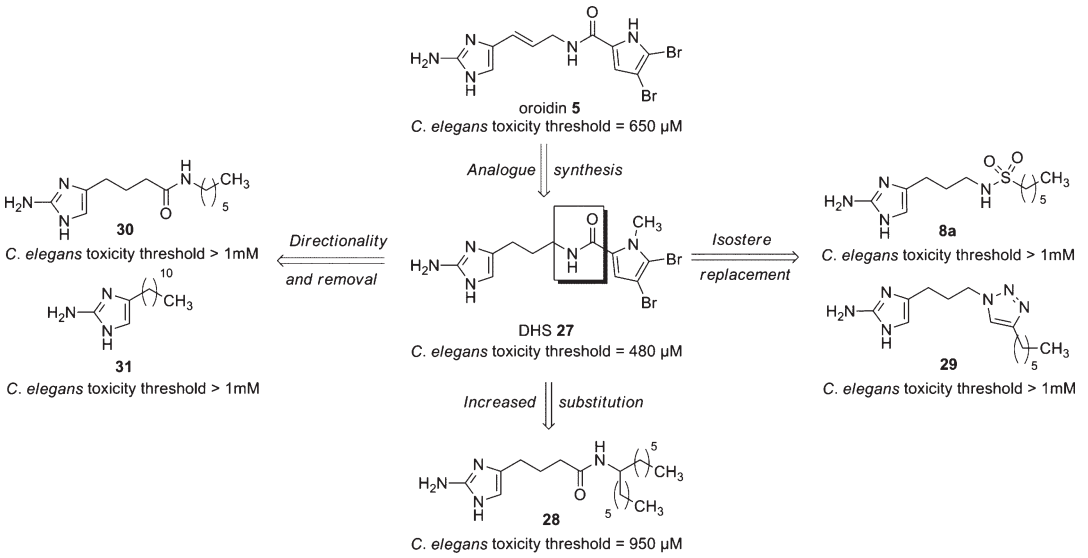


Figure 2. *C. elegans* toxicity thresholds of selected hexyl chain based isosteric oroidin analogues.

The continued investigation into the development of antibiofilm analogues derived from the oroidin scaffold has inevitably led us to further question the possible utilization of these molecules as therapeutic treatments in biofilm remediation efforts. Ultimately, the molecules must retain their noncytotoxic biofilm-modulating properties without affecting the surrounding cellular environments. Preliminary cytotoxicity experiments involving GH4C1 rat pituitary cells and N2A mouse neuroblastoma cells have shown that select 2-aminoimidazole (2-AI) analogues possessed nontoxic activity at concentrations up to 600 μM.[27,28] To further investigate the toxicity profiles of a specific class of 2-AI derivatives, we analyzed the effects they had on the development of the multicellular eukaryote *Caenorhabditis elegans*.

The overall goal of the assay was to examine what effect various concentrations of 2-AI molecules would have on late larval stage *C. elegans* development (Supporting Information for detailed assay). The National Institute of Environmental Health and Safety (NIEHS) has validated preliminary screens for eukaryotic toxicity in model organisms such as *C. elegans* as a means for the strategic testing of possible toxic substances.[29,30] Briefly, in 96-well microtiter plates, the nematodes were monitored for their ability to develop into egg-laying adults, for the eggs to hatch into early stage larvae, and for these larvae to subsequently feed on a bacterial suspension. Observations were made through visualization under a dissecting microscope, with the results being interpreted in a binary manner. Either the wells remained turbid and opaque signaling that the worms had not fed on the bacterial suspension at the given concentration of the 2-AI compound (toxic) or the wells became transparent indicating that the 2-AI derivative had not interfered with developmental activities at that particular concentration (nontoxic). Ivermectin, a potent nematocide, was employed as a positive control. Using this experimental design, thresholds of toxicity toward *C. elegans* development were then determined.

A logical starting point for the assay was to first assess the toxicity of oroidin and dihydrosventrin (DHS) **27**, which was one of the first 2-AI analogues assembled from the oroidin skeleton that was found to possess biofilm modulating properties.[14,26] In addition to assaying the most active member of the present isosteric library (**8a**), it was decided to also examine a number of other hexyl aliphatic chain 2-AI analogues in which the amide bond was modified in an analogous fashion (Figure 2).[25] The results from the toxicity screens are summarized in Figure 2. Oroidin **5** was found to display a threshold of 650 μM, meaning that any concentration over that mark was found to be toxic to the worms. DHS **27** was slightly more toxic, exhibiting a detrimental effect at concentrations greater than 480 μM. However, many of the aliphatic derivatives chosen for analysis were found to be nontoxic at much higher concentrations (**8a**, **29**, **30**, **31**), with most even exceeding the maximum concentration of 1 mM used in the study. Analogue **28** was close to the 1 mM mark, eliciting a toxic effect at concentrations greater than 950 μM. These results are the first to demonstrate the associated toxicity of oroidin and related analogues to the development of *C. elegans*. The relative lack of toxicity at such high concentrations undoubtedly provides a suitable foundation for the continued exploration of the affects of these compounds in other model organisms.

In conclusion, employment of a reductive acylation strategy to incorporate sulfonamide, urea, or thiourea functionalities into the oroidin backbone has led to the discovery that other amide isosteres have the potential to be potent and selective modulators of *P. aeruginosa* biofilm development. Furthermore, related isosteric analogues that bear aliphatic side chains are reported to be highly nontoxic in nature to the development of *C. elegans* in comparison to the natural product oroidin. Ultimately, it is envisioned that the reductive acylation reaction will prove amenable for the synthesis of a variety of biotinylated conjugates to help further probe the mechanism of action of these biofilm modulating nontoxic compounds.

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Supporting Information Available: Antibiofilm activity charts, growth curves, *C. elegans* toxicity assay procedures, characterization data, and representative spectra from each class of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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