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Letter

Total Synthesis and Microbiological Evaluation of Leopolic Acid A and Analogues

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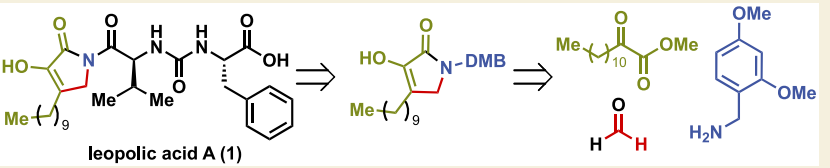

ABSTRACT: New antimicrobial scaffolds are scarce, and there is a great need for the development of novel therapeutics. In this study, we report a convergent 9-step synthesis of leopolic acid A and a series of targeted analogues. The designed compounds allowed for incorporation of non-natural ureido dipeptide moieties and 4- and 5-position substituents around the 2,3-pyrrolidinedione of leopolic acid A. Leopolic acid A displayed modest antimicrobial activity (32 μg/mL) against MRSA, while the most active analogues displayed slightly improved activity (8−16 μg/mL). Additionally, several of the leopolic acid A analogues displayed promising antibiofilm activity, most notably having an MBEC:MIC ratio of ∼1. Overall, this work represents an initial SAR of the natural product and a framework for further optimization of these bioactive scaffolds within the context of bioactive pyrrolidinediones.

KEYWORDS: *antibiotics, antibiofilm, natural products, multicomponent reactions, heterocycles*

Natural products constitute a valuable source of bioactive molecules and have been the source of many FDA approved drugs, especially antibiotics. Since penicillin was first discovered, around three-quarters of all antibiotics in use have been developed from natural products.[1−3] In addition, natural products are a promising source for antibiofim agents.[4−6] Biofilms are challenging to treat, with limited options available. Due to the current lack of novel antimicrobial scaffolds, accessing natural products with antimicrobial activity is a worthwhile path to enable new antimicrobial development.[7] Leopolic acid A is a terrestrial natural product first isolated from *Streptomyces* sp. found in the rhizosphere of *Juniperus excels* in the Crimean mountains. The isolation team reports modest antimicrobial activity (16 μg/mL against *S. aureus*) of the natural product via an unknown mechanism of action.[8] Structurally, leopolic acid A contains a 2,3-pyrrolidinedione headgroup bearing an aliphatic chain appended to a urea containing dipeptide composed of L-valine and L-phenylalanine. These structural features make leopolic acid A a promising scaffold for antimicrobial development, since there have been several reports of oligo-, cyclic-, and polypeptides that have displayed novel antimicrobial properties.[9−14]

To date, only one total synthesis of leopolic acid A has been reported in 11 steps.[15] The previous approach by Dallavalle and co-workers utilizes a Michael addition followed by a Dieckmann cyclization to form the 2,3-pyrrolidinedione, followed by subsequent elongation of the aliphatic chain. The dipeptide fragment was installed stepwise (Figure 1A) and

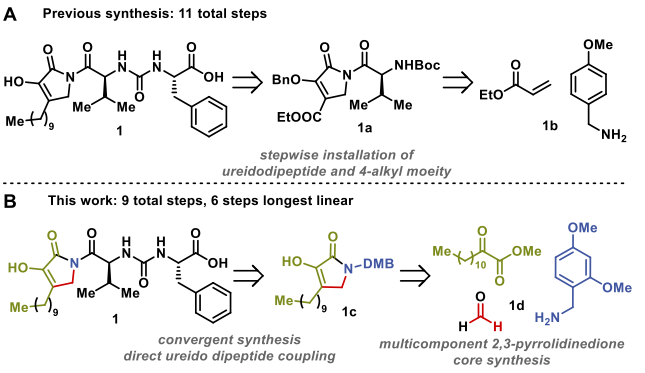

Figure 1. (A) Previous synthetic strategy used to synthesize leopolic acid A; (B) this work.

no structure activity relationship (SAR) studies were explored. To the best of our knowledge, only one natural product analogue has been reported, which displayed slightly improved antimicrobial activity compared to the parent compound (8 μg/mL against *S. aureus*).[16]

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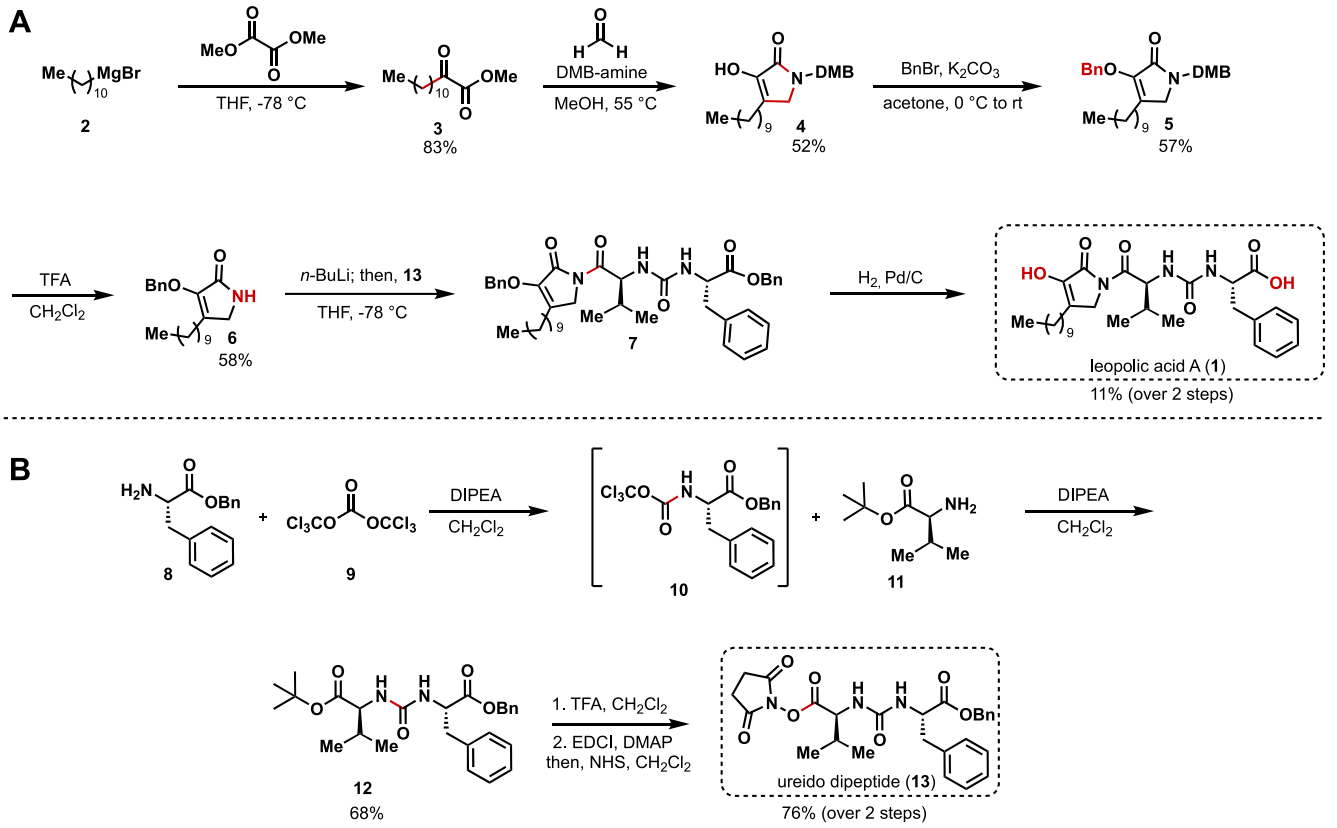

Figure 2. (A) Synthetic route to prepare leopolic acid A (**1**); (B) synthesis of ureido dipeptide fragment.

While developing multicomponent approaches to bioactive heterocycles, we have developed methods for the rapid synthesis of 2,3-pyrrolidinediones and leveraged them, and their regioisomeric oxazolidinone counterparts, as leads for antibiotic discovery.[17−24] Given our rapid access to diverse 2,3-pyrrolidinedione scaffolds, we envisioned a one-pot synthesis of the requisite headgroup of leopolic acid A followed by convergent coupling to the dipeptide fragment (Figure 1B), thereby providing an analogue-friendly route that would allow diversification around the natural product scaffold.

Herein, we report the total synthesis of leopolic acid A in 9 steps (6 longest linear) using a versatile and convergent synthetic strategy. This route allowed for the synthesis and SAR studies of 9 novel leopolic acid A analogues to evaluate their antimicrobial and antibiofilm properties.

As noted, we envisioned accessing leopolic acid A by a convergent strategy to bring together the 2,3-pyrrolidinedione core with the ureido dipeptide fragment (Figure 1B). Our forward route began with the synthesis of the pyruvic ester derivative via Grignard addition of freshly prepared *n*-undecane magnesium bromide and dimethyl oxalate to provide compound **3** (Figure 2A). With the pyruvic ester **3** in hand, we performed the multicomponent reaction using DMB-amine and formaldehyde, to access compound **4**. While this reaction proceeded as expected, slight heating was required to drive the reaction forward, unlike previous aldehyde substrates that convert quickly at room temperature. The 2,3-pyrrolidinedione was benzyl protected to prevent the degradation of the core when deprotecting the 2,4-dimethoxybenzylamine with TFA. Overall, the methylene bearing 2,3-pyrrolidinediones such as **4** were much more labile than their substituted counterparts prepared previously as deprotection of the unprotected enol resulted in significant decomposition even at reduced temperatures.

The ureido dipeptide fragment required for coupling with compound **6** was prepared by reaction of L-phenylalanine benzyl ester hydrochloride with L-valine *tert*-butyl ester hydrochloride promoted by triphosgene, to access the doubly protected ureido dipeptide **12**. The *tert*-butyl group was removed with TFA to access the corresponding carboxylic acid necessary for the coupling reaction with 2,3-pyrrolidinedione core **6**. *N*-Hydroxysuccimide, EDCI, and DMAP were used to install the *N*-hydroxysuccinimide activating group in ureido dipeptide **13**. To complete the synthesis of leopolic acid A, several coupling conditions were explored, revealing *n*-BuLi and LiHMDS mediated coupling to be the most effective to access the dibenzyl-protected peptide **7**. Finally, hydrogenation with Pd/C and H_2 was used to cleave the benzyl protecting groups and provide access to leopolic acid A (**1**). While the yields of this 2-step procedure are low, there is significant recovery of starting material, and the convergent nature of the route was prioritized at this stage of the project.

The versatile synthetic route developed for the synthesis of leopolic acid A allows access to a wide variety of analogues of this natural product. We started by focusing our efforts on the effects of modifying the 4- and 5-position of the pyrrolidinedione, as our multicomponent reaction made for facile derivatization (Figure 3). By employing various aldehydes in the multicomponent reaction, we were able to access methyl (**14**), ethyl (**15**), propyl (**16**), and phenyl (**17**) analogues, while keeping the 4-*n*-decane and the ureido dipeptide moieties in place. Substituted derivatives were isolated as mixtures of diastereomers in some cases. With this initial set of compounds, we screened leopolic acid A and analogues **14**−**17**

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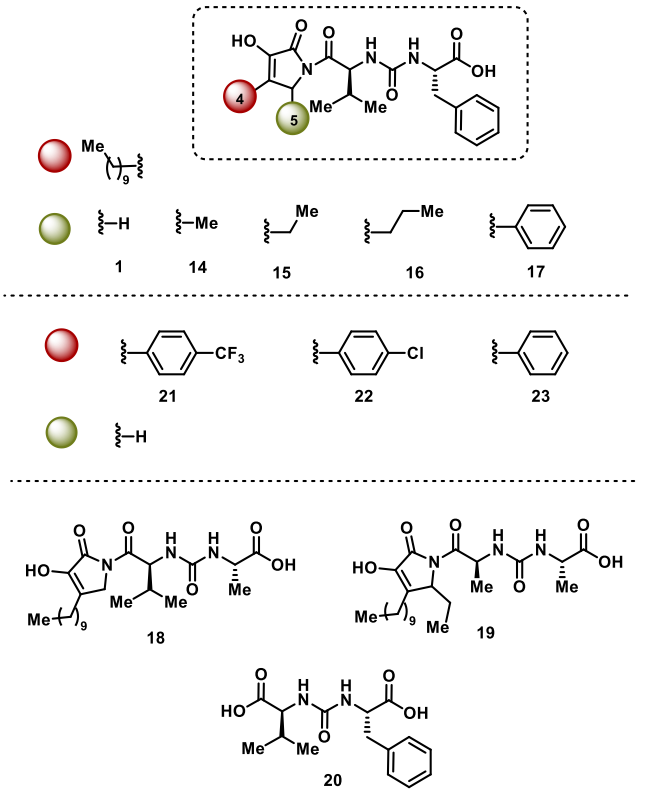

Figure 3. Analogues prepared via the developed route.

for antimicrobial and antibiofilm activity against Gram-positive pathogen *S. aureus* (MSSA 29213, MSSA 25923), methicillin resistant *S. aureus* (MRSA 33591) and we performed biofilm eradication assays using a robust biofilm forming strain of *S. aureus* (MSSA 25923) (Table 1). We observed that leopolic acid A displayed a consistently moderate activity against all three *S. aureus* strains tested (MIC = 32 μg/mL), while it displayed potent antibiofilm activity (MBEC), as it eradicated the preformed bacterial biofilm at concentrations as low as 16 μg/mL). The substitution of the methylene in the 5-position by a methyl (**14**) and ethyl (**15**) group was favorable for slight improvement in antimicrobial activity, while the antibiofilm activity was negatively impacted by this change. Strikingly, the 5-propyl substituted analogue (**16**) displayed the most potent antibiofilm activity (8 μg/mL), while the antimicrobial activity of this analogue was >128 μg/mL for the different *S. aureus* strains tested. The presence of a phenyl in the 5-position (**17**) almost completely turned off the antimicrobial activity (with the exception of the MRSA strain) and negatively impacted the antibiofilm properties of this scaffold.

We next explored how changes in the ureido dipeptide affected leopolic acid A's antimicrobial activity. We elected to change the L-phenylalanine moiety to an L-alanine in the ureido dipeptide, preserving the rest of the natural product core to access compound **18**. That modest structural change resulted in a complete loss of antimicrobial activity; however, it was unexpected that this compound still displayed some promising level of biofilm disruption properties, similar to the natural product. We then decided to substitute the dipeptide with L-alanine in both positions in compound **15**, since **15** displayed the most promising antimicrobial activity in our initial screening. We thereby prepared the ureido dialanine analogue **19**, which displayed no antimicrobial activity and reduced antibiofilm activity, corroborating that the ureido dipeptide fragment is crucial for this compound's activity, and thus we continued with the SAR studies without altering this fragment. We also explored the antimicrobial activity of the isolated dipeptide fragment (**20**), and we found that it was completely inactive against the bacterial strains tested.

Finally, we targeted modification to the 4-position of leopolic acid A by using different phenyl pyruvic esters. We accessed *p*-CF_3-phenyl (**21**), *p*-Cl-phenyl (**22**), and phenyl (**23**) analogues. We have reported previously that phenyl pyruvic esters enhance 2,3-pyrrolidinedione antimicrobial activity.[25] Surprisingly, none of the phenyl pyruvic esters improved or even retained the antimicrobial activity observed for leopolic acid A. These findings demonstrated that both the 4-*n*-decane tail and the ureido-L-valine-L-phenylalanine dipeptide were important for preserving the antimicrobial activity of the natural product. Our limited SAR revealed that **1** tolerates modifications performed at the 5-position, but demonstrated very low tolerance to other modifications in the ureido dipeptide or at the 4-position.

While no compound demonstrated potent activity, it is of note that analogues **16**−**19** displayed low MBEC:MIC ratios against the three strains tested, considering that these ratios tend to be 100−1000× for frontline FDA-approved antimicrobial agents. In all cases, we confirmed that the MBEC values were smaller than the corresponding MIC values. Although the MBEC assays were conducted by the standard protocol, we followed this assay up with quantification of viable bacteria and demonstrated that the compounds were dispersing, and not killing the bacteria at concentrations lower than MIC (see the Supporting Information).[26,27] There were no viable bacteria at concentrations in line with the

Table 1. MIC and MBEC Results[a]

Compound	V	1	14	15	16	17	18	19	20	21	22	23
MIC (MSSA 25923)	1	32	16	16	>128	128	>128	>128	>256	>256	>256	>256
MIC (MSSA 29213)	1	32	32	32	>128	>128	>128	>128	>256	>256	>256	>256
MIC (MRSA 33591)	1	32	16	16	>128	16	>128	>128	>256	>256	>256	>256
MBEC (*S. aureus* 25923)	2048	16	64	64	8	64	32	64	>256	>256	>256	>256
MBEC (*S. aureus* 29213)	2048	16	-	-	8	-	32	-	-	-	-	>128
MBEC (*S. aureus* 33591)	2048	16	-	-	8	-	8	-	-	-	-	>128
(MBEC/MIC) ratio	2048	0.5	4	4	<0.06	<0.5	<0.25	<0.5	NA	NA	NA	NA

[a]V = vancomycin; see the Supporting Information for complete procedures and expanded discussion. MIC = Minimum inhibitory concentration; MBEC = minimum biofilm eradication concentration.

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compound's MIC, highlighting that even if this metric is used, the compounds are still equipotent against planktonic and biofilm embedded bacteria. While we do not yet understand the mechanism of these effects, they suggest that there is potential to further exploit this dual activity in targeting treatments of tolerant, biofilm implicated infections. We are actively investigating the potential mechanism of action of these 2,3-pyrrolidinediones for both their antimicrobial and antibiofilm activities.

In conclusion, we have developed a facile and versatile strategy to synthesize leopolic acid A and its derivatives. We encountered that leopolic acid A displayed a low tolerance to alterations made either to the 4-*n*-decane or the ureido dipeptide substituents. Leopolic acid A's antimicrobial activity was improved by adding short *n*-alkyl substituents at the 5-position, and some of these analogues displayed surprisingly low MBEC/MIC ratios, showing potential as antibiofilm agents. More research is needed to further assess the antimicrobial potential of these 2,3-pyrrolidinedione scaffolds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.3c00068.

Detailed experimental procedures, spectroscopic data and ^{1}H and ^{13}C NMR spectra; purity of lead compounds > 95% as assessed by ^{1}H NMR and LC analyses (PDF)

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Notes

The authors declare the following competing financial interest(s): J.G.P. is founder of Synoxa Sciences, Inc., a biotechnology company developing next-generation antimicrobial agents and antibiofilm agents.

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