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Letter

Investigation of Isobactin Analogues of Teixobactin

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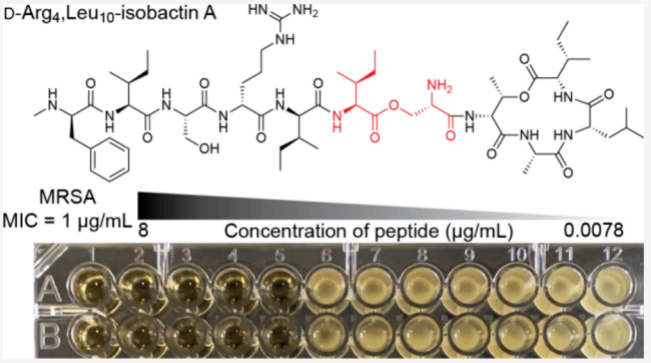
ABSTRACT: Although teixobactin is a promising antibiotic drug candidate against Gram-positive bacteria, it aggregates to form gels that may limit intravenous administration. We previously reported *O*-acyl isopeptide prodrugs of teixobactin analogues that address the problem of gel formation while retaining antibiotic activity. We termed these compounds *isobactins*. In the current Letter, we present nine new isobactin analogues that exhibit a reduced propensity to form gels in aqueous conditions while maintaining potent antibiotic activity against MRSA, VRE, and other Gram-positive bacteria. These isobactin analogues contain commercially available amino acid residues at position 10, replacing the synthetically challenging L-*allo*-enduracididine residue that is present in teixobactin. The isobactins undergo clean conversion to their corresponding teixobactin analogues at physiological pH and exhibit little to no hemolytic activity or cytotoxicity. Because isobactin analogues exhibit enhanced solubility, delayed gel formation, and are more synthetically accessible, it is anticipated that isobactin prodrug analogues may be superior drug candidates to teixobactin.



KEYWORDS: *teixobactin, prodrug, antibiotic, peptide, MRSA, VRE*

Teixobactin is a nonribosomal depsipeptide with potent antibiotic activity against Gram-positive bacteria, including drug-resistant strains such as MRSA and VRE.[1] This promising antibiotic candidate has limited solubility in serum or buffer and aggregates to form gels in the physiological conditions needed for intravenous administration.[2−5] The limited solubility and propensity to form gels has the potential to jeopardize its promise as a clinically useful intravenous antibiotic against drug-resistant Gram-positive pathogens by limiting dosing to low concentrations that do not form gels or aggregates.

Our laboratory recently introduced *O*-acyl isopeptide prodrug analogues of teixobactin, termed isobactins, that are stable and nongelating in acidic solution but convert to the corresponding active teixobactin analogue at neutral pH (Figure 1).[6] We studied analogues with arginine, lysine, and leucine at position 10, because the native *allo*-enduracididine is not commercially available. The isobactin analogues exhibited improved solubility in aqueous conditions and delayed gel formation over the corresponding peptides. These isobactin analogues exhibit comparable if not slightly improved antibiotic activity compared to their corresponding teixobactin analogues. Leu_{10}-isobactin A, for example, exhibits *in vitro* activity against MRSA at 0.5 μg/mL and *in vivo* activity in a neutropenic mouse thigh infection assay at 3−10 mg/kg. It is stable as the trifluoroacetate or hydrochloride salt but converts to Leu_{10}-teixobactin at neutral pH (Figure 2).

Leu_{10}-teixobactin and the corresponding Leu_{10}-isobactins are not as active as teixobactin itself, because the *allo*-enduracididine (*allo*-End) residue at position 10 makes critical contacts with the MurNAc residue in lipid II and related cell-wall precursors.[7,8] Singh and co-workers have reported that teixobactin analogues with cyclohexylglycine (Chg) at position 10 exhibit good antibiotic activity and have suggested that the Chg group can better interact with the MurNAc residue of lipid II.[9−11] The increased hydrophobicity and lack of charge of Chg greatly limits the solubility of Chg_{10}-teixobactin. For this reason, Singh and co-workers have further pursued analogues in which D-Gln_4 is replaced with D-Arg_4 to offset the loss of the charge provided by *allo*-End_{10}. The commercial availability of Chg and D-Arg building blocks render these teixobactin analogues potential alternatives to teixobactin that can easily be prepared by chemical synthesis.

In spite of the charge provided by the D-Arg residue, D-Arg_4,Chg_{10}-teixobactin has only modest solubility and forms gels, both of which may limit its utility as an intravenous antibiotic. In the current paper, we set out to prepare isobactin prodrugs of Chg_{10}-teixobactin, D-Arg_4,Chg_{10}-teixobactin, and

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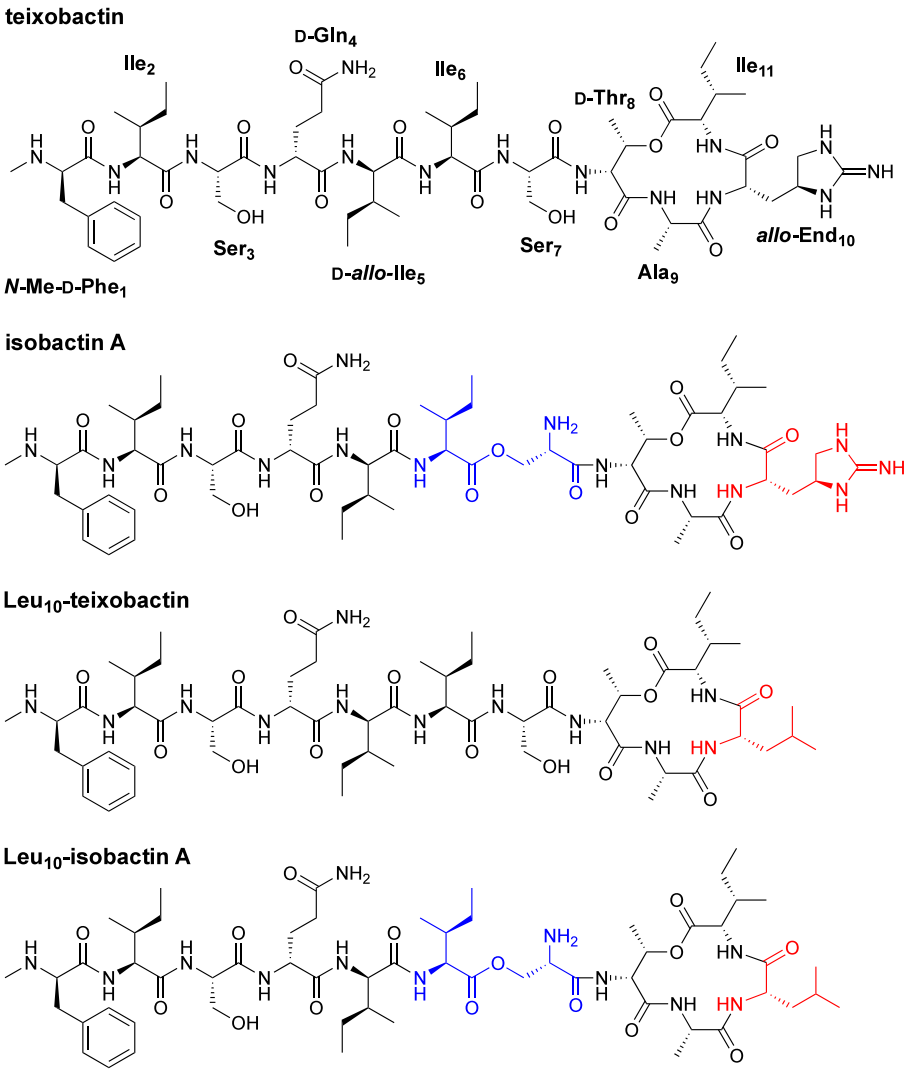

Figure 1. Structures of teixobactin, isobactin A, Leu_{10}-teixobactin, and Leu_{10}-isobactin A. Leu_{10}-teixobactin and Leu_{10}-isobactins A, B, and C were previously reported by Jones et al.[6]

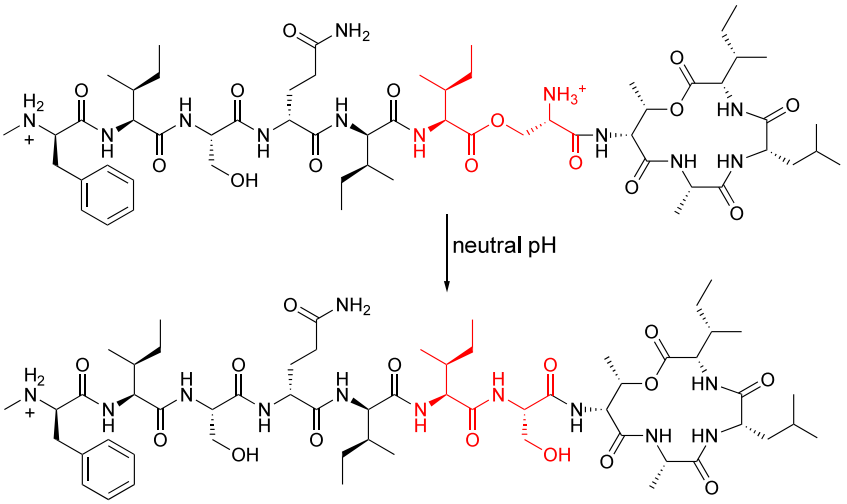

Figure 2. Conversion of Leu_{10}-isobactin A to Leu_{10}-teixobactin at neutral pH.

D-Arg_4,Leu_{10}-teixobactin and compare their solubility and antibiotic activity to Chg_{10}-teixobactin, D-Arg_4,Chg_{10}-teixobactin, and D-Arg_4,Leu_{10}-teixobactin. Our working hypothesis was that the corresponding isobactin prodrugs would have enhanced solubility and diminished propensity to form gels, while exhibiting comparable or improved antibiotic activity.

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We have introduced three different *O*-acyl isopeptide linkages, resulting in isobactins A, B, and C.[6,12−14] These isobactin prodrugs vary in the position at which the *O*-acyl isopeptide linkage is present — between Ile_6 and Ser_7, between Ile_2 and Ser_3, or between both Ile_6 and Ser_7 and Ile_2 and Ser_3. We synthesized the isobactin prodrug analogues as the trifluoroacetate (TFA) salts using our previously reported synthesis that uses Fmoc-based solid-phase peptide synthesis (SPPS) and the commercially available Boc-Ser(Fmoc-Ile)−OH *O*-acyl isodipeptide building block (Figure 3). Yields for all the new isobactin analogues prepared in this study are reported in Table S2.

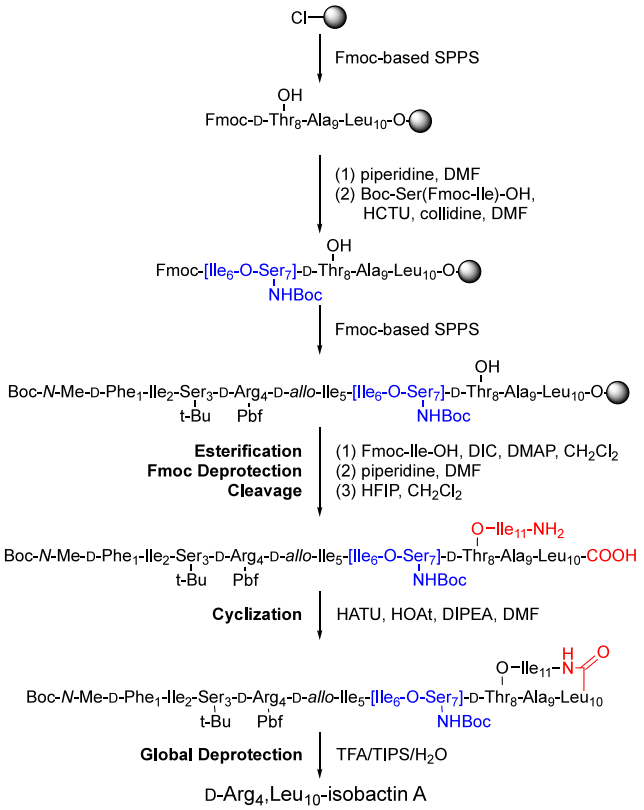

Figure 3. Synthesis of D-Arg_4,Leu_{10}-isobactin A.

Using this approach, we synthesized isobactin prodrugs of Chg_{10}-teixobactin, D-Arg_4,Chg_{10}-teixobactin, and D-Arg_4,Leu_{10}-teixobactin (Figure 4A). The Chg_{10}-isobactins replace the native *allo*-End with the commercially available cyclic hydrophobic residue cyclohexylglycine (Figure 4B). The D-Arg_4,Chg_{10}-isobactins again replace the native *allo*-End with cyclohexylglycine and also replace the native D-Gln_4 with D-Arg to restore the charge lost by the substitution at position 10 (Figure 4B). The D-Arg_4,Leu_{10}-isobactins replace the native *allo*-End with an uncharged residue and also replace the native D-Gln_4 with D-Arg to restore the natural charge of teixobactin (Figure 4B).

We evaluated the antibiotic activity of the isobactin analogues using minimum inhibitory concentration (MIC) assays with five Gram-positive bacteria and compared the MIC values to those of the parent teixobactin analogues. We also used *E.* coli as a Gram-negative control. We compared the activities of the teixobactin analogues and prodrugs to those of teixobactin and vancomycin. We performed these MIC assays in the presence of 0.002% polysorbate 80, as this additive is thought to prevent teixobactin and its derivatives from being adsorbed by 96-well polystyrene plates and has been shown to significantly affect MIC results.[1,15−17]

Each of the teixobactin analogues proved active against the Gram-positive bacteria, and the corresponding isobactin analogues exhibited comparable or slightly better antibiotic activity than the teixobactin analogues (Table 1). The D-Arg_4,Leu_{10}-isobactins showed equal or slightly improved activity to D-Arg_4,Leu_{10}-teixobactin. Thus, the D-Arg_4,Leu_{10}-isobactins exhibited MICs of 0.0078−1 μg/mL, while D-Arg_4,Leu_{10}-teixobactin exhibited MICs of 0.0078−2 μg/mL. D-Arg_4,Leu_{10}-teixobactin, for example, exhibited an MIC of 1 μg/mL against MRSA, while the D-Arg_4,Leu_{10}-isobactins A, B, and C exhibited MICs of 1, 0.5−1, and 1 μg/mL, respectively. The D-Arg_4,Leu_{10}-isobactins also exhibited similar activities to those that we had previously observed for the Leu_{10}-isobactins.[6]

The Chg_{10}-isobactins showed equal or slightly improved activity to Chg_{10}-teixobactin. Thus, the Chg_{10}-isobactins exhibited MICs of 0.0625−1 μg/mL and Chg_{10}-teixobactin exhibited MICs of 0.125−1 μg/mL. Chg_{10}-teixobactin, for example, exhibited an MIC of 1 μg/mL against MRSA, while the Chg_{10}-isobactins A, B, and C exhibited MICs of 0.5, 0.5, and 1 μg/mL, respectively. The D-Arg_4,Chg_{10}-isobactins exhibited similar activities to D-Arg_4,Chg_{10}-teixobactin, with MICs of 0.125−2 μg/mL. The D-Arg_4,Chg_{10}-isobactins were generally slightly less active than the corresponding Chg_{10}-isobactins.

The isobactin analogues convert cleanly to the corresponding teixobactin analogues under physiological conditions, with pH being the trigger that allows deprotonation of the α-amino group of serine and rearrangement of the ester linkage to an amide linkage. Thus, D-Arg_4,Leu_{10}-isobactins A and B convert to D-Arg_4,Leu_{10}-teixobactin with half-lives of 41 and 27 min respectively at pH 7.4 and ambient temperature, and half-lives of 16 and 9 min at 37 °C. D-Arg_4,Leu_{10}-isobactin C converts to D-Arg_4,Leu_{10}-teixobactin via D-Arg_4,Leu_{10}-isobactins A and B, and the overall conversion is 93.5% complete at 60 min at 37 °C. The Chg_{10}-isobactins and D-Arg_4,Chg_{10}-isobactins also undergo clean conversion to the corresponding teixobactin analogues. The rates of conversion are similar to those of the D-Arg_4,Leu_{10}-isobactins, however precise measurement of the rates of conversion was impeded by precipitation of the Chg_{10}-teixobactin and D-Arg_4,Chg_{10}-teixobactin products, which are poorly soluble at 0.5 mg/mL in the phosphate buffer used for the conversion assay.

The isobactin analogues are more soluble and have a diminished propensity to form gels, making them attractive alternatives to the corresponding teixobactin analogues. We evaluated gel formation of isobactin analogues and the corresponding teixobactin analogues by adding DMSO solutions of the peptide TFA salts to phosphate buffered saline (PBS) at pH 7.4 and observing gel formation over time.[6] When D-Arg_4,Leu_{10}-teixobactin is added to PBS, moderate sized gelatinous aggregates form immediately (Figure 5). In contrast, when the D-Arg_4,Leu_{10}-isobactins are added, no immediate gel formation occurs. After 5 min, almost no gelatinous aggregates are observed. After 15 min, the number of aggregates increases but the size of the aggregates does not. By 60 min, gel formation increases but only small gelatinous aggregates are observed. Although we had previously observed delayed gel formation in the Leu_{10}-isobactins, the reduction in gel formation is even more pronounced in the D-Arg_4,Leu_{10}-

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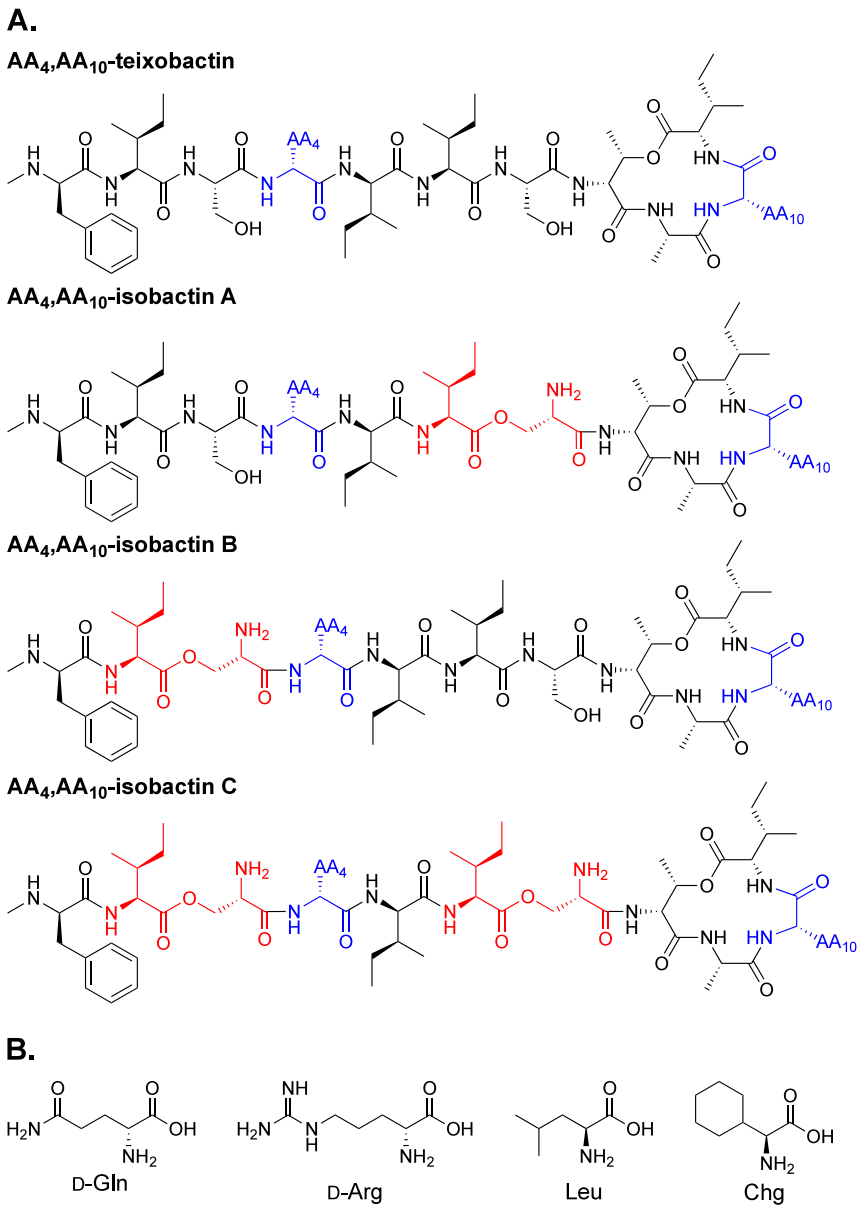

Figure 4. (A) Structures of new teixobactin analogues and isobactins A, B, and C prepared in this study. AA_4 is D-Gln or D-Arg; AA_{10} is Leu or Chg. (B) Structures of D-Gln, D-Arg, Leu, and Chg.

isobactins. Thus, it appears that the additional positive charge associated with the replacement of D-Gln_4 with D-Arg is responsible for further diminishing gel formation.

The Chg_{10}-isobactins exhibit greater gel formation than the Leu_{10}-isobactins but still exhibit substantially less gel formation than Chg_{10}-teixobactin, Leu_{10}-teixobactin, or teixobactin itself. Chg_{10}-teixobactin forms large gelatinous aggregates immediately upon addition to PBS. When the Chg_{10}-isobactins are added to PBS, a few small aggregates form immediately, and the quantity of gelatinous aggregates increases over time, with moderate levels of aggregates being visible at 60 min (Figure S2). Fewer aggregates are observed for Chg_{10}-isobactin C than for Chg_{10}-isobactins A and B at each time point of comparison (0, 5, 15, and 60 min). D-Arg_4,Chg_{10}-teixobactin also forms large gelatinous aggregates immediately upon addition to PBS. The D-Arg_4,Chg_{10}-isobactins exhibit delayed gel formation and less gel formation over 60 min than the corresponding Chg_{10}-isobactins (Figure S3).

Collectively, these observations indicate that the isobactin analogues exhibit substantially less gel formation than teixobactin or the corresponding teixobactin analogues. Furthermore, the observations suggest that isobactin and teixobactin analogues with greater hydrophobicity or less net positive charge exhibit more gel formation than those with less hydrophobicity or greater net positive charge. The D-Arg_4,Leu_{10}-isobactins emerge from these comparisons as having the best balance of activity and delayed gel formation. Although the Chg_{10}-isobactins exhibit somewhat greater activity against MRSA and VRE, we believe the greater gel formation of these compounds make them less attractive to pursue for drug development than the D-Arg_4,Leu_{10}-isobactins.

In synthesizing and purifying these isobactin and teixobactin analogues, we have observed similar differences in solubility. We prepare all of these peptides as the TFA salts and purify them by reverse-phase HPLC in acetonitrile−water mixtures. The D-Arg_4,Leu_{10}-isobactins exhibit good solubilities in 20%

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Table 1. MIC values of isobactin analogues, teixobactin analogues, teixobactin, and vancomycin in μg/mL with 0.002% polysorbate 80

	Bacillus subtilis ATCC 6051	*Staphylococcus epidermidis* ATCC 14990	*Staphylococcus aureus* (MSSA) ATCC 29213	*Staphylococcus aureus* (MRSA) ATCC 700698	*Enterococcus faecalis* (VRE) ATCC 51299	*Escherichia coli* ATCC 10798
Chg_{10}-teixobactin	0.125	0.5	1	1	1	>8
Chg_{10}-isobactin A	0.0625	0.25	0.5	0.5	0.5	>8
Chg_{10}-isobactin B	0.0625	0.25	0.25	0.5	0.5	>8
Chg_{10}-isobactin C	0.125	0.5	0.5	1	1	>8
D-Arg_4,Chg_{10}-teixobactin	0.125	0.125	2	0.5	1	>8
D-Arg_4,Chg_{10}-isobactin A	0.125	0.25	1	1	1	>8
D-Arg_4,Chg_{10}-isobactin B	0.125	0.25	2	1	1	>8
D-Arg_4,Chg_{10}-isobactin C	0.125	0.125	1	1	1	>8
D-Arg_4,Leu_{10}-teixobactin	≤0.0078	0.0625	0.25−0.5	1	2	>8
D-Arg_4,Leu_{10}-isobactin A	≤0.0078	0.0156−0.03125	0.125−0.25	1	1	>8
D-Arg_4,Leu_{10}-isobactin B	0.0078−0.0156	0.03125−0.0625	0.125	0.5−1	1	>8
D-Arg_4,Leu_{10}-isobactin C	0.0156−0.03125	0.0625	0.25	1	1−2	>8
Teixobactin	0.0078	0.0078	0.5	0.25	0.25	>8
Vancomycin	0.5	2	1	2	>8	>8

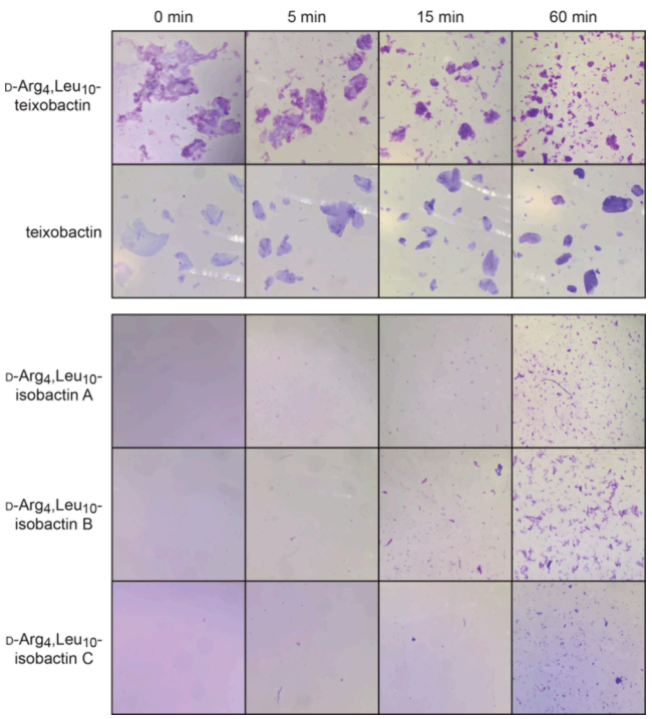

Figure 5. Gel formation of D-Arg_4,Leu_{10}-teixobactin and teixobactin and delayed gel formation of D-Arg_4,Leu_{10}-isobactin A, B, and C.

acetonitrile in water, which is the solvent we typically use for injecting samples onto the preparative HPLC column. For D-Arg_4,Leu_{10}-teixobactin, a higher concentration of acetonitrile is required, typically 35%. The D-Arg_4,Chg_{10}-isobactins and Chg_{10}-isobactins typically require 30% acetonitrile. Even higher concentrations of acetonitrile are required to dissolve D-Arg_4,Chg_{10}-teixobactin and Chg_{10}-teixobactin for HPLC injection, typically 40%.

We evaluated the cytotoxicity of the isobactin analogues and their corresponding teixobactin analogues in HeLa cells using a Promega Cytotox-Glo assay (Figures S4−S9). In these experiments, all the isobactin analogues tested exhibited no cytotoxicity at concentrations up to 25 μM (36−43 μg/mL) and slight cytotoxicity at 50 μM (73−86 μg/mL), with the D-Arg_4,Chg_{10}-isobactins exhibiting the most cytotoxicity at 50 μM. The parent teixobactin analogues generally exhibited no significant cytotoxicity at concentrations as high as 50 μM. Thus, it appears that the extra ammonium group associated with the isobactins imparts slightly enhanced cytotoxicity to the isobactins, perhaps by promoting interaction with cell membranes.

We further evaluated the isobactin analogues and the corresponding teixobactin analogues in hemolytic assays with human red blood cells in the absence and presence of 0.002% polysorbate 80 (Figures S10−S15). We used Triton X-100 and water (vehicle) as positive (100% lysis) and negative (0% lysis) controls in the hemolysis assays.[3,6,18,19] We used 1.25 μM (3.6 μg/mL) melittin as an additional positive control. In the absence of polysorbate 80, all the isobactin analogues exhibited no hemolytic activity at concentrations as high as 25 μg/mL and mild hemolytic activity at higher concentrations, with values between 2−5% at 100 μg/mL. In the presence of polysorbate 80, all the isobactin analogues exhibited even lower hemolytic activity with hemolysis between 0−2% at 100 μg/mL. We observed 20−50% hemolysis with 1.25 μM melittin in the absence and presence of polysorbate 80. These studies suggest that the isobactin analogues are suitable for intravenous administration at concentrations well above the MIC values.

In conclusion, the isobactin prodrug analogues have good antibiotic activity against Gram-positive pathogens while overcoming the problems of poor solubility and gel formation of teixobactin and teixobactin analogues that are likely to limit their preclinical development. The isoacyl peptide linkage of

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these prodrugs enhances solubility and delays gel formation, allowing the prodrugs to disperse before undergoing rearrangement to the active peptide drugs. Although the isobactin prodrugs described herein do not contain the natural *allo*-End residue at position 10, they still exhibit excellent antibiotic activity against the pathogens MSSA, MRSA, and VRE and are generally more active than vancomycin. Incorporation of the hydrophobic residues Leu or Chg at position 10 in place of the synthetically challenging *allo*-End allows for potent analogues for preclinical development that are readily accessible through chemical synthesis. Replacement of D-Gln with D-Arg at position 4 further enhances solubility and compensates for the increased hydrophobicity associated with the loss of the charged residue *allo*-End at position 10.

The isobactin prodrugs are stable at acidic pH but undergo clean conversion to the active drugs at physiological pH. They exhibit little to no hemolysis or cytotoxicity. The D-Arg_4,Leu_{10}-isobactins exhibit the best balance of delayed gel formation and antibiotic activity, making them superior candidates for intravenous administration. The Chg_{10}-isobactins and D-Arg_4,Chg_{10}-isobactins also exhibit good antibiotic activity, but have limited solubility when compared to the D-Arg_4,Leu_{10}-isobactins. The D-Arg_4,Leu_{10}-isobactins are more soluble and less prone to gel formation than the Leu_{10}-isobactins and may thus be superior candidates for preclinical development.

EXPERIMENTAL SECTION

Materials and Methods. Materials and methods for the synthesis, purification, and analysis of the isobactin analogues and teixobactin analogues are the same as previously described.[6]

Synthesis of the Isobactin Analogues and Their Corresponding Teixobactin Analogues. All isobactin analogues and teixobactin analogues were prepared as the trifluoroacetate salts by solid-phase peptide synthesis followed by solution phase cyclization, as previously described.[6] For all isobactin prodrug analogues, Boc-Ser(Fmoc-Ile)−OH was coupled in place of the desired Ile and Ser residues. Syntheses on a 0.1−0.2 mmol scale afforded 8−101 mg (6−52%) of the isobactins and corresponding teixobactin analogues (Table S2 in the Supporting Information.) The A-series prodrugs typically afforded the highest yields (33−52%). All isobactins and teixobactin analogues prepared were >95% pure by HPLC. Characterization data are reported in the Supporting Information.

Conversion Kinetics Studies of the Isobactin Analogues at Room Temperature and 37 °C. Conversion kinetics studies at room temperature and 37 °C were performed as previously described.[6] Data from these studies are summarized in Table S1 and Figure S1 in the Supporting Information.

MIC Assays of the Isobactin Analogues and Their Corresponding Teixobactin Analogues (Ref 6). All bacterial cultures and MIC assays were performed in media containing 0.002% polysorbate 80. *Bacillus subtilis* (ATCC 6051), *Staphylococcus epidermidis* (ATCC 14990), *Staphylococcus aureus* (ATCC 29213), and *Escherichia coli* (ATCC 10798) were cultured from glycerol stocks in Mueller-Hinton broth overnight in a shaking incubator at 37 °C. *Staphylococcus aureus* (ATCC 700698) was cultured from a glycerol stock in brain heart infusion broth overnight in a shaking incubator at 37 °C. *Enterococcus faecalis* (ATCC 51299) was cultured from a glycerol stock in brain heart infusion broth containing 4 μg/mL of vancomycin overnight in a shaking incubator at 37 °C.

An aliquot of a 1 mg/mL antibiotic stock solution in DMSO was diluted with appropriate culture medium to make a 16 μg/mL solution. A 200-μL aliquot of the 16 μg/mL solution was transferred to a sterile, polystyrene 96-well plate. 2-fold serial dilutions were made with media across a 96-well plate to achieve a final volume of 100 μL in each well. These solutions had the following concentrations: 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, and 0.0156 μg/mL.

An OD_{600} value was obtained for the overnight cultures of each bacterium as measured for 200 μL in a 96-well plate. The background absorbance of each culture medium was then subtracted from the OD_{600} obtained for each bacterium using its corresponding culture medium. An appropriate volume of each overnight cultures was then diluted in the correct medium to create a 1 mL stock solution with an OD_{600} of 0.075. Each diluted stock was then further diluted (to approximately 1×10^6 CFU/mL) by adding 130 μL to 15.4 mL of the correct culture medium. A 100-μL aliquot of the diluted bacterial solution was added to each well in the 96-well plates, resulting in final bacteria concentrations of approximately 5×10^5 CFU/mL in each well. With the addition of 100-μL of bacteria in broth to each well, the teixobactin analogues and isobactin analogues were thus diluted to the following concentrations: 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156, and 0.0078 μg/mL.

The plate was covered with a lid and incubated at 37 °C for 16 h. The OD_{600} were then measured using a 96-well UV/vis plate reader. The MIC values were taken as the lowest concentration that had no bacterial growth. Each MIC assay was run in quadruplicate (technical replicates). Several of the MIC assays were repeated to ensure reproducibility.

Gel Formation Studies of the Isobactin Analogues and Their Corresponding Teixobactin Analogues. Gel formation studies were performed as previously described.[6] Data from these studies are summarized in Figure 5, and in Figures S2 and S3 in the Supporting Information.

Hemolytic Assay of the Isobactin Analogues and Their Corresponding Teixobactin Analogues. Hemolytic assays of the isobactin analogues and their corresponding teixobactin analogues were performed as previously described.[6] Data from these studies are summarized in Figures S10−S15 of the Supporting Information.

Cell Culture and Cytotoxicity Assays of the Isobactin Analogues and Their Corresponding Teixobactin Analogues. Cell culture and cytotoxicity assays of the isobactin analogues and their corresponding teixobactin analogues were performed as previously described.[6] Data from these studies are summarized in Figures S4−S9 of the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.4c00215.

Supplementary figures, RP-HPLC analytical traces, and mass spectra (PDF)

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Author Contributions

C. R. J., G. H. L, and M. S. T. L. P synthesized the peptides and C. R. J. purified and characterized the peptides. C. R. J. and G. H. L. performed conversion studies. C. R. J., G. H. L, and M. S. T. L. P. performed MIC studies. C. R. J. performed gelation, hemolytic, and cytotoxicity experiments. J. S. N. and C. R. J. wrote and edited the manuscript.

Notes

The authors declare the following competing financial interest(s): J. S. N. and C. R. J. have filed a patent application on prodrugs of teixobactin and teixobactin analogues through the Regents of the University of California. The authors have collaborated with and received subcontracts from NovoBiotic Pharmaceuticals, LLC, albeit not for this project.

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