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Authors
Brunicardi, Jackson
Griffin, James
Ferracane, Michael
et al.

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Structure–Activity Relationship Studies of the Peptide Antibiotic Clovibactin

Jackson E. H. Brunicardi,[‖] James H. Griffin,[‖] Michael J. Ferracane, Adam G. Kreutzer, Jeramiah Small, Ana-Teresa Mendoza, Joseph W. Ziller, and James S. Nowick*

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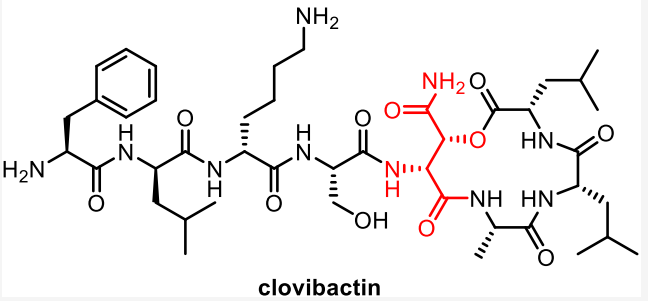
ABSTRACT: Our laboratory reported the chemical synthesis and stereochemical assignment of the recently discovered peptide antibiotic clovibactin. The current paper reports an improved, gram-scale synthesis of the amino acid building block Fmoc-(2*R*,3*R*)-3-hydroxyasparagine-OH that enables structure–activity relationship studies of clovibactin. An alanine scan reveals that residues Phe_1, D-Leu_2, Ser_4, Leu_7, and Leu_8 are important for antibiotic activity. The side-chain amide group of the rare D-Hyn_5 residue is not essential to activity and can be replaced with a methyl group with a moderate loss of activity. An acyclic clovibactin analogue reveals that the macrolactone ring is essential to antibiotic activity. The enantiomer of clovibactin is active, albeit somewhat less so than clovibactin. A conformationally constrained clovibactin analogue retains moderate antibiotic activity, while a backbone *N*-methylated analogue is almost completely inactive. X-ray crystallography of these two analogues reveals that the macrolactone ring adopts a crown-like conformation that binds anions.

Improved synthesis, SAR studies, X-ray crystallography



clovibactin

INTRODUCTION

Clovibactin is a newly reported antibiotic that is active against Gram-positive bacteria, including drug-resistant human pathogens such as MRSA and VRE, without detectable resistance.[1] It is an eight-residue depsipeptide comprising a linear tail (residues 1–4) and a macrolactone ring (residues 5–8). Clovibactin kills bacteria by inhibiting cell-wall biosynthesis, specifically targeting the pyrophosphate moiety of multiple peptidoglycan precursors. Shukla and co-workers have recently reported solid-state NMR studies of clovibactin binding lipid II pyrophosphate. On the basis of these studies, they propose a preliminary model of target binding in which clovibactin forms antiparallel β-sheet assemblies upon binding to the pyrophosphate group. This model resembles the mechanism of action of the related antibiotic teixobactin, which we have also studied.[2−8]

We previously reported the chemical synthesis of clovibactin and a stereochemical assignment of the rare noncanonical amino acid hydroxyasparagine at position 5.[9] In this paper, we improve upon our previous synthesis, enabling an alanine scan and other structure–activity relationship studies. These studies allow us to identify critical amino acid residues and thus to better understand how clovibactin interacts with Gram-positive bacteria. We obtained X-ray crystallographic structures of two clovibactin analogues designed to probe the relationship between the conformation and supramolecular assembly of clovibactin and its antibiotic activity, and we show that both analogues bind anions in the crystallographic structures.

RESULTS AND DISCUSSION

Improved Route to Fmoc-(2*R*,3*R*)-3-Hydroxyasparagine-OH. To facilitate the preparation of clovibactin derivatives, we shortened our synthesis of Fmoc-(2*R*,3*R*)-3-hydroxyasparagine-OH from 13 to 7 steps, allowing its preparation on a gram scale (Figure 1).[9] Application of published syntheses of (2*R*,3*R*)-3-hydroxyaspartic acid shortened our synthesis by two steps.[10,11] Thus, our improved synthesis now begins with D-aspartic acid rather than (+)-diethyl L-tartrate. D-Aspartic acid is converted to benzamide **1** by Fischer esterification with CH_3OH to give the dimethyl ester, followed by benzoylation of the α-amino group with BzCl using modified Schotten–Baumann conditions.[12,13] Benzamide **1** is converted to oxazoline **2** by treatment with 2.1 equiv. LHMDS followed by addition of I_2. The reaction proceeds via an enolate dianion, which reacts stereoselectively with I_2 and then cyclizes by an intramolecular

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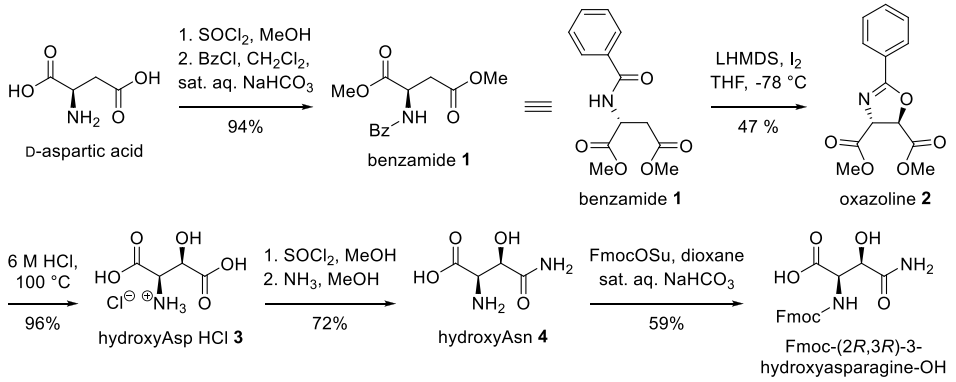

Figure 1. Improved synthesis of Fmoc-(2*R*,3*R*)-3-hydroxyasparagine-OH.

S_N2 reaction (Figure S1).[10] Subsequent hydrolysis with HCl cleaves the methyl ester and oxazoline groups to give (2*R*,3*R*)-3-hydroxyaspartic acid as the hydrochloride salt (**3**).

The synthesis was shortened by an additional four steps through the elimination of Boc and benzyl ester protection and deprotection steps.[9,14] Hydroxyaspartic acid hydrochloride (**3**) was converted to hydroxyasparagine (**4**) by a regioselective Fischer esterification of the γ-carboxylic acid, followed by ammonolysis of the methyl ester group to give a carboxamide group. Protection of the α-amino group with FmocOSu gives Fmoc-(2*R*,3*R*)-3-hydroxyasparagine-OH in a 18% overall yield over seven steps.

Using this same route, we also prepared the enantiomer Fmoc-(2*S*,3*S*)-3-hydroxyasparagine-OH from L-aspartic acid. This building block enabled the synthesis of the enantiomer of clovibactin, *ent*-clovibactin.

Structure−Activity Relationship Studies. We performed structure−activity relationship (SAR) studies to identify the features of clovibactin that contribute to its strong antibiotic activity. We first performed an alanine scan to identify individual amino acid residues in clovibactin that contribute to its antibiotic activity. Each residue was sequentially replaced with alanine while maintaining the native stereochemistry: L-alanine for positions 1, 4, 7, and 8 and D-alanine for positions 2 and 3. Position 6 was not modified, as it is already alanine. Additionally, we prepared D-Thr_5-clovibactin, in which the primary amide group of D-Hyn_5 is replaced with a methyl group (Figure 2).[15] This substitution is analogous to an alanine mutation in determining the significance of the side-chain amide group of hydroxyasparagine. To probe the importance of the absolute configuration of clovibactin, we also prepared the enantiomer of clovibactin, *ent*-clovibactin. To probe the importance of the macrolactone ring, we prepared an acyclic analogue, *seco*-clovibactin.

We synthesized these analogues as the trifluoroacetate (TFA) salts by solid-phase peptide synthesis and solution-phase cyclization as described previously, and we assessed their antibiotic activities against the Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus epidermidis*, methicillin-susceptible *Staphylococcus aureus* (MSSA), and methicillin-resistant *Staphylococcus aureus* (MRSA).[9] We used natural clovibactin and vancomycin in these experiments as positive controls and the Gram-negative bacterium *Escherichia coli* as a negative control. Table 1 summarizes the minimum inhibitory concentrations (MICs) of the different analogues.

Clovibactin is highly active against these Gram-positive bacteria, exhibiting MIC values of 0.0625−0.5 μg/mL. Alanine

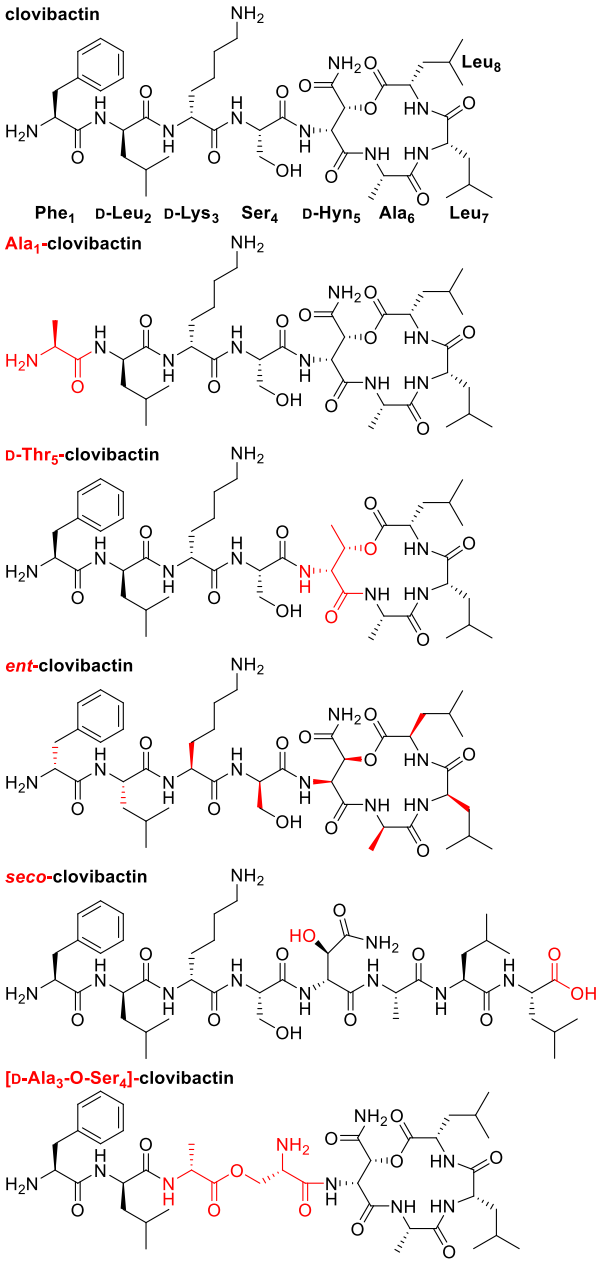

Figure 2. Clovibactin and representative analogues used for SAR studies.

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Table 1. MIC Values of Clovibactin and SAR Analogues of Clovibactin in μg/mL

	Bacillus subtilis ATCC 6051	*Staphylococcus epidermidis* ATCC 14990	*Staphylococcus aureus* (MSSA) ATCC 29213	*Staphylococcus aureus* (MRSA) ATCC 700698	*Escherichia coli* ATCC 10798
natural clovibactin	0.0625−0.125	0.5	0.5	0.25	16
synthetic clovibactin	0.125	0.25−0.5	0.5	0.25−0.5	16
Ala_1-clovibactin	2−4	8−16	16	16	>32
D-Ala_2-clovibactin	4−8	16	16−32	32	>32
Ala_4-clovibactin	2	4−8	8	8	32
D-Thr_5-clovibactin	1	2	8	8	>32
Ala_7-clovibactin	0.5−1	4	4	4	>32
Ala_8-clovibactin	16	32	>32	>32	>32
ent-clovibactin	0.5−1	1	1	1−2	16
seco-clovibactin	>32	>32	>32	>32	>32
[D-Ala_3-O-Ser_4]-clovibactin	>32	>32	>32	>32	>32
D-Thr_5-clovibactin diene	0.5	2	4	4	32
stapled D-Thr_5-clovibactin	4	8	16	16	>32
N-Me-D-Leu_2,D-Thr_5-clovibactin	32	>32	>32	>32	>32
vancomycin	0.125−0.25	2	1	4	>32

mutation of clovibactin residues 1, 2, 4, 7, or 8 reduces antibiotic activity in all cases, but none of the resulting analogues are completely inactive (Figure 3). Leu_8, D-Leu_2, and

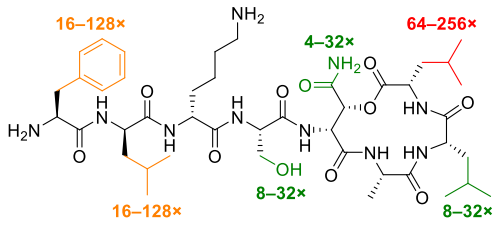

Figure 3. Effect of alanine (residues 1, 2, 4, 7, and 8) or threonine (residue 5) substitution of clovibactin residues on MIC values. Ranges represent fold changes in activity compared to natural clovibactin.

Phe_1 are most sensitive to alanine replacement. Furthermore, the primary amide group of the unusual D-Hyn_5 residue is not necessary for antibiotic activity.[14] The macrolactone ring of clovibactin is critical for antibiotic activity: The acyclic analogue *seco*-clovibactin is completely inactive against all bacteria tested (MIC > 32 μg/mL).

The enantiomer of clovibactin exhibits MICs of 0.5−2 μg/mL against the Gram-positive bacteria studied. Although *ent*-clovibactin is 2−8-fold less active than clovibactin, these MIC values are comparable to vancomycin. The activity of *ent*-clovibactin indicates that it is still capable of recognizing the achiral pyrophosphate group of lipid II and related cell-wall precursors; however, interactions with chiral components (such as *N*-acetylmuramic acid, MurNAc) must also be important for recognition of the target.[1]

We observed modest activity (MIC 16−32 μg/mL) against the Gram-negative bacterium *E. coli* for clovibactin, Ala_4-clovibactin, and *ent*-clovibactin. We have previously observed both natural and synthetic clovibactin to have an MIC of 8 μg/mL against *E. coli* ATCC 10798.[9] Shukla and co-workers reported natural clovibactin to have an MIC of 64 μg/mL against this same strain of *E. coli*.[1] These differing results may reflect subtle differences in the procedures used for the MIC assays.

Shukla and co-workers recently suggested a model for the mechanism of action of clovibactin.[1] In this model, the four tail residues form an amphiphilic β-strand, in which the polar residues (D-Lys_3 and Ser_4) and the nonpolar residues (Phe_1 and D-Leu_2) are displayed on opposite faces. D-Lys_3 and Ser_4 then make hydrogen bond contacts with the pyrophosphate group of lipid II, while Leu_2 anchors clovibactin in the bacterial cell membrane. The amide NH groups of the macrolactone ring of clovibactin form hydrogen bonds to lipid II pyrophosphate in a fashion similar to teixobactin.[16−19] The hydrophobic side chains Ala_6, Leu_7, and Leu_8 on the macrolactone ring are envisioned to form a "hydrophobic glove" that wraps around the pyrophosphate group and facilitates its desolvation while making hydrophobic contacts with the MurNAc group of lipid II and related cell-wall precursors.

The structure−activity relationships we have observed against Gram-positive bacteria are largely consistent with the model proposed by Shukla and co-workers. The reduction in activity upon alanine mutation of Leu_8 and Leu_7 is consistent with the reduced ability to form a "hydrophobic glove" and desolvate lipid II pyrophosphate and participate in hydrophobic interactions with the MurNAc group. The decrease in activity upon alanine mutation of Ser_4 is consistent with the importance of proposed polar interactions of the serine OH group with the pyrophosphate group. The reduction in activity upon the D-alanine mutation of D-Leu_2 is consistent with the proposed role of D-Leu_2 in membrane anchoring. The reduction in activity upon alanine mutation of Phe_1 suggests that Phe_1 may also be involved in membrane anchoring or important hydrophobic interactions.

Despite repeated attempts, we were unable to synthesize or purify D-Ala_3-clovibactin. We hypothesized that removal of a positive charge renders the peptide insoluble in the aqueous conditions necessary for purification and subsequent MIC assays. Following a strategy previously employed by our laboratory to prepare tractable prodrugs of aggregation-prone teixobactin peptides, we prepared [D-Ala_3-O-Ser_4]-clovibactin, in which the D-Ala_3-Ser_4 peptide linkage is replaced with an *O*-acyl linkage (Figure 2).[6,7] Peptides containing *O*-acyl linkages

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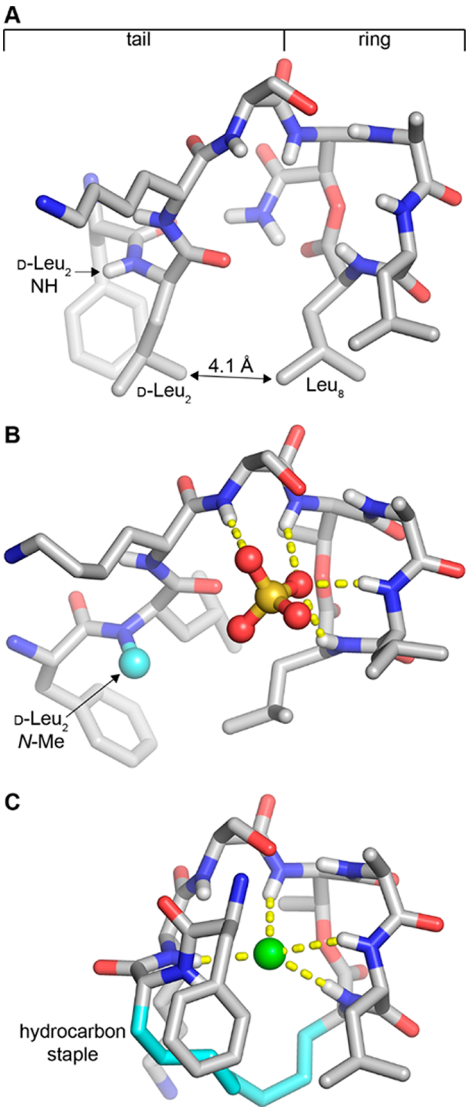

Figure 4. Crystallographically based molecular model of clovibactin and X-ray crystallographic structures of *N*-methylated and stapled clovibactin analogues. (A) Molecular model of clovibactin based on the X-ray crystallographic structure of an analogue (PDB 8CUG).[8] (B) X-ray crystallographic structure of *N*-Me-D-Leu_2,D-Thr_5-clovibactin bound to a sulfate anion. Eight peptide molecules comprise the asymmetric unit; a representative molecule is shown. The *N*-Me carbon of *N*-Me-D-Leu_2 is highlighted in cyan. Hydrogen bonds are shown as yellow dashed lines. (C) X-ray crystallographic structure of stapled D-Thr_5-clovibactin bound to a chloride anion. Three peptide molecules comprise the asymmetric unit; a representative molecule is shown. The hydrocarbon staple is highlighted in cyan.

to serine are stable at acidic pH but convert quantitatively to the corresponding amide-linked peptides at neutral pH.[20,21] Thus, we observed that [D-Ala_3-O-Ser_4]-clovibactin rapidly converts to D-Ala_3-clovibactin in phosphate buffer at pH 7.4 (Figure S2). [D-Ala_3-O-Ser_4]-Clovibactin proved to be completely inactive against all bacteria in the concentration range tested (Table 1), which suggests that D-Lys_3 may be important to the antibiotic activity of clovibactin. [D-Ala_3-O-Ser_4]-Clovibactin exhibited limited solubility upon conversion to D-Ala_3-clovibactin, which suggests that its inactivity in MIC assays may result from its poor solubility, rather than inherent importance of the charged D-Lys_3 group.

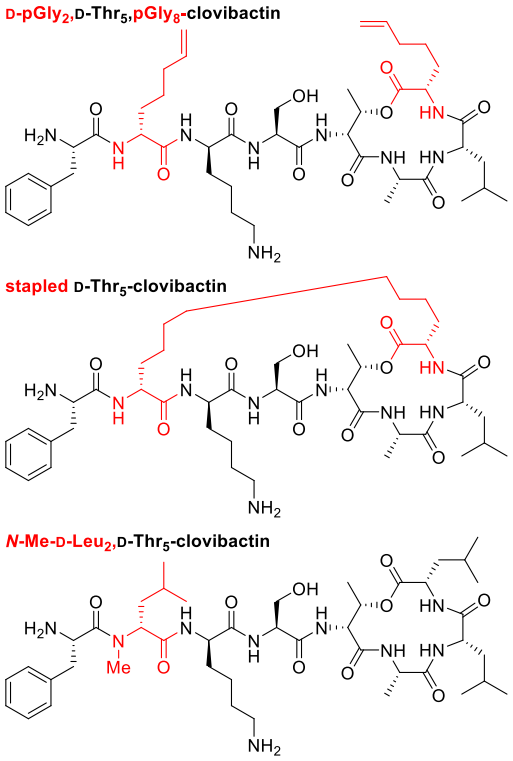

Figure 5. D-Thr_5-Clovibactin analogues.

X-ray Crystallography. We previously reported a model for the conformation and supramolecular assembly of clovibactin based on the X-ray crystallographic structure of a clovibactin epimer.[9] In this model, clovibactin adopts an amphiphilic conformation with the hydrophobic side chains of Phe_1, D-Leu_2, Ala_6, Leu_7, and Leu_8 on one face of the molecule and the hydrophilic side chains of D-Lys_3, Ser_4, and D-Hyn_5 on the opposite face (Figure 4A). The molecules assemble in a linear head-to-tail fashion, with the amide NH groups of D-Leu_2 and D-Lys_3 in one molecule hydrogen bonding to the carbonyl groups of Ala_6 and Leu_7 in an adjacent molecule. We have now prepared an analogue of clovibactin designed to probe the relationship between its conformation and antibiotic activity (stapled D-Thr_5-clovibactin) and a second analogue designed to probe the relationship between its supramolecular assembly and antibiotic activity (*N*-Me-D-Leu_2,D-Thr_5-clovibactin) (Figure 5). We incorporated commercially available D-threonine at position 5 instead of (2*R*,3*R*)-3-hydroxyasparagine in these analogues to expedite their syntheses. We obtained X-ray crystallographic structures of both analogues and determined their MIC values.

In our molecular model of clovibactin, the side chains of D-Leu_2 and Leu_8 are proximal to one another (Figure 4A). To help enforce this conformation, we prepared a stapled analogue in which positions 2 and 8 are linked by an eight-carbon chain (stapled D-Thr_5-clovibactin, Figure 5). We crystallized stapled D-Thr_5-clovibactin in conditions containing $MgCl_2$ and determined its X-ray crystallographic structure (Figure 4C). Three peptide molecules comprise the asymmetric unit, and each molecule forms a cage that binds a chloride anion. The macrolactone ring adopts a crown-like conformation in which the amide NH groups of D-Lys_3, D-Hyn_5, Leu_7, and stapled residue 8 form hydrogen bonds with the chelated chloride anion. The amide NH group of Ala_6 hydrogen bonds to the

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side-chain oxygen atom of Ser_4. We have observed similar ring conformations and anion binding in the X-ray crystallographic structures of four different analogues of teixobactin.[2,4,5,7]

Stapled D-Thr_5-clovibactin is 2−4-fold less potent than D-Thr_5-clovibactin in MIC assays against the Gram-positive bacteria tested (Table 1). The retention of antibiotic activity despite a dramatic change in the overall flexibility of the molecule indicates that a conformation in which residues 2 and 8 are proximal is active. The diene precursor to stapled D-Thr_5-clovibactin (D-$pGly_2$,D-Thr_5,$pGly_8$-clovibactin, Figure 5) is 2-fold more potent than D-Thr_5-clovibactin. These results suggest that enforcing proximity between positions 2 and 8, while tolerated, does not enhance antibiotic activity.

In our model of clovibactin assembly, the molecules align such that the amide NH group of D-Leu_2 forms an intermolecular hydrogen bond with the carbonyl group of Leu_7 in an adjacent molecule (Figure 4A).[9] To probe whether the D-Leu_2 amide NH group is important for antibiotic activity, we prepared a clovibactin analogue in which the amide NH group of D-Leu_2 is *N*-methylated. We crystallized *N*-Me-D-Leu_2,D-Thr_5-clovibactin in conditions containing $CdSO_4$ and determined its X-ray crystallographic structure (Figure 4B). Eight peptide molecules comprise the asymmetric unit (Figure S3A, B). The molecules pack through hydrophobic interactions among their side chains and coordinate to Cd^{2+} ions through their *N*-terminal amino groups.

In each of the molecules, the macrolactone ring adopts a crown-like conformation, and the NH group of Ala_6 hydrogen bonds to the side chain oxygen atom of Ser_4 (Figure S3C). Three of the molecules bind sulfate anions, with the backbone NH groups of Ser_4, D-Thr_5, Leu_7, and Leu_8 hydrogen bonding to the oxygen atoms of the sulfate anion. These interactions are similar to how teixobactin binds to the phosphate group of lipid II and related cell-wall precursors.[19] All eight molecules share identical conformations of residues 3−8 in the peptide backbone. This backbone conformation is similar to that observed in our previous model of clovibactin assembly, differing only at Phe_1 and D-Leu_2, presumably due to the *N*-methyl group (Figure S4).

In an MIC assay, *N*-Me-D-Leu_2,D-Thr_5-clovibactin is weakly active against *B. subtilis* (MIC = 32 ug/mL) and inactive against all other bacterial strains tested (Table 1). This inactivity is consistent with a model in which supramolecular assembly of clovibactin through intermolecular hydrogen bonding of the D-Leu_2 amide NH group is important for its antibiotic activity.[22] These findings are consistent with both a model in which clovibactin assembles in a linear head-to-tail fashion[9] and a model in which clovibactin forms antiparallel dimers.[1]

CONCLUSION

A gram-scale synthesis of Fmoc-protected (2*R*,3*R*)-3-hydroxyasparagine enables structure−activity relationship studies of the peptide antibiotic clovibactin. These studies provide insights into the roles of various residues in its activity. Replacement of Phe_1, D-Leu_2, Ser_4, D-Hyn_5, Leu_7, or Leu_8 with Ala, D-Ala, or D-Thr indicates that the side chains of these residues are important, but not essential, to the antibiotic activity of clovibactin. The nonpolar residue Leu_8, and to a lesser extent Phe_1 and D-Leu_2, is most important for antibiotic activity. The enantiomer of clovibactin is less active than clovibactin but still exhibits good antibiotic activity against Gram-positive bacteria, while a linear clovibactin analogue with no macrolactone ring is completely inactive. X-ray crystallography of clovibactin analogues shows that the backbone amide NH groups of residues D-Hyn_5, Leu_7, and Leu_8 chelate anions and suggests that clovibactin binds to the pyrophosphate group of lipid II and related cell-wall precursors through similar hydrogen-bonding interactions. The improved synthetic route to Fmoc-(2*R*,3*R*)-3-hydroxyasparagine-OH and results of the alanine scan and other SAR studies should facilitate the development of additional analogues of clovibactin with improved pharmacological properties.

ASSOCIATED CONTENT

Data Availability Statement

The data supporting this article are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.4c01414.

X-ray crystallographic procedure and properties, amino acid and peptide syntheses, characterization data, and MIC assay procedure (PDF)

Accession Codes

Crystallographic coordinates of stapled D-Thr_5-clovibactin were deposited into the Cambridge Crystallographic Data Centre (CCDC) with code 2358085.

Crystallographic coordinates of *N*-Me-D-Leu_2,D-Thr_5-clovibactin were deposited into the Protein Data Bank (PDB) with code 9B1C.

AUTHOR INFORMATION

Corresponding Author

James S. Nowick − *Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States; Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, California 92697, United States;* orcid.org/0000-0002-2273-1029; Email: jsnowick@uci.edu

Authors

Jackson E. H. Brunicardi − *Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States;* orcid.org/0000-0002-3661-9476

James H. Griffin − *Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States;* orcid.org/0009-0004-9448-1526

Michael J. Ferracane − *Department of Chemistry, University of Redlands, Redlands, California 92373, United States*

Adam G. Kreutzer − *Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States;* orcid.org/0000-0002-9724-6298

Jeramiah Small − *Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States*

Ana-Teresa Mendoza − *Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States;* orcid.org/0009-0000-3799-1897

Joseph W. Ziller − *Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States;* orcid.org/0000-0001-7404-950X

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.joc.4c01414

Author Contributions

[‖]J.E.H.B. and J.H.G. contributed equally to this work. J.E.H.B. synthesized Fmoc-(2*R*,3*R*)-3-hydroxyasparagine-OH. J.E.H.B. and J.S. synthesized Fmoc-(2*S*,3*S*)-3-hydroxyasparagine-OH. J.E.H.B., J.H.G., M.J.F., J.S., and A.M. synthesized the peptides. J.E.H.B., J.H.G., and M.J.F. performed the experiments and analyzed the results. J.E.H.B., J.H.G., and M.J.F. prepared the manuscript with J.S.N. A.G.K. and J.W.Z. collected X-ray crystallographic data and solved the crystal structures of *N*-Me-D-Leu_2,D-Thr_5-clovibactin and stapled D-Thr_5-clovibactin, respectively. J.S.N. supervised the project and assisted in the experimental design and writing of the manuscript.

Notes

The authors declare the following competing financial interest(s): Initial support for work on clovibactin was provided by a subaward from NovoBiotic Pharmaceuticals LLC.

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