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Potent Analogues of Clovibactin from Commercially Available Amino Acid Building Blocks

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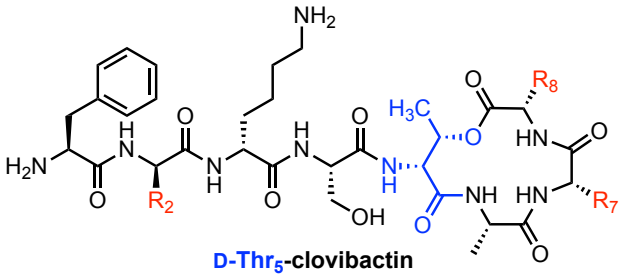


Abstract

This paper reports highly active analogues of clovibactin in which the rare, non-canonical amino acid D-hydroxyasparagine is replaced with the commercially available amino acid D-threonine. Sequential mutation of leucines 2, 7, and 8 to the more hydrophobic homologue cyclohexylalanine

dramatically increases the antibiotic activity of D-Thr_5-clovibactin. The resulting analogues (D-Cha_2,D-Thr_5-clovibactin, Cha_7,D-Thr_5-clovibactin, and Cha_8,D-Thr_5-clovibactin) are readily prepared by standard peptide synthesis techniques and exhibit excellent activity (≤ 1 μg/mL) against the Gram-positive, drug-resistant pathogens MRSA and VRE.

New antibiotics are desperately needed to treat antibiotic-resistant infections.[1,2,3] Antibiotic-resistant bacteria cause more than 2.8 million illnesses and 35,000 deaths in the United States each year. Gram-positive pathogens — including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), antibiotic-resistant *Streptococcus pneumoniae*, and three others — cause more than 54% of these illnesses and 60% of these deaths.[4] Clovibactin has recently been reported as a promising antibiotic against Gram-positive bacteria, including drug-resistant human pathogens such as MRSA and VRE, without detectable resistance.[5] Clovibactin is a cyclodepsipeptide composed of eight amino acids (Figure 1). Residues 1–4 comprise a linear tail and residues 5–8 comprise a macrocycle. The most notable feature of clovibactin is the rare, non-canonical amino acid D-hydroxyasparagine (D-Hyn) at position five, which serves as the linchpin in the macrolactone ring. Clovibactin kills bacteria by binding lipid II and related cell wall precursors and inhibiting cell-wall biosynthesis. Clovibactin is able to evade bacterial resistance because it binds to the immutable pyrophosphate group of lipid II and its precursors.

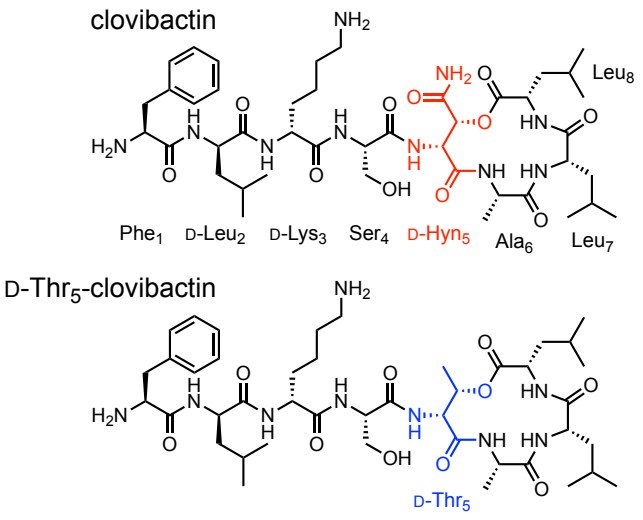
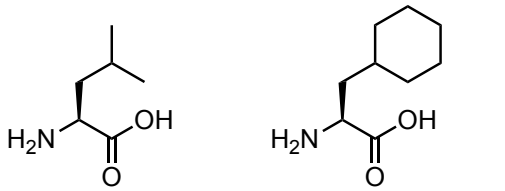


Figure 1. The chemical structures of clovibactin and D-Thr$_5$-clovibactin. The rare, non-canonical amino acid D-hydroxyasparagine (D-Hyn) is highlighted in red. D-Thr$_5$-clovibactin is a synthetic analogue of clovibactin in which D-Hyn is replaced by D-threonine (highlighted in blue).

In the current study, we set out to develop potent analogues of clovibactin in which D-hydroxyasparagine (D-Hyn) is replaced with the commercially available amino acid D-threonine (D-Thr, Figure 1). Replacement of D-Hyn with D-Thr eliminates the need to prepare D-Hyn through a multistep synthesis and opens the possibility of large-scale preparation of clovibactin analogues for preclinical studies.[6,7] Our laboratory recently reported a series of structure-activity relationship (SAR) studies of clovibactin in which D-Thr$_5$-clovibactin (NB-018) — an analogue pioneered by NovoBiotic Pharmaceuticals — was introduced.[6,8,9] This study demonstrated that replacement of D-Hyn$_5$ in clovibactin with D-Thr results in a 4–32-fold decrease in activity. Herein, we report potent analogues of clovibactin containing D-Thr that are similar in *in vitro* antibiotic activity to clovibactin.

In our initial report on the synthesis and stereochemical determination of clovibactin, we observed through X-ray crystallography of a clovibactin epimer that the residues Phe$_1$, D-Leu$_2$, Leu$_7$, and Leu$_8$ align on one side of the molecule to create a hydrophobic surface.[7] We postulated that this hydrophobic surface may be important to interactions with the bacterial cell membrane. Solid-state NMR studies by Weingarth and coworkers would later determine the specific contacts that D-Leu$_2$, Ala$_6$, Leu$_7$, and Leu$_8$ make with lipid II and the bacterial cell membrane.[5] We then reported an alanine scan of clovibactin which determined the importance of each side chain to the antibiotic activity of clovibactin.[6] The sequential mutation of Phe$_1$, D-Leu$_2$, Leu$_7$, and Leu$_8$ to alanine had resulted in an 8–256-fold decrease in antibiotic activity — suggesting that these hydrophobic side chains are very important for antibiotic activity. Collectively, the X-ray crystallographic, NMR, and SAR studies suggest that the hydrophobic interactions of D-Leu$_2$, Leu$_7$, and Leu$_8$ with lipid II and the bacterial cell membrane are especially important in the exceptional antibiotic activity of clovibactin.

We hypothesized that increasing the hydrophobicity of residues 2, 7, and 8 may increase the antibiotic activity of D-Thr$_5$-clovibactin. To test this hypothesis, we sequentially mutated leucines 2, 7, and 8 to the more hydrophobic homologue cyclohexylalanine. Cyclohexylalanine is commercially available, and it contains three more carbon atoms than leucine, making it substantially more hydrophobic (ΔCLogP = +1.2, Figure 2). We thus prepared the three Cha analogues (D-Cha$_2$,D-Thr$_5$-clovibactin, Cha$_7$,D-Thr$_5$-clovibactin, and Cha$_8$,D-Thr$_5$-clovibactin; Figure 3) by solid-phase peptide synthesis and assessed their antibiotic activity against MRSA, VRE, and other Gram-positive bacteria through minimum inhibitory concentration (MIC) assays.



Leucine (Leu) CLogP: -1.667 Cyclohexylalanine (Cha) CLogP: -0.474

Figure 2. Leucine and cyclohexylalanine. CLogP values were calculated using ChemDraw.

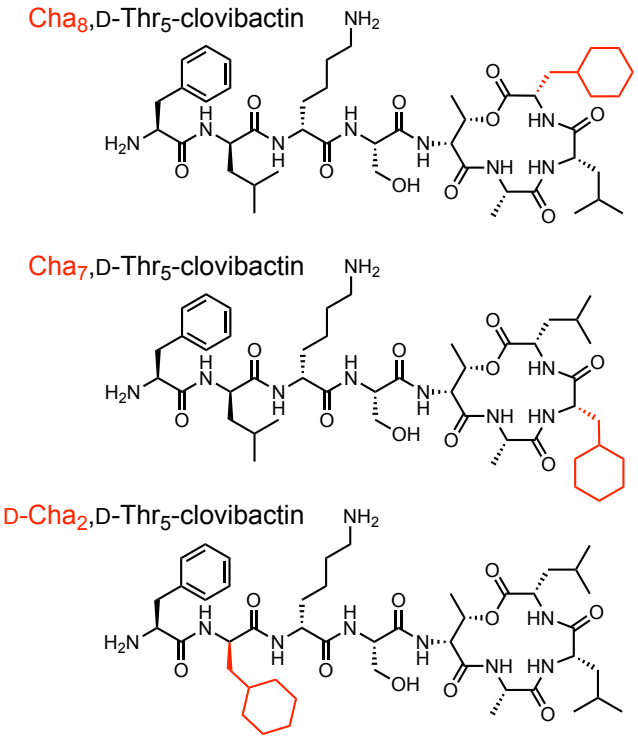


Figure 3. Cyclohexylalanine (Cha) analogues of D-Thr$_5$-clovibactin.

Synthesis of the Cyclohexylalanine Analogues of D-Thr$_5$-clovibactin. We synthesized D-Cha$_2$, Cha$_7$, and Cha$_8$,D-Thr$_5$-clovibactin by Fmoc-based solid-phase peptide synthesis of the branched precursors on 2-chlorotrityl chloride resin followed by solution-phase macrocyclization (Figure 4).[6] Residue 7 is first loaded on the resin, and residues 6 through 1 are sequentially coupled. Residue 8 is then introduced by Steglich esterification to the β-hydroxy group of D-Thr$_5$. Residue 8 is deprotected and the otherwise protected peptide is cleaved from the resin with 20% HFIP (hexafluoroisopropanol) in CH_2Cl_2. Solution-phase macrocyclization, global deprotection, and preparative reverse-phase chromatographic purification yields D-Cha$_2$, Cha$_7$, and Cha$_8$,D-Thr$_5$-clovibactin as the trifluoroacetate (TFA) salts.

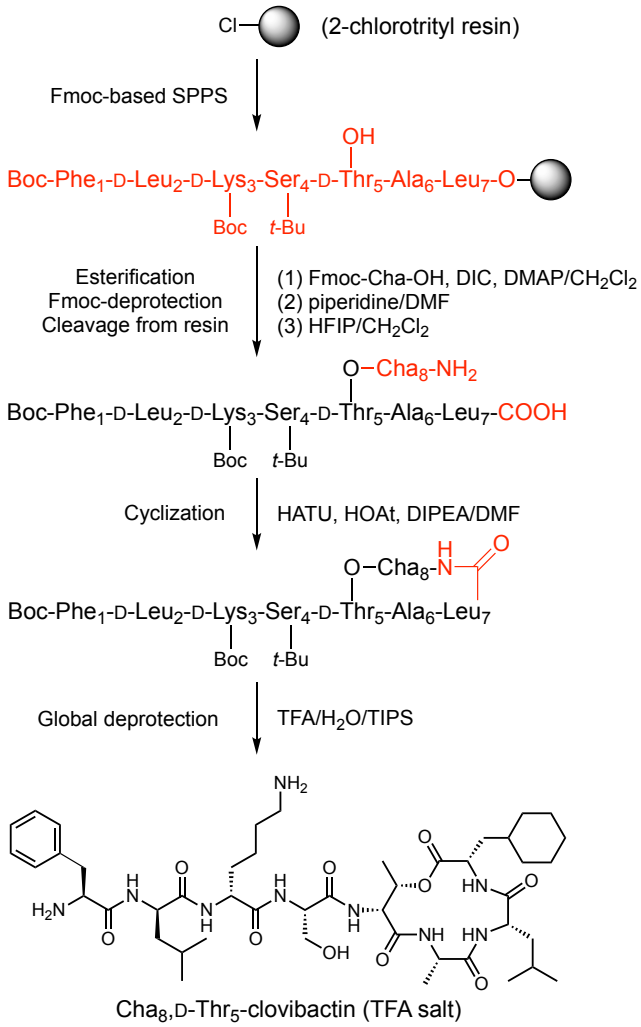


Figure 4. Synthesis of Cha$_8$,D-Thr$_5$-clovibactin.

Minimum Inhibitory Concentration (MIC) Assays. We tested the three cyclohexylalanine analogues in minimum inhibitory concentration (MIC) assays against a panel of five Gram-positive bacteria (*Bacillus subtillis*, *Staphylococcus epidermidis*, methicillin-susceptible *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus faecalis*). The Gram-negative bacterium (*Escherichia coli*) was used as a negative control, as little or no activity against Gram-negative bacteria is expected. We compared the antibiotic activity of the Cha analogues to clovibactin, D-Thr$_5$-clovibactin, and vancomycin. The MIC assays were performed with and without 0.002% v/v polysorbate 80 (PS-

80), which is expected to help prevent the adsorption of the peptides the 96-well polystyrene plates that are used for the assays.[5,10,11] Table 1 summarizes the minimum inhibitory concentrations (MICs) of the different analogues.

Table 1. MIC values of clovibactin and hydrophobic analogues of clovibactin in μg/mL

	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
clovibactin	0.125[a] 0.125[b]	0.5[a] 0.125–0.25[b]	0.5[a] 0.5[b]	0.25[a] 0.5[b]	2[a] 1[b]	16[a] 8[b]
D-Thr$_5$-clovibactin	1–2[a] 1–2[b]	2–4[a] 4[b]	8[a] 4–8[b]	4–8[a] 4[b]	8[a] 8[b]	>32[a] >32[b]
D-Cha$_2$,D-Thr$_5$-clovibactin	0.25–0.5[a] 0.125[b]	0.5–1[a] 0.125–0.25[b]	1[a] 1–2[b]	1–2[a] 1[b]	2[a] 0.5–1[b]	16[a] 16[b]
Cha$_7$,D-Thr$_5$-clovibactin	0.25–0.5[a] 0.125–0.25[b]	1[a] 0.5[b]	1[a] 1[b]	1[a] 1[b]	2[a] 1[b]	16–32[a] 8–16[b]
Cha$_8$,D-Thr$_5$-clovibactin	0.25–0.5[a] 0.125–0.25[b]	0.5[a] 0.125–0.25[b]	1[a] 0.5–1[b]	1[a] 0.5–1[b]	1–2[a] 0.5[b]	8–16[a] 8[b]
vancomycin	0.125–0.25[a] 0.25[b]	2[a] 1–2[b]	1[a] 2[b]	4[a] 4[b]	>32[a] >32[b]	>32[a] >32[b]

[a] Culture media containing 0% polysorbate 80. [b] Culture media containing 0.002% polysorbate 80.

D-Cha$_2$, Cha$_7$, and Cha$_8$,D-Thr$_5$-clovibactin all exhibited potent activity against the Gram-positive bacteria — with activities of 0.5–2 μg/mL against MSSA, MRSA, and VRE and activities of 0.125–1 μg/mL against *B. subtilis* and *S. epidermidis*.[12] In some cases, slightly better MIC values were observed in the presence of 0.002% PS-80. Each of the cyclohexylalanine analogues exhibited MICs of ≤ 1 μg/mL in the presence of PS-80. The analogues also exhibited comparable or greater activity than the clinically used antibiotic vancomycin and were active against VRE, against which vancomycin is inactive. The analogues were generally 4–16-fold more active than D-Thr$_5$-clovibactin against MSSA, MRSA, and VRE, but generally slightly less active than clovibactin with the exception of VRE. The enhanced activity of the

cyclohexylalanine analogues over D-Thr$_5$-clovibactin confirms the hypothesis that increasing the hydrophobicity of residue 2, 7, or 8 increases the antibiotic activity of clovibactin analogues.

Cha$_8$,D-Thr$_5$-clovibactin appears to be slightly more active than D-Cha$_2$,D-Thr$_5$-clovibactin and Cha$_7$,D-Thr$_5$-clovibactin, with MIC values against MSSA, MRSA, and VRE that are either equal or 2–4-fold lower in this twofold-dilution-based experiment. This observation is consistent with our previous finding that position eight of clovibactin is most sensitive to mutation in an alanine scan.[6] The greatly enhanced activity of D-Cha$_2$, Cha$_7$, and Cha$_8$,D-Thr$_5$-clovibactin over D-Thr$_5$-clovibactin is consistent with a model in which the side chains of D-Leu$_2$, Leu$_7$, and Leu$_8$ promote important interactions between clovibactin and the bacterial cell membrane.[5] The cyclohexylalanine analogues exhibit modest activity (8–32 μg/mL) against the Gram-negative bacterium *E. coli*. This activity is greater than that of D-Thr$_5$-clovibactin (>32 μg/mL) and may reflect a greater propensity of the more lipophilic analogues to interact with a variety of types of cells.

Hemolysis Assays. We tested and compared the three cyclohexylalanine analogues to D-Thr$_5$-clovibactin in a hemolysis assay to help assess the potential utility of these compounds as intravenous antibiotics (Figure 5). D-Thr$_5$-clovibactin showed no hemolytic activity at concentrations as high as 100 μg/mL. Each of the cyclohexylalanine analogues showed some hemolytic activity at the highest concentrations tested, but none at concentrations as high as 12.5 μg/mL. D-Cha$_2$,D-Thr$_5$-clovibactin showed no hemolysis at 25 μg/mL and 1.6% and 6.9% hemolysis at 50 and 100 μg/mL. Cha$_7$,D-Thr$_5$-clovibactin showed 0.8%, 3.4%, and 10.2% hemolysis at 25, 50, and 100 μg/mL. Cha$_8$,D-Thr$_5$-clovibactin was the most hemolytic, with 2.9%, 7.3%, and 15.0 % hemolysis at 25, 50, and 100 μg/mL. These levels of hemolysis suggest that these antibiotics could be administered intravenously at concentrations 10–20 times the MIC

values without exhibiting significant hemolytic activity. Nevertheless, the greater hemolytic activity of the cyclohexylalanine analogues over D-Thr$_5$-clovibactin suggests that both antibiotic activity and hemolytic activity increase with increased hydrophobicity and their non-specific interactions with lipid bilayer membranes may be significant.

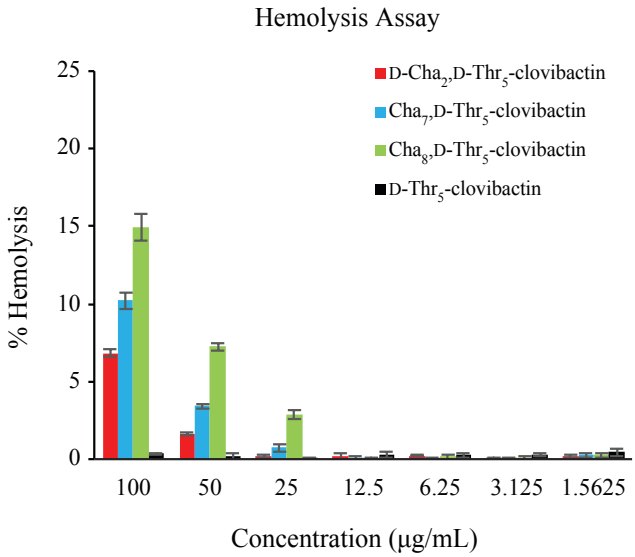


Figure 5. Hemolysis of human red blood cells by clovibactin analogues at varying concentrations. Hemolysis assays were performed on 5% blood in PBS at pH 7.4 in the presence of 0.002% polysorbate 80, which was added to minimize adsorption of the peptides to the assay plates.

Cytotoxicity Assays. We evaluated the cytotoxicity of the three cyclohexylalanine analogues toward HeLa cells to further assess the potential utility of these antibiotics, and we compared the analogues to D-Thr$_5$-clovibactin to further evaluate the effect of their increased hydrophobicity. The cytoxicity was assessed using the Promega Cytotox-Glo assay, with 24-hour exposure of the HeLa cells to the antibiotics. The cyclohexylalanine analogues showed no significant cytoxicity at concentrations as high as 50 µg/mL (Figure S2, S3). At 100 µg/mL, the analogues showed moderate cytotoxicity. In comparison, D-Thr$_5$-clovibactin showed no cytotoxicity at concentrations as high 100 µg/mL. These studies further suggest that the cyclohexylalanine analogues could be administered at concentrations well above the MIC values.

Conclusion. D-Cha$_2$, Cha$_7$, and Cha$_8$,D-Thr$_5$-clovibactin exhibit excellent antibiotic activity against MSSA, MRSA, and VRE and other Gram-positive bacteria. Replacement of the rare, non-proteinogenic amino acid D-hydroxyasparagine (D-Hyn) in clovibactin with commercially available D-threonine (D-Thr) results in a substantial decrease in antibiotic activity; however, replacement of any of the leucine residues with the more hydrophobic, commercially available amino acid cyclohexylalanine restores much of the antibiotic activity. The resulting cyclohexylalanine analogues are somewhat more lytic and cytotoxic than D-Thr$_5$-clovibactin. Nevertheless, the antibiotic activity occurs at concentrations substantially below those that are damaging to mammalian cells. For these reasons, D-Cha$_2$, Cha$_7$, and Cha$_8$,D-Thr$_5$-clovibactin warrant further investigation through preclinical studies in rodents or other animal models.[13]

Author Contributions

J.E.H.B. and J.J.S. synthesized the peptides. J.E.H.B. and J.J.S. performed the MIC assays and interpreted the results. J.E.H.B. and M.S.T.L.P. performed the hemolytic experiments. J.E.H.B., M.S.T.L.P., and J.I.C.P. performed the cytotoxicity experiments. J.E.H.B. and J.S.N. conceived the project idea and prepared the manuscript. J.S.N. supervised the project.

Conflict of Interest

None.

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 cost at https://pubs.acs.org/doi/10.1021/#####.

Supplementary figures; procedures for synthesis, MIC assays, hemolytic assays, and cytotoxicity assays; and characterization data.

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References

1. *World Health Organization Antimicrobial Resistance Webpage*. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance (accessed 2024-10-10).
2. *Center for Disease Control and Prevention Antimicrobial Resistance Home Page*. https://www.cdc.gov/antimicrobial-resistance/ (accessed 2024-10-10).
3. Naghavi, M.; Vollset, S. E.; Ikuta, K. S.; Swetschinski, L. R.; Gray, A. P.; Wool, E. E.; Aguilar, G. R.; Mestrovic, T.; Smith, G.; Han, C.; et al. Global Burden of Bacterial Antimicrobial Resistance 1990–2021: A Systematic Analysis with Forecasts to 2050. *The Lancet* **2024**, *404*, 1199–1226
4. U.S. Department of Health and Human Services Centers for Disease Control and Prevention. *Antibiotic resistance threats in the United States 2019*. Centers for Disease control and

Prevention, 2019. https://www.cdc.gov/antimicrobial-resistance/media/pdfs/2019-ar-threats-report-508.pdf (accessed 2024-10-10).

5. Shukla, R.; Peoples, A. J.; Ludwig, K. C.; Maity, S.; Derks, M. G. N.; De Benedetti, S.; Kreuger, A. M.; Vermeulen, B. J. A.; Harbig, T.; Lavore, F.; Kumar, R.; Honorato, R. V.; Grein, F.; Nieselt, K.; Liu, Y.; Bonvin, A. M. J. J.; Baldus, M.; Kubitscheck, U.; Breukink, E.; Achorn, C.; Nitti, A.; Schwalen, C. J.; Spoering, A. L.; Ling, L. L.; Hughes, D.; Lelli, M.; Roos, W. H.; Lewis, K.; Schneider, T.; Weingarth, M. An antibiotic from an uncultured bacterium binds to an immutable target. *Cell* **2023**, *186*, 1−15.
6. Brunicardi, J. E. H.; Griffin, J. H.; Ferracane, M. J.; Kreutzer, A. G.; Small, J.; Mendoza, A.-T.; Ziller, J. W.; Nowick, J. S. Structure–Activity Relationship Studies of the Peptide Antibiotic Clovibactin. *J. Org. Chem*. **2024**, *89*, 12479–12484.
7. Krumberger, M.; Li, X.; Kreutzer, A. G.; Peoples, A. J.; Nitti, A. G.; Cunningham, A. M.; Jones, C. R.; Achorn, C.; Ling, L. L.; Hughes, D. E.; Nowick, J. S. Synthesis and Stereochemical Determination of the Peptide Antibiotic Novo29. *J. Org. Chem.* **2023**, *88*, 2214–2220.
8. Peoples, A. J.; Hughes, D.; Ling, L. L.; Millett, W.; Nitti, A. G.; Spoering, A.; Steadman, V. A.; Chiva, J. C.; Lazarides, L.; Jones, M. K.; Poullennes, K. G.; Lewis, K.; Epstein, S. "Depsipeptides and Uses Thereof.". US11,203,616, 2021.
9. Personal communications with NovoBiotic Pharmaceuticals.
10. Arhin, F. F.; Sarmiento, I.; Belley, A.; McKay, G. A.; Draghi, D. C.; Grover, P.; Sahm, D. F.; Parr, T. R.; Moeck, G. Effect of Polysorbate 80 on Oritavancin Binding to Plastic Surfaces: Implications for Susceptibility Testing. *Antimicrob. Agents Chemother.* **2008**, *52*, 1597–1603.

11. Kavanagh, A.; Ramu, S.; Gong, Y.; Cooper, M. A.; Blaskovich, M. A. T. Effects of Microplate Type and Broth Additives on Microdilution MIC Susceptibility Assays. *Antimicrob. Agents Chemother.* **2018**, *63*, e01760-18.
12. Singh recently reported similar activity against MRSA for these and other analogues of clovibactin in a patent. Singh, I. New Antibacterial Products. WO2024100391, **2024**.
13. Brunicardi, J.; Small, J.; Padilla, M. S. T. L.; Carrera Plancarte, J.; Nowick, J. Potent Analogues of Clovibactin from Commercially Available Amino Acid Building Blocks. *ChemRxiv* **2024**. DOI:10.26434/chemrxiv-2024-57p0l. This content is a preprint.