


Blueprints for the rational design of therapeutic mutacin 1140 variants

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Abstract

Lantibiotics represent a large untapped pipeline of attractive scaffolds for the development of novel antibiotics. Saturation mutagenesis was employed to substitute every amino acid of a lantibiotic called mutacin 1140 (MU1140), creating an unbiased expression library of 418 variants that was used to study the permissiveness to mutagenesis and the “drugability” of several compounds. Contrasting previous reports, the results from this study supported that not all residues involved in lanthionine bridge formation were critical for maintaining optimal activity. While substitutions in lanthionine bridges in Ring A, C, and D invariably lead to inactive variants, permissive substitutions in Abu8 and Ala11 (Ring B) were observed, albeit infrequently. Further, the data generated suggested that the unsaturated bond from Dha5 (Ser5) may not be critically involved in Lipid-II binding but still important for conferring optimal activity. This study identified additional permissive mutations of Ser5, including Ser5His, Ser5Met, Ser5Gln, and Ser5Leu. In contrast, no permissive substitutions were identified for Dhb14, which suggested that this residue may be critical for optimal activity. Novel blueprints are proposed for directing further development of MU1140 variants and other lantibiotics, which may enable the rational design, development, manufacture, and formulation of an entirely new class of anti-infectives.

KEYWORDS

antibiotic, bacteriocin, lanthipeptide, lantibiotic, mutacin

1 | INTRODUCTION

By convention, lanthipeptides with antimicrobial activities are called lantibiotics.^[1] Type A (I) lantibiotics are cationic polycyclic lanthipeptides that derive their names from the thioether ring containing amino acids lanthionine (Lan, Ala-S-Ala) and/or 3-methyl-lanthionine (MeLan, Abu-S-Ala).

These classes of compounds are generally produced by Gram-positive organisms and often include other posttranslationally unsaturated amino acids such as 2,3-didehydroalanine (Dha), 2,3-didehydrobutyrine (Dhb), and the unsaturated lanthionine derivatives aminovinyl-D-cysteine (AviCys) at their C-terminus (reviewed in^[2,3,4] see Table 1 and Figure 1). The widespread use of the lantibiotic nisin as a food preservative has driven

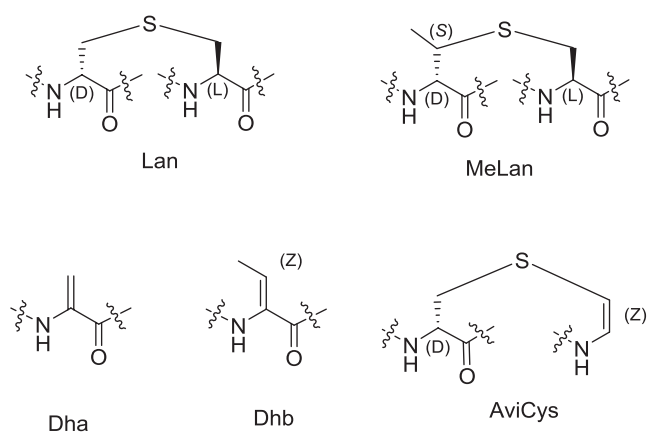
Abbreviations: Abu, aminobutyric acid; AviCys, aminovinyl-D-cysteine; Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; MU1140, Mutacin 1140; PK, pharmacokinetic; PD, pharmacodynamic.

TABLE 1 Amino acid sequence of the core peptide precursor and posttranslationally modified MU1140

Precursor	Ser3	Ser5	Cys7	Thr8	Cys11	Thr14	Ser16	Ser19	Cys21	Cys22
Modified	Ala3	Dha5	Ala7	Abu8	Ala11	Dhb14	Ala16	Ala19	Ala21	Avi-Cys22

Notes. Only posttranslationally modified residues are displayed. Bolded residues are involved in a lanthionine (Lan, Ala-S-Ala) and/or 3-methyl-lanthionine (MeLan, Abu-S-Ala) bridges.

Avi-Cys: aminovinyl-D-cysteine; Dha: 2,3-didehydroalanine; Dhb: 2,3-didehydrobutyrine.

**FIGURE 1** The structure of posttranslationally modified residues in lanthipeptides

much of the research in this field and as a consequence, most of our understanding of the structure-activity of lantibiotics has been derived from studies on nisin.^[5] Nevertheless, seminal work on related compounds has supported the potential usefulness of lantibiotics as therapeutic agents in several lantibiotics other than nisin, including gallidermin,^[6] haloduracin,^[7] lactacin 3147,^[8,9] and others (reviewed in^[3]). Despite a wealth of knowledge and many supporting in vitro and animal studies, only a few lantibiotics have been fully evaluated in preclinical and clinical studies and none have advanced to widespread clinical testing.^[10,11] This may be explained in part by economic considerations as well as technical challenges for manufacturing sufficiently pure lantibiotics in sufficient quantity for human clinical trials. Despite these challenges, several lantibiotics have successfully progressed into preclinical and clinical testing, including NVB302 for the treatment of *Clostridium difficile* infections, NVB333 for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcal (VRE) infections, microbisporicin for the treatment of MRSA, and others.^[10–12] Preclinical data generated thus far continue to support the concept that lantibiotics are efficacious in vitro and in vivo in Gram-positive models of infection. Also, clinical data obtained from the successful Phase-1 trial with NVB302 (MJ Dawson, Novacta Biosystems, personal communication), and successful Phase-2 with duramycin (for cystic fibrosis,^[13])

imply that this class of compounds should be well tolerated in humans.

Mutacin 1140 (MU1140) is naturally produced by the Gram-positive bacterium *Streptococcus mutans*^[14–16] and has historically received considerable attention as a potentially useful and novel antimicrobial because of its spectrum of activity, potency, low frequency of antimicrobial resistance, limited cytotoxicity, and overall pharmacological profile^{[(17,18], and Oragenics, unpublished)}. The genetic properties and characteristics of the *lan* polycistronic operon have been well described and are derived from both partial sequencing of the *lan* operon,^[15] and the recently completed entire genome sequence of the host strains, JH1140^[19] and JH1000 (Oragenics, unpublished). Much is also known about the structure and novel mechanism of action of MU1140. Based on NMR analysis and computational modeling, MU1140 has a U-shaped conformation, where Rings A/B and Rings C/D are facing one another and are separated by a relatively flexible “hinge region”.^[4,20] Rings A/B of nisin have been shown to bind to the pyrophosphate moiety of lipid-II,^[21] while the C-terminal lanthionine rings are thought to be involved in self-assembly, leading to a pore-like organization. The high degree of sequence and structural similarity between MU1140 and nisin suggest that MU1140 binds lipid-II and self-assembles in a similar fashion to nisin.^[20] The essential role of Lipid-II in cell wall synthesis makes it a target for many antimicrobial peptides.^[22] While MU1140 does not appear to span the entire bi-lipid outer-membrane to create a functional pore,^[20] the mechanism of action of MU1140 is thought to be similar to nisin (which also binds Lipid-II at the pyrophosphate moiety) in that it disrupts the peptidoglycan synthesis of Gram-positive bacteria via Lipid-II abduction.^[23–25] In contrast, vancomycin binds to Lipid-II at the terminal part of the pentapeptide. Replacement of terminal D-Ala in the pentapeptide of Lipid-II is possible and changes the composition of the pentapeptide without interfering with cell wall synthesis, thereby selecting for vancomycin-resistant bacteria.^[23] For example, vancomycin-resistant enterococci and staphylococci that acquire *vanA* develop vancomycin-resistance by substituting D-Ala-D-Ala for D-Ala-D-Lactate in Lipid-II.^[26]

Because a substantial amount of information exists for MU1140, it is a compelling model to study the

structure–function attributes of naturally occurring lantibiotics and their derivatives. While the structure-function of key positions has been previously studied for MU1140,^[27,28] and other archetype lantibiotics,^[3,29] the use of saturation mutagenesis as a means to comprehensively study the structure-function of every amino acids constituting a lantibiotic, in an unbiased and addressable fashion, has not been previously undertaken. Therefore, the work presented in this report provides the blueprints enabling further development of MU1140-variants, and other lantibiotics, as they are being rationally designed, developed, manufactured, and formulated as novel anti-infectives.

2 | METHODS AND MATERIALS

2.1 | Strain construction

To generate *S. mutans* SM126 (JH1000 Δ lanAA'), the 5' and 3' flanking regions of lanAA' were PCR amplified from *S. mutans* JH1140 genomic DNA using primers JK433/JK442 and JK445/JK446, generating 1,002 and 1,030 bp amplicons, respectively. The erythromycin resistance (Erm^R) gene was PCR amplified from vector pVA891^[30] using primers JK440 and JK441, generating 988 bp amplicon. The pVA891 plasmid backbone was amplified using inverse-PCR with primers JK447 and JK432, generating an amplicon of 2,106 bp. Individual amplicons were purified using a clean and concentrate kit (Zymo Research) and then assembled using In-Fusion (Clontech) and subsequently transformed into *E. coli* NEB turbo cells (New England Biolabs). The resulting construct was streaked-purified to assure that a single clone was used and then named pLAN075 (Supporting information Figure S1). The 5'lanAA' flanking—Erm^R—3' lanAA' flanking sequence of pLAN075 was PCR amplified using JK452 and JK498. Amplification of the 2,960 bp PCR product was confirmed by visualization on a 1.2% agarose gel then transformed into *S. mutans* JH1000 using standard techniques^[31] with selection for the integration event plating onto Petri dishes containing 3 μ g/ml erythromycin. Colonies were genotyped by PCR amplification of the lanAA' locus using primers JK452 and JK498, and sequence identity was confirmed by Sanger DNA sequencing of the resulting amplicon (Quintara Biosciences). Refer to Table 2 for details.

S. mutans SM152 (JH1140::Erm^R) was generated by integration of the pVA891 erythromycin resistance selectable marker^[30] in the lanA'-lanB intergenic region of JH1140 using Splicing by Overlap Extension (SOE)-PCR. Briefly, 1,000 bp of JH1140 genomic DNA 5' of lanA' was PCR amplified using primers JC023 and JC012. 1,000 bp of JH1140 genomic DNA 3' of lanA' was PCR amplified using primers JC014 and JC024. The erythromycin resistance gene was

PCR amplified from plasmid pVA891 using primers JC011 and JC013. Amplification of the correct sized PCR products was confirmed by visualization on a 0.8% agarose gel. The PCR products were then purified using a Zymo clean and concentrate kit (Zymo Research). Splicing by Overlap Extension (SOE) PCR was used to generate integration cassettes to integrate the erythromycin resistance gene into the JH1140 genome downstream of lanA'. The 5' and 3' lanA' integration arms and erythromycin resistance gene were pooled in equal volume, and 1 μ l was used for the SOE-2 reaction. Primers JC023 and JC024 were used to amplify the final assembled integration cassettes with 1,000 bp recombination arms on each flank of the erythromycin resistance gene. Transformation into *S. mutans* was performed as per the lanAA' deletion procedure (as described above for SM126). The verification of correct vector integration was confirmed using colony PCR amplification of the lanAA' locus using primers JK433 and JK168. Refer to Table 2 for details. DNA sequencing was performed to confirm nucleotide identity and appropriate integration of the vector cassette (Quintara Biosciences).

2.2 | Library construction of mutacin 1140 (MU1140) variants

2.2.1 | Plasmid library

A saturated plasmid-based lanA library was first constructed by substituting 19 amino acids codons at each of the 22 amino acid codon positions on a plasmid vector encoding lanA (the structural gene encoding MU1140) and resulted in a total of 418 amino acid variants of MU1140 (refer to Table 2 for details). Briefly, the episomal plasmid was constructed into shuttle plasmid pMK4, which confers ampicillin resistance in *E. coli*, and chloramphenicol resistance in *S. mutans*. A 1,065 bp fragment of *S. mutans* JH1140 genomic DNA encoding lanAA' was PCR amplified using primers JK374 and JK376 using Phusion polymerase (NEB). A 4,801 bp fragment of pMK4 was PCR amplified using primers JK378 and JK379 using Phusion polymerase (NEB). The two amplicons were cloned using the In-Fusion cloning system, and the resulting 5,866 bp vector was verified by DNA sequencing of the lanAA' DNA using JK374 and JK376 and named pLAN042 (Supporting information Figure S2). Plasmid pLAN042 was used as a template to build lanA variants using In-Fusion HD Cloning Plus PCR mutagenesis.^[32] The library was constructed in an addressable fashion, where the predicted identity of each lanthipeptide is known prior to any biological assay, with each vector containing a single codon mutation to confer a designated amino acid change, in the context of the codon usage preference of *S. mutans*. Confirmation of the codon change was confirmed by DNA sequencing (data not shown). The lanA variant library in pMK4 was transformed into *S. mutans* JH1140 by natural

transformation^[31] and transformants selected on trypticase soy yeast extract (TSYEX) agar plates containing 3 µg/ml chloramphenicol after incubation (in a candle jar) for 3 days at 37°C. All media were from Difco, and all chemicals and antibiotics were from Fisher Scientific.

2.2.2 | Chromosomal library

Selected MU1140 variants in *S. mutans* JH1140 were reconstructed by allelic replacement where the native chromosomal *lanA* gene was replaced with *lanA* variants encoding codon substitutions. Splicing by Overlap Extension (SOE) PCR was used to construct DNA vectors for integration of *lanA* variants into the JH1140 chromosome. Briefly, oligonucleotides JC023 and JC051 were used to amplify 1000 bp of SM152 genomic DNA 5' of *lanA* for use as a homologous recombination arm. Oligonucleotides JC052 and JC024 were used to amplify 1,000 bp of SM152 genomic DNA 3' of *lanA*' and a selectable erythromycin resistance marker (from pVA891,^[30] for use as a homologous recombination arm. Oligonucleotides JC047 and JC048 were used to amplify 554 bp of DNA encoding *lanA* variants and flanking sequence from the 418 *lanA* variant plasmid library. DNA amplification of PCR products of appropriate size was confirmed by agarose gel electrophoresis and purified using a Zymo ZR96 PCR clean up kit (Zymo Research). The three amplicons were pooled, amplified, and nested; oligonucleotides JC027 and JC028 were used to amplify the 3,509 bp final PCR product containing 750 bp recombination arms, *lanA* variant, and erythromycin selectable marker. DNA vectors were transformed into *S. mutans* JH1140 by natural transformation.^[31] Transformants were plated onto TSYEX agar containing 3 µg/ml erythromycin and incubated (in a candle jar) for 3 days at 37°C. Colony PCR and Sanger DNA sequencing was utilized to confirm replacement of the chromosomal copy of *lanA* with the *lanA* variant encoded on the integration vector.

2.3 | Colony PCR and DNA Sequencing

A 2 µl aliquot of overnight cell culture was added to 25 µl PCR Lyse (Epicentre) and incubated at 99°C for 5 min. 2 µl of lysed sample was added to Phusion PCR master mix (NEB) and amplified with primers JK499 and JK500. DNA sequencing of each clone was performed to verify codon mutation using JK374 and JK376.

2.4 | High-throughput *Micrococcus luteus* bioassay

S. mutans colonies expressing the individual 418 variants were manually picked into 2.2-ml deep well plates (E&K Scientific) containing 1 ml of TSYEX and 3 µg/ml chloramphenicol. SM152 (high producer) and SM126 (*lanA*⁻)

were used as positive and negative controls, respectively. The plates were sealed with PCR film (VWR) and grown overnight to saturation at 37°C without shaking. 100 µl of overnight culture was transferred to a second 2.2 ml deep well plates containing 900 µl of growth media containing 5% yeast extract, 4% glucose, 0.1% CaCl₂, 100 mM bis-tris buffer pH 6.7, and 0.5 µg/ml chloramphenicol. Microbiological media were from Difco, antibiotics, and chemicals were from Fisher Scientific. The expression plates were sealed with porous seals (VWR) and shaken for 20 hr at 37°C and 900 rpm. The cultures were centrifuged for 10 min at 3,220 × *g*, and the supernatant was removed for testing.

M. luteus ATCC 272 was streaked onto a Luria-Bertani (LB) agar plate and grown at 30°C for 48 h. 5 ml of LB broth was transferred to a 14-ml culture tube. *M. luteus* cells were inoculated from plate cultures and grown overnight at 30°C in a shaker at 250 rpm. Cells were diluted 1:100 in 50 ml LB broth in a 250 ml Erlenmeyer flask and grown for 2 hours at 30°C. Following this, *M. luteus* was centrifuged for 5 min at 3,220 × *g*, resuspended in 5 ml of fresh LB, and inoculated into 45°C molten 125 ml soft agar (5 g/l Bacto agar, 8 g/l Difco nutrient broth) at OD₆₀₀ 0.1, and poured into a Q-tray (Genetix). The uncovered plate was dried in a laminar flow hood for 30 min. A QPix 450 colony picking robot (Molecular Devices) equipped with a fixed 96 metal pin head was used to spot replicate 10 times from a 96-well source plate (shallow Greiner U-bottom) containing 150 µl medium to the Q-tray. The plates were grown overnight at 30°C and imaged using an Epson Expression 10,000 XL optical scanner. Zones of clearing were compared to the positive control (SM152) and electronically scored from 0-3. Zones were scored as 0 (no zone of clearing), 1 (zone of clearing < positive control), 2 (zone of clearing = as positive control), or 3 (zone of clearing > than positive control). Specifically, each image of a zone of clearing assay was evaluated by determining the size of the zone of clearing relative to a graphic circle representing the mean image size of the control zones for each assay plate. This allowed to empirically determine zones that were less than, equal to, or greater than the zone of clearing for the positive control treatment. The final data were reported as the average of two independent experiments.

2.5 | Medium-scale production, purification and characterization of selected compounds

MU1140 chromosomal *lanA* variant strains were grown in 1 L bioreactor fermentors to generate sufficient quantities of compounds to purify and characterize. Briefly, *S. mutans* strain glycerol stock from -80°C freezer was used to aseptically inoculate 60 ml stock TSYEX medium into disposable sterile 125-ml shake flask, and incubated in a

TABLE 2 Strains, plasmids, and DNA oligonucleotides used in this study

Name	Property, genotype or sequence	Source or reference
Strains		
JH1000	MU1140 producing strain of <i>S. mutans</i> (WT)	[15]
JH1140	MU1140 hyperproducing strain of <i>S. mutans</i> derived from JH1000	[15]
SM152	JH1140::Erm (intergenic <i>lanA'</i> – <i>lanB</i>)	This work
SM126	<i>S. mutans</i> JH1000 Δ <i>lanAA'</i> (<i>lanA</i> [−])	This work
Plasmids		
pMK4	Amp ^R in <i>E. coli</i> ; Cam ^R in <i>S. mutans</i>	GenBank: EU549778.1
pLAN042	pMK4:: <i>lanAA'</i>	This work Supporting information Figure S2
pLAN075	pVA891:: <i>lanAA'</i>	This work Supporting information Figure S1
pVA891	Source of Erm ^R cassette	[30]
DNA primers ^a		
JK374	5'-TCCGCTCATGAGACAGTGATTAGAAGCTATGTTATCATGG AGTTATAGTAATC	This work
JK376	5'-ATTTATCAGGGTTATAATATCAACATATGATAATGTCACG ACCAAGTTTC	This work
JC023	5'-GAACTTTATCGAGAATTAAGAATTGCTAGAGG	This work
JC024	5'-CTGAAGAGAATCCTTGATATGATGAGATAACTCCAA	This work
JC027	5'-GTTATCAGAATTATATGTCAGAAAAGATATCAAAGG	This work
JC028	5'-CAAATAAATTTAACTCAGTGATCAAAAACCTTCTTTG	This work
JC047	5'-AATATCAACATATGATAATGTCACGACCAAG	This work
JC048	5'-ATGTTTCAGTTACACACATGAAAAAGGAGG	This work
JC051	5'-CCTCCTTTTTCATGTGTGTAAGTGAACAT	This work
JC052	5'-CTTGTCGTGACATTATCATATGTTGATATT	This work
JK378	5'-ATAACCCTGATAAATGCTTCAATAATTTGAAAAAGG	This work
JK379	5'-TGTCTCATGAGCGGATACATATTTGAATGTATTTAG	This work
JK432	5'-AGGCGTTTAAGGGCACCAATAAC	This work
JK433	5'-TGCCCTTAAACGCCTGTAGAACGTGATAAGTTGTATATAT CTCTCCTAGTCA	This work
JK440	5'-GTTCGTGCTGACTTGCACCATATC	This work
JK441	5'-TCTACATTCCTTTAGTAACGTGTAACCTTCC	This work
JK442	5'-CAAGTCAGCACGAACAATATCCTCCTTTTTCATGTGTGTAA CTGAAC	This work
JK445	5'-TAAAGGGAATGTAGACTATAAATTACTTAAATTGATAG GAAACTTGGTCGTGAC	This work
JK446	5'-CAAGTCAGCACGAACATTGTTGCCCTCACCAATGTCAC	This work
JK447	5'-GTTCGTGCTGACTTGTAAATTATTAGGTATACTACTGACAG CTTCCAAGG	This work
JK452	5'-GTAGAACGTGATAAGTTGTATATATCTCTCCTAGTCA	This work
JK498	5'-ATTGTTGCCCTCACCAATGTCAC	This work
JK499	5'-AGTGAGCGAGGAAGCGGAAG	This work
JK500	5'-GGAATAAGGGCGACACGGAAATG	This work

^aPrimers used for site-directed mutagenesis are proprietary (Intrexon)

shaker at 125 rpm and 37°C overnight (to a final OD₆₀₀ of 1.5–2.0). A Sartorius Biostat A plus 1 L was used to conduct fed-batch fermentations under aerobic stirred-tank conditions with automated temperature/pH/dissolved oxygen controls, with an initial culture volume of 800 ml. The growth media consisted of 200 ml of 200 g/l yeast extract, 8 ml of 300 g/l glucose, and 21.6 ml of 1 M CaCl₂ solution. The feed solution used in exponential growth phase was 300 g/l glucose, the base used for pH control was 5 N NaOH, and Antifoam B was used as needed. The following control loops and setpoints were used during the batch growth phase: set temperature to 37°C, airflow to 0.25 l/min; DO = 15%, controlled by agitation, 150–800 rpm; pH set to 6.5. The volume of inoculum needed was normalized to 20 ml of OD₆₀₀ 1.75. 15 ml of 1 M CaCl₂ solution was added as a bolus into fermentor after 5 hr of batch fermentation. The exponential fed-batch stage was initiated at ca. 6–8 hr postinoculation, at which time, the automated glucose feed was initiated. Antifoam B addition was fixed at 1 drop per 30 min, and the addition rate of feed solution was based on the following equation:

$$F(t) = \frac{\mu_c \cdot X_0 \cdot V_0 \cdot e^{\mu_c \cdot t}}{S_{in} \cdot Y_{X/S}}$$

F , Pump rate (l/hr); μ_c : Specific growth rate = 0.10 h⁻¹; V_0 : Initial working volume = 0.8 L; t : Fermentation time (hr), reset to zero when exponential fed-batch starts; X_0 : Initial dry cell weight = 1.6 (g/l); S_{in} : Glucose concentration in Feed solution = 300 (g/l); $Y_{X/S}$: Dry cell weight yield on glucose = 0.35 (g/g).

At 28 hr postinoculation, the fermentation broth was removed from each fermentor and poured into 1-L HDPE bottles and centrifuged at 7,500 rpm (12,300 \times g) for 15 minutes at room temperature. Supernatant was removed and stored in 1-L glass bottles at 4°C for processing within 7 days or -20°C for longer term storage.

Culture supernatants were extracted using chloroform extractions (modified from 15) to isolate the compounds of interest. Briefly, culture liquors were extracted twice using chloroform at a 1:1 medium:chloroform ratio and shaken for 10 min per extraction on a flat orbital shaker. The chloroform emulsion between the two solvent layers was filtered on Grade 1 Whatman qualitative filter paper (Fisher Scientific), and the solid retained on the filter was lyophilized to dryness. Following postextraction resolubilization of variants, titers were determined and were compared to values obtained during titer determination of fermentation medium prelarge scale chloroform extraction. Recovery was approximately 100% of theoretical based on initial titers for the majority of variants. Compounds were further purified to $\geq 90\%$ purity by reverse-phase flash chromatography on C18 Aq RediSep RF Gold, 150 g, over 10 min using a gradient with 0.1% formic acid containing 5% acetonitrile (ACN)/95% H₂O and 0.1%

formic acid containing ACN at a flow rate of 85 ml/min. The identity, purity, and quantity of each variant were determined by UPLC and UPLC/MS on Waters ACQUITY UPLC BEH C18 column, particle size 1.7 μ m, 2.1 \times 50 mm. Buffer A was 0.1% formic acid in H₂O, and buffer B was 0.1% formic acid in ACN. The gradient was 5% buffer B to 40% buffer B over 3 min at a flow rate of 0.5 ml/min. Injection volume was 5 μ l. The mass of peak of interest was analyzed by electrospray ionization mass spectrometry with source temperature of 150°C, and desolvation temperature was 500°C. Cone gas flow was 50 l/hr, and desolvation gas flow was 1,000 l/hr.

2.6 | Normalized specific activity of selected compounds

In the high-throughput bioassay presented above, the precise concentration of compound was not known in the culture liquor prior to MIC testing. Therefore, a minimum inhibitory concentration (MIC) assay was used to provide specific activity testing on purified ($\geq 90\%$ purity) lantibiotics and included steps of normalization for concentration and for purity. *M. luteus* ATCC 272 was streaked onto an LB agar plate from a freezer stock and grown at 30°C for 48 hr. A single colony was transferred into 5 ml of LB broth in a 14-ml culture tube and grown overnight at 30°C in a shaker at 250 rpm. Cells were diluted 1:100 in 50 ml Mueller Hinton II broth in a 250-ml Erlenmeyer flask and grown shaking for 2 hr at 30°C, 200 rpm. *M. luteus* was transferred to a 50-ml screw cap tube, centrifuged for 5 min at 3220 \times g , resuspended in fresh Mueller Hinton II at an OD₆₀₀ of 0.05, mixed by vortexing at high speed for 30 s, poured into a 50 ml sterile reservoir, and inoculated into 96-well plates in 100 μ l volumes using a 12-channel pipette. MU1140 and variants were serially diluted twofold in 50% ethanol, and 1 μ l volumes were added to each well using a 12-channel pipette. Serial dilutions were performed starting at ~1 mg/ml for the highest concentration tested. The plates were shaken for 16 hr at 30°C and quantified using a plate reader to determine well OD₆₀₀ values. OD₆₀₀ values were normalized by wells containing medium only. Normalized MIC was defined as the MIC that is purity-adjusted and back-calculated once the concentration and purity are precisely determined by HPLC. MIC determinations were made following normalized wells that contained OD₆₀₀ \leq 0.1 after 16 hr of growth.

3 | RESULTS

3.1 | Screening of permissive substitutions with an episomal expression library

The involvement of individual amino acids to the biological activity of MU1140 was investigated by permuting all 22 amino acids of the core lantibiotic with every other 19

naturally occurring amino acids. The resulting saturation mutagenesis library comprised 418 theoretical possible variants of MU1140. The episomal library was initially constructed in a shuttle called pLAN042, which combined the ability to perform cloning in *E. coli* and expression in *S. mutans* at high-throughput.

Following successful episomal library construction, a high-throughput and semi-quantitative activity screening was performed using *Micrococcus luteus* as a reporter strain. This bioassay assessed the antimicrobial activity the supernatant of each variant strain in two replicates (on two different plates) by monitoring biological activity (zone of clearing) of culture supernatants. Zones were scored as 0 (no zone of clearing), 1 (zone of clearing < positive control), 2 (zone of clearing = positive control), or 3 (zone of clearing > positive control). This semi-quantitative assay was used to eliminate poor performing MU1140 variants and those that are either not biosynthesized or transported. Positive control (MU1140) and negative control (SM126 containing the empty plasmid pMK4 that does not express a lantibiotic) were spotted on several plates. Also selected compounds were spotted on several plates and in multiple locations. It was noted that zone sizes varied between plates but remained relatively consistent between replicates. Final scores were expressed as the average of two independent assays.

The impact of substitutions on the biological activity of variants (*M. luteus* as a reporter strain) is depicted in Figure 2. Several of those non-permissive positions are involved in lanthionine bridge formation (Ala3, Ala7, Ala16, Ala19, and Ala21), C-terminal vinyl group (AviCys22), or other functional elements (Dhb14).

3.2 | Normalized activity testing and characterization of selected variants

While the high-throughput bioassay enables rapid screening of hundreds of strains expressing lanA variants and detects those with no or significantly reduced activity (as compared to the natural compound (see Figure 2), this assay was not normalized by lantibiotic concentration, and as such it can only provide qualitative data. Plasmid-borne libraries can be compromised by variable copy number, genetic reorganization, and potential pleiotropic effects. To provide quantitative data and to decouple activity from potential biosynthesis or transport defects, the variants that retained the highest levels of activity in the episomal library were reconstructed by allelic replacement. The mutation was integrated in a single copy at the *lan* locus in the chromosome of the host cell. In addition, other selected variants were chosen based on positional diversity. A total of 32 variants were reconstructed, produced by fermentation at the 1 L scale, and purified to homogeneity. This process

generated sufficient amounts of material to enable the characterization of the lantibiotic variants, including the precise and accurate measurement of the specific activity of each compound. LC/MS was used to confirm the mass of the predicted products and matched the computed values, except for (Leu6Thr) which appeared to be dehydrated (-18 Daltons by LC/MS, data not shown). The normalized MIC of every 32 reconstructed variants against *M. luteus* is presented in Figure 3 alongside their parent, MU1140. While eight variants led to a relatively lower activity (>0.04 µg/ml), the great majority of compounds (MIC between 0.01 and 0.04 µg/ml) were within one dilution of MU1140 (MIC 0.021 µg/ml). Six compounds were identified as having an apparently lower MIC than MU1140 on *M. luteus* and included Phe1Ile, Phe1Thr, Lys2Met, Leu6Ala, Arg13Asp, and Phe17Leu. The mass and chromatography data are presented for these selected variants in Supporting information Figure S3.

4 | DISCUSSION

4.1 | MIC activity determination

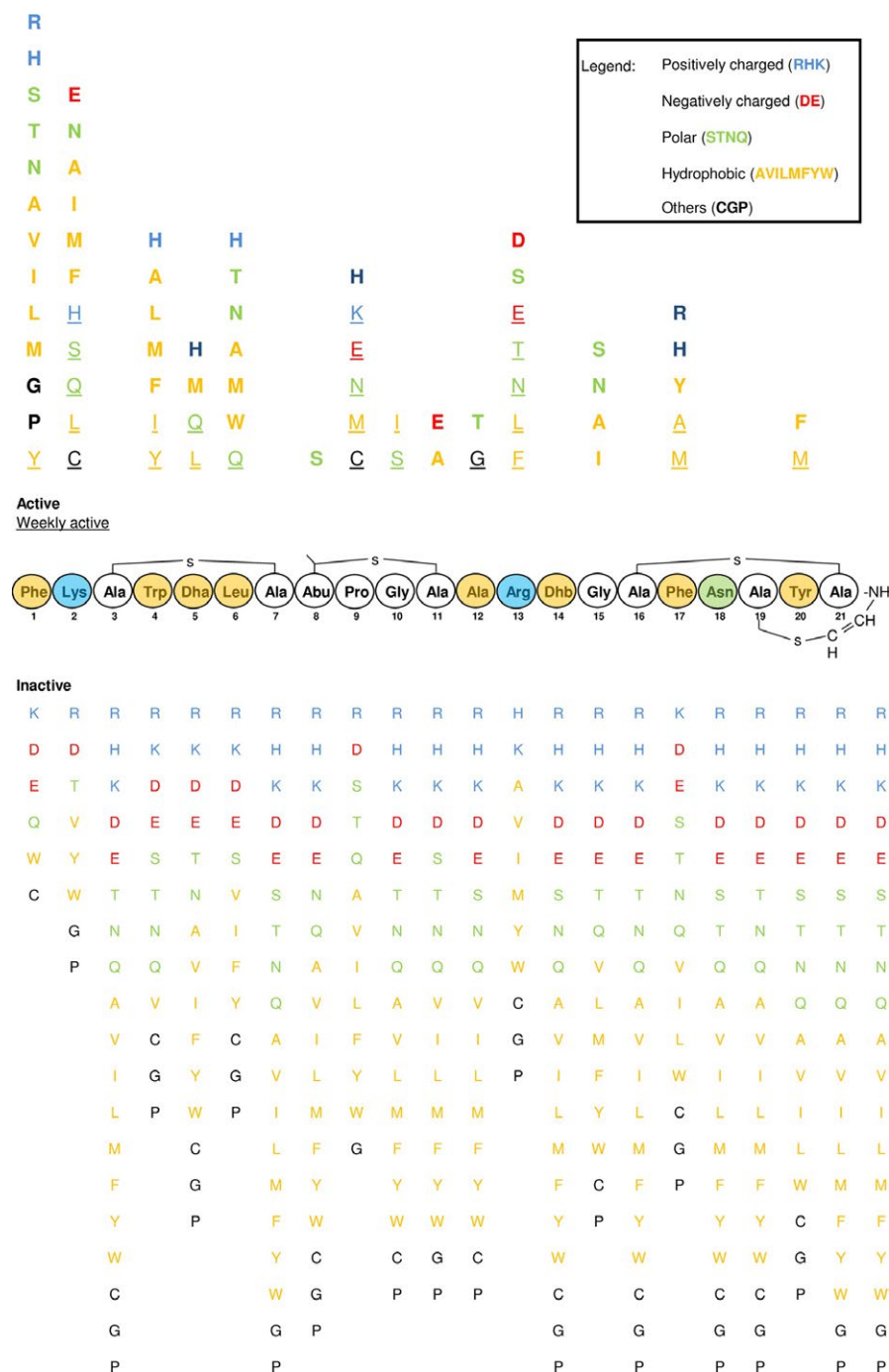
This is the first exhaustive study focused on the structure-activity relationships of mutacin 1140. Seminal work in this field investigated key substitutions in nisin and closely related molecules such as lacticin 481 and 3147, nukacin ISK-1, mersacidin, haloduracin, and others (reviewed in^[3]). To date, no single study has provided a comprehensive evaluation of all possible amino acid permutations side-by-side and provided a full picture of their potential impact on biological activity. The construction and testing of a saturated mutagenesis library enabled unbiased sampling of the MU1140 amino acid sequence. High-throughput screening allowed the initial library to be triaged into a small subset of mutants, conferring structural diversity, and potentially different chemical and/or biological properties.

While the episomal expression system enabled high-throughput screening, it presented obvious limitations and substantial variability. This high-throughput method was not adequate to quantify subtle changes of activity between variants. More importantly, the high-throughput screening method used to sieve through non-permissive mutations did not allow the distinction between variants that may have interfered with the biosynthesis or transport of the desired compounds, or between mutations that simply rendered the compound biologically inactive. While recent evidence supports that single point mutations rarely result in compounds that are significantly affected in their biosynthesis or transport, unless they are located to Ring D,^[28] this potential source of bias cannot entirely be ruled out. Titer variability was observed among all compounds targeted for specific activity testing (see Figure 2), but

none of the producing strains were totally unable to synthesize or transport the compounds of interest (see Figure 3). Regardless of the underlying basis for strain inactivity, all non-permissive variants would ultimately be less “druggable” considering the implications on manufacturing. Nevertheless, this method remained sufficiently sensitive to identify those variants with little to no activity, which ultimately comprised 83% of the library. Efforts to perform well-to-well normalization were difficult due to low amounts of lantibiotic produced, leading to challenging quantification of the MICs. Similar strain to strain optical densities were observed following growth of the episomal

lanA variant library in 96-well microwell plates, and as such inhibitory activity of one or more lantibiotic peptides to the *S. mutans* production host was unlikely and ruled out (data not shown). It is well recognized that activity testing derived from plasmid-borne libraries can never completely eliminate the confounding variability related to copy number, genetic reorganization, or pleiotropic effects.^[33]

The use of a deferred antagonism assay as means to assess differences of activity between variants is widespread and has been used exhaustively because of its ease in differentiating strain or molecule performance in a biological assay context. In this method, bacterial colonies expressing



lantibiotics are grown on a Petri dish, instead of spotting purified lantibiotic on a plate, and the zone of inhibition that results from the diffusing secreted peptides can be quantified after the colony has formed on a lawn of sensitive reporter strain.^[27] Data derived from deferred antagonism assays must be interpreted with care as they suffer from limitations and do not measure the specific activity of a given compound; a substitution that modifies the overall charge, lipophilicity, or hydrophobicity of a variant may also affect the diffusion rate, stability, and solubility of a given variant, as compared to MU1140.^[34–36] More importantly, this method assumes that all mutant strains express each compound with identical levels of transcription, post-translational processing, and secretion, which may confound analysis. To avoid this and other potential sources of bias, the episomal library in pLAN042 was transformed into *S. mutans* SM126 ($\Delta lanAA'$) to generate an allelic replacement library (*lanA* variants integrated on the chromosome in single copy). Recognizing the inherent limitations of high-throughput bioassays and the deferred antagonism assay, a more traditional MIC approach was also used. This method provided normalized and specific activity testing on select purified lantibiotics (>90% purity by HPLC) which were isolated from the chromosomal library. It was noted that the data derived from this method provided more consistency between replicates, and between wells, and hence served as a more reliable assay to determine the specific effect of a given amino acid substitution on antimicrobial activity.

Despite the inherent limitations discussed above, the episomal high-throughput method allowed triage of hundreds of variants and detection with a high degree of confidence those with no, or significantly less activity, as compared

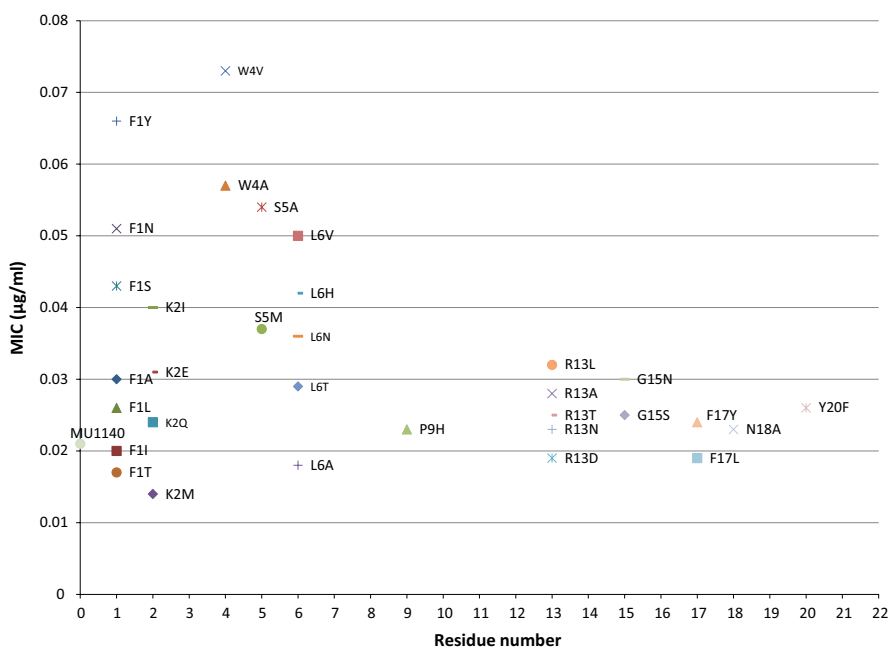
to the naturally occurring parent molecule compound (see Figure 2) and derive useful manufacturing and structure–activity relationship information. Specifically, 73 variants of 418 (17%) showed similar or improved antimicrobial activity compared to MU1140 against *M. luteus*. Due to the different platform used to assess activity, it remains challenging to directly compare the normalized data presented here with previously published data from others due to differences in MIC determination methodology. That the great majority (75%) of compounds in this study were within a dilution of MU1140 (MIC between 0.01 and 0.04 $\mu\text{g}/\text{ml}$) and that only six compounds had a lower MIC than MU1140 (on *M. luteus*, see Figure 3) suggest that single amino acid substitutions may not be sufficient to design variants with markedly increased potency. This remains to be confirmed across several other bacterial species.

4.2 | Structure–activity relationships and permissive substitutions

4.2.1 | N-terminus substitutions

It was found that the first two *N*-terminal amino acids (Phe1 and Lys2) were very permissive to substitution, with a few exceptions. For example, substitutions of Phe1 did not permit acidic (negatively charged) substitution with Asp, although the Lys2Glu substitution was found to be permissive (see Figure 2). Why Lys2Glu is permissive and Lys2Asp is not remains unclear. On the one hand, it may be related to the fundamental difference between Asp and Glu; there is an extra $-\text{CH}_2-$ in Glu, which may provide sufficient distance between the negative charge of Glu and the Lipid-II binding domain in Ring A/B not to interfere

FIGURE 3 Normalized Minimum Inhibitory Concentration (MIC) of Selected MU1140 Variants. The compounds were chosen based on the high-throughput screening and normalized activity testing to cull down the saturated library to a smaller manageable subset of mutations that confer (a) structural diversity, (b) different chemical properties, and/or (c) improved therapeutic profile. Substitution follows standard amino acid nomenclature, and the normalization procedure is described in the Section 2



with Lipid-II interaction. On the other hand, the proximity of mutations in positions 1 and 2 relative to the Ring A, or the nature of the native residues (Phe1 and Lys2), may prove more important than previously appreciated. This observation remains to be verified by determining the solution structure of the MU1140 and lipid-II complex.^[21] The binding interactions between nisin and the pyrophosphate moiety of lipid-II have been well characterized.^[21] The amide (NH) of residues 2–5 and 8 from the peptide backbone form hydrogen bonds with the oxygen from the pyrophosphate.^[21,23] Because of the structural and sequence similarities, it has been suggested that MU1140 would bind in a similar manner to the pyrophosphate of Lipid-II.^[4]

While the permissiveness of the first two *N*-terminal residues cannot entirely be attributed to the acidic nature of the amino acids at these positions, the permissive characteristics of those amino acids are critical from a drug design standpoint as they may be particularly susceptible to degradation by exoproteases. New evidence suggests that the metabolic stability of MU1140 variants developed for the treatment of *C. difficile* enteritis correlates with their intrinsic susceptibility to proteolytic degradation in the GI tract (pepsin, trypsin, and chymotrypsin). A correlation was observed between the stability of MU1140 variants *in vitro* (in biologically relevant fluids) and their efficacy *in vivo*.^[37,38] We have recently reported that variants of MU1140 that contain *N*-terminal amino acids that are “stabilizing” according to the *N*-end rule, which dictates the half-life of proteins intracellularly,^[37,39,40] were also fortuitously characterized by a greater metabolic stability *in vitro* and a greater efficacy *in vivo*. In contrast, those variants that contained “destabilizing” amino acids at their *N*-terminus were characterized by a poor metabolic stability *in vitro* and a lower efficacy *in vivo*.^[37] To our knowledge, there is no satisfactory explanation or hypothesis to explain why there is such a high degree of correlation between *in vitro* half-life in biologically relevant simulated fluids and half-life as determined by the *N*-end rule and it cannot be completely ruled out that this may be a serendipitous finding.

4.2.2 | Lanthionine bridges substitutions

Only two (Ala3 and Ala7) of the nine amino acids constituting Ring A/B were not permissive to substitutions. In contrast, five of the seven amino acids constituting Ring C/D were not permissive, including every position involved in lanthionine formation. As presented in Figure 2, the data confirm previously published reports that substitutions in lanthionine bridges in positions Ala3 and Ala7 (Ring A), Ala16 and Ala21 (Ring C), and Ala19 and AviCys22 (Ring D) invariably lead to inactive variants.^[41] One explanation for this finding may be that those essential amino acids are involved in maintaining the correct conformation of active MU1140

variants, leading to efficient binding to Lipid-II via Ring A/B, and proper self-assembly via Rings C/D.^[4] Another explanation is that the formation of Ring D may be necessary for transport.^[41] Therefore, substitutions in the A and C/D Rings are much less permissive to mutations and conserved within this class of compounds.^[2]

Unexpectedly, permissive substitutions in Abu8 and Ala11 (Ring B) were observed, albeit infrequently. Earlier literature suggests that Ring B is involved in binding to and abduction of Lipid-II, and as such would be expected to be non-permissive to mutation. There is some degree of discrepancy between the current report and some of the previously published data. Specifically, a Cys11Ala substitution led to a 65% decreased in activity as assessed by a deferred antagonism assay^[41] while our high-throughput screening did not detect any effect on activity for this substitution. In addition, a Cys21Ala substitution led to a compound with a 35% reduction in activity^[41] while our screening detected a total loss of activity for this substitution. It needs to be pointed out that there was a methodological difference between published work and the work resented here. The discrepancy observed between this report and other previously published reports may be a reflection of the different methodologies used. The mechanism whereby Glu or Ala may be able to replace Cys11 (in precursor) in Ring B function remains unclear and is under investigation.

In this study, the Abu8Ser variant retained biological activity, as judged by non-normalized activity testing, suggesting that small modification, (*e.g.*, the deletion of a methyl group) can in fact be tolerated without substantial loss in antimicrobial activity. This observation was somewhat unexpected considering that Abu8 (MeLan) is conserved in the Ring B of several structurally related lantibiotics including nisin, subtilin, epidermin, and gallidermin.^[2]

Altogether, these observations support the concept that the lipid-II binding domain (Rings A/B) of MU1140 may tolerate more sequence heterogeneity than previously noticed for other members of the epidermin family,^[3,21,42] without negatively impacting lipid-II abduction. Experiments are ongoing to quantify the association and dissociation constants of various homologs of MU1140 to purified lipid-II, which may further substantiate the specific role of key residues on binding to lipid-II.

4.2.3 | Non-lanthionine rings A/B substitutions

It was observed that Trp4 was permissive to most hydrophobic amino acids but was not permissive for most amino acids with polar functional groups (like amides and hydroxyl groups). This observation is consistent with a previous report where a Trp4Ala maintained bioactivity.^[27] It is unknown why a high degree of hydrophobicity in position 4 may be important for

activity. It may well be that hydrophobicity is necessary to strengthen interactions with the hydrophobic core of the lipid bilayer membrane once lipid-II has been bound. This hypothesis would only be plausible if the molecule inserts into the membrane as does nisin, which contradicts the prevailing model of membrane-bound MU1140, which suggested that a monomer cannot span the entire length of a lipid bilayer.^[20] In this model, the type II β turn structure found in ring B is positioned at the surface of the membrane. This allows to position of the charged amino terminus, the Lys2 side chain, and the Arg13 side chain into a common plane that could associate with the negative charge headgroups and anchor the molecule to the surface of the membrane. In that model, the polar side chain of Arg13 is submerged into the bilayer and is easily accessible for complex formation and the Trp4 side chain is positioned away from the rest of the molecule, suggesting that it may be involved in oligomerization.^[20]

Serine in position 5 is dehydrated to Dha during posttranslational modification and is one of the moieties commonly found in numerous lantibiotics (e.g., subtilin and nisin have a Dha5, but epidermin and gallidermin do not (reviewed in^[2]). The conservation of this amino acid in several compounds long suggested that it may confer an important functional role in MU1140 and nisin A and subtilin. However, we and others have previously reported that Dha5 in MU1140 and nisin A can be substituted by several other amino acids including Phe, Ala, Val, Lys, Gly, and Leu without significantly affecting activity.^[27,42] Dha5 in Mutacin 1140 can be substituted with Gly, Thr or Glu without affecting transport, while only a Dha5Gly substitution appears to retain full activity as measured by a deferred antagonism assay.^[28] Consistent with those reports, the present study identified several additional permissive mutations of Ser5, which included Ser5His, Ser5Met, Ser5Gln, and Ser5Leu (Figure 2). It is speculated here that although the Dha5 amine group may be forming a hydrogen bond with lipid-II, the unsaturated Dha5 side chain group may be facing away from the pyrophosphate of lipid-II, which, in turn, may allow some degree of functional group modifications. There is some discrepancy between the current report and some of the previously published data on Ser5Ala substitutions. Specifically, we found that this substitution leads to an active compound in our normalized MIC assay while Chen et al. previously reported that this substitution leads to a compound with a greater activity (vs wild-type MU1140), as assessed by a deferred antagonism assay.^[27,28] It is hypothesized that the apparent discrepancy observed may also be a reflection of the different methodologies used. Altogether, the data above suggest that the unsaturated bond from Dha5 may not be critically involved in Lipid-II binding but still important for conferring optimal activity.

Substitution of Gly10 was only permissive for Ile and Ser. Hydrophobic and relatively smaller amino acids (e.g., Ala and Val) would have been expected to be permissive if steric

hindrance was the primary mechanism involved in the substitution. Our observation suggests a more complex structure–activity relationship and remains under further investigation.

The rationale for the conservation of the lantionine rings among several lipid-II binding lantibiotics has been described in some detail. A “cage structure” has been proposed by Hsu and colleagues where the pyrophosphate moiety of lipid II is presumably coordinated by the *N*-terminal backbone on nisin via intermolecular hydrogen bonds and has been shown to be able to accommodate a variety of side chain compositions.^[21] Because of the striking sequence similarity between member of the epidermin family^[2,28] and because lipid-II binding appears to be a realistic common killing mechanism, it is not unreasonable to propose that a similar structure–function model would exist between the lipid-II binding domain of nisin and mutacin 1140.^[25] However, it is becoming evident that the choice of lipid-II content of artificial membranes may affect the results and the conclusions drawn from of lipid-II studies.^[43] Further, it is important to bare in mind that while both nisin and mutacin 1140 present a core lipid-II domain that only differs by 1 residue, the rest of the molecule is very different. In fact, both gallidermin and epidermin are more homologous to mutacin 1140 than nisin is, despite presenting 2 and 3 substitutions in the core lipid-II binding site, respectively.^[2,28] It has been proposed that the binding mode of lantibiotics to lipid-II incorporated in a biological membrane may differ from those obtained in structural modeling studies based on the isolated molecule in solution, in artificial bilayers or in unilamellar vesicles^[21,43] and that the membrane surface surrounding lipid-II may be contributing to the binding.^[43] If this hypothesis holds, then it is not unreasonable to propose that the structure of the mutacin/lipid-II complex in biological membranes might differ from the canonical nisin model, and consequently tolerate more sequence heterogeneity than previously noticed for nisin. This explanation may serve as the basis to hypothesize why certain of the permissive residues found in this study apparently contradicts the current model for nisin/lipid-II interactions.

4.2.4 | Hinge region substitutions (residues 12–15)

Position 12–15 is designated the hinge region and is a relatively flexible linear region that connects Rings A/B to Rings C/D, resulting in the characteristic U-shape of MU1140.^[20] Ala12 and Gly15 were not particularly permissive and only tolerated two and four substitutions, respectively. There appeared to be a trend toward smaller amino acids, and Gly15 seemed to only tolerate substitutions with residues that are polar, uncharged, or hydrophobic.

Arg13 is promiscuous to substitutions in the hinge region. Interestingly, no other positively charged amino acid substitutions were tolerated. This is surprising as one would expect



that the overall charge of this molecule would be a critical parameter to maintain optimal cell surface interaction and Lipid-II binding and consequently that a deletion of a positively charged residue or the substitution with a negatively charged residue would be detrimental to biological activity.

Arg13 is a particularly important residue to consider during drug design as this amino acid can be cleaved by enzymes of the digestive tract (e.g., trypsin and chymotrypsin). Furthermore, MU1140, nisin, Pep5, and Epidermin are known to be sensitive to proteolytic degradation.^[14,43,44] The work presented here confirms that Arg13 offers several opportunities for productive substitutions that may be less prone to proteolytic degradation. Furthermore, we have recently demonstrated that single and multiple amino acid substitutions of MU1140 that substitute Arg13 with residues that are less sensitive to trypsin or chymotrypsin invariably lead to more stable compounds in biologically relevant simulated fluids, and to more efficacious antibiotics in a hamster models of *Clostridium difficile* infection.^[37,38]

The Thr in position 14 is naturally dehydrated to a Dhb during posttranslational modifications.^[2] Interestingly, no permissive substitutions were identified for Dhb14 in the present study, which suggests that this residue may be critical for antimicrobial activity. To our knowledge, this observation has not been previously reported and is in contradiction with the recent report of Geng and Smith^[28] who reported that Dhb14Gly and Dhb14Ala variants are not affected in their transport across the membrane, and of a similar bioactivity, as compared to MU1140. Again, the different methodological approaches used may explain some of the observed discrepancies.

4.2.5 | Non-lanthionine rings C/D substitutions

It has previously been reported that Rings C/D of MU1140 are involved in self-assembly of MU1140 and provide a critical structure-function for potency.^[4] All acidic substitutions (negatively charged), amidated, polar, or hydroxyl amino acids substitutions were consistently non-permissive in C/D Rings. Phe17 substitutions only included positively charged or hydrophobic substitutions, while Tyr20 could only be substituted by hydrophobic Phe or Met. Recently, substitution in Ring D with an amidated Cys22 has been reported, in which a carboxyl analog of MU1140 (MU1140-COOH) leads to loss of antimicrobial activity, while the substitution with a methyl amidated MU1140 (Mu1140-NHMe) restores biological activity.^[19]

One notable residue for substitution or modification is Asn18. Others have previously reported that Asn side chains mediate the association of transmembrane peptides into homo- or heterooligomers,^[45] and an Asn residue is found at position 18 in the lantibiotics epidermin, gallidermin,

staphylococcin T, mutacin b-NY266, and mutacin. As MU1140 is too small to form a functional transmembrane pore, it has been suggested that its oligomerization is essential for function and that a substitution in Asn18 would result in loss of function.^[20] While that position was not permissive in the high-throughput screening assay (see Figure 2), further characterization using a normalized assay supported that Asn18Ala has a similar potency to MU1140 (see Figure 3).

4.3 | Posttranslational dehydration of Thr

During production and purification, LC/MS confirmed the identity of all compounds under investigation. While a great majority of constructions proved correct, the product of an unexpected posttranslational dehydration was observed with the identification of a Leu6Dhb (Ring A) instead of the intended Leu6Thr, which suggested that Leu6Thr was further dehydrated to Dhb during posttranslational modifications. A total of nine variants were reconstructed with substitutions with Ser or Thr, which would be expected to be permissive to further posttranslational modifications. It is interesting to note that Leu6Thr was the only variant that was further dehydrated to Leu6Dhb. Others have previously reported that lantibiotics can be hydrolyzed but to our knowledge, the dehydration of a Ser or Thr at different positions than those occurring naturally has not been previously reported for MU1140^[27] and will need to be confirmed by NMR analysis.

5 | CONCLUSIONS

The structure–activity relationship of MU1140 and the “drugability” of several variants were investigated in this study using a saturated expression library and characterized with a normalized MIC dataset. In contrast to previous reports, most but not all residues comprised in lanthionine bridges in MU1140 appeared to be critical to maintain biological activity. Rings A and C/D did not tolerate substitutions of lanthionines while Ring B did, in a few instances. Dhb14 and the AviCys22 precursor were essential to preserve function, while Dha5 was not. These data provide further insight on the structure–function roles of several critical amino acids to assure proper binding to Lipid-II and efficient self-assembly of MU1140.

While seminal work on lanthipeptides established the potential of this novel class of molecules to serve as useful antimicrobial therapeutics (reviewed in^[43]), only a few compounds have actually been tested in clinical trials and none have been approved for human use. As new approaches are being developed to address manufacturing (fermentation and purification) issues shared among those molecules and formulation challenges (i.e., stability and solubility issues), a framework for the drug development of MU1140-related

compounds is emerging. While mutacin 1140 is active via a novel mechanism of action,^[24] presents limited potential for resistance development,^[18] and shows an excellent safety profile,^[17] it is also becoming apparent that naturally occurring mutacin 1140 has several limitations that hinder its further advancement as a drug candidate and ultimately its “drugability”. The blueprints developed in the current report enabled the conceptualization of compounds with improved properties, without negatively impacting their potency. The framework that is emerging predicts that no single amino acid substitutions will be sufficient by itself to produce variants with all of the improved characteristics needed for a “drugable” lantibiotic, including: (a) a longer half-life, (b) a greater potency, (c) limited sensitivity to proteolytic degradation, (d) greater solubility, and (e) greater stability.^[37,38] However, it is also becoming clear that variants with markedly improved PK/PD properties can be engineered, but have to combine several substitutions to present all of the desired properties necessary to justify later stages of clinical development.^[38]

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CONFLICT OF INTEREST

The authors (JHP and MH) declare they were employees and stock-holders of Oragenics, a for-profit organization, during data collection, analysis, and writing of the manuscript. JC is an employee of Intrexon, a for-profit organization. All other co-authors were employees of Oragenics (AWD) or Intrexon Corporation (JAK, RES, SM, MM, and YZ) during data collection and initial analysis of the results.

AUTHOR CONTRIBUTIONS

JAK conceptualized, designed, and executed experiments. RES designed methods. SM developed analytical methods. MM developed extraction methods. JC designed DNA vectors and constructs. Yihui Zhu performed fermentation

process optimization. AWD contributed to design and management. JHP analyzed data and drafted the manuscript. MH conceptualized, designed the experiments, supervised the study, analyzed data, and prepared the manuscript.

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REFERENCES

- [1] P. G. Arnison, M. J. Bibb, G. Bierbaum, A. A. Bowers, T. S. Bugni, G. Bulaj, J. A. Camarero, D. J. Campopiano, G. L. Challis, J. Clardy, P. D. Cotter, D. J. Craik, M. Dawson, E. Dittmann, S. Donadio, P. C. Dorrestein, K. D. Entian, M. A. Fischbach, J. S. Garavelli, U. Göransson, C. W. Gruber, D. H. Haft, T. K. Hemscheidt, C. Hertweck, C. Hill, A. R. Horswill, M. Jaspars, W. L. Kelly, J. P. Klinman, O. P. Kuipers, A. J. Link, W. Liu, M. A. Marahiel, D. A. Mitchell, G. N. Moll, B. S. Moore, R. Müller, S. K. Nair, I. F. Nes, G. E. Norris, B. M. Olivera, H. Onaka, M. L. Patchett, J. Piel, M. J. Reaney, S. Rebuffat, R. P. Ross, H. G. Sahl, E. W. Schmidt, M. E. Selsted, K. Severinov, B. Shen, K. Sivonen, L. Smith, T. Stein, R. D. Süßmuth, J. R. Tagg, G. L. Tang, A. W. Truman, J. C. Vederas, C. T. Walsh, J. D. Walton, S. C. Wenzel, J. M. Willey, W. A. van der Donk, *Nat. Prod. Rep.* **2013**, *30*(1), 108.
- [2] C. Chatterjee, M. Paul, L. Xie, W. A. van der Donk, *Chem. Rev.* **2005**, *105*, 633.
- [3] A. C. Ross, J. C. Vederas, *J Antibiot (Tokyo)*. **2011**, *64*(1), 27.
- [4] L. Smith, J. Hillman, *Curr. Opin. Microbiol.* **2008**, *11*, 401.
- [5] D. Field, P. D. Cotter, R. P. Ross, C. Hill, *Bioengineered* **2015**, *6*, 187.
- [6] F. Götz, S. Perconti, P. Popella, R. Werner, M. Schlag, *Int. J. Med. Microbiol.* **2014**, *304*(1), 63.
- [7] T. J. Oman, W. A. van der Donk, *ACS Chem. Biol.* **2009**, *4*, 865.
- [8] C. Piper, P. G. Casey, C. Hill, P. D. Cotter, R. P. Ross, *Int J Microbiol.* **2012**, *2012*, 806230.
- [9] S. Suda, P. D. Cotter, C. Hill, R. P. Ross, *Curr. Protein Pept. Sci.* **2012**, *13*, 193.
- [10] E. L. Ongey, H. Yassi, S. Pflugmacher, P. Neubauer, *Biotechnol. Lett.* **2017**, *39*, 473.
- [11] S. K. Sandiford, *Expert Opin. Drug Discov.* **2015**, *10*, 315.
- [12] S. Boakes, W. J. Weiss, M. Vinson, S. Wadman, M. J. Dawson, *J Antibiot (Tokyo)*. **2016**, *69*, 850.
- [13] H. Grasemann, F. Stehling, H. Brunar, R. Widmann, T. W. Laliberte, L. Molina, G. Döring, F. Ratjen, *Chest* **2007**, *131*, 1461.
- [14] J. D. Hillman, K. P. Johnson, B. I. Yaphe, *Infect. Immun.* **1984**, *44*(1), 141.
- [15] J. D. Hillman, J. Novák, E. Sagura, J. A. Gutierrez, T. A. Brooks, P. J. Crowley, M. Hess, A. Azizi, K. Leung, D. Cvitkovitch, A. S. Bleiweis, *Infect. Immun.* **1998**, *66*, 2743.
- [16] H. G. Sahl, R. W. Jack, G. Bierbaum, *Eur. J. Biochem.* **1995**, *230*, 827.
- [17] O. Ghobrial, H. Derendorf, J. D. Hillman, *J. Pharm. Sci.* **2010**, *99*, 2521.
- [18] O. G. Ghobrial, H. Derendorf, J. D. Hillman, *Int. J. Antimicrob. Agents* **2009**, *33*(1), 70.

- [19] J. Escano, P. Deng, S. E. Lu, L. Smith, *Genome Announc.* **2016**, 4, e00472-16.
- [20] L. Smith, C. Zachariah, R. Thirumoorthy, J. Rocca, J. Novák, J. D. Hillman, A. S. Edison, *Biochemistry* **2003**, 42, 10372.
- [21] S. T. Hsu, E. Breukink, E. Tischenko, M. A. Lutters, B. de Kruijff, R. Kaptein, A. M. Bonvin, N. A. van Nuland, *Nat. Struct. Mol. Biol.* **2004**, 11, 963.
- [22] H. Brötz, M. Josten, I. Wiedemann, U. Schneider, F. Götz, G. Bierbaum, H. G. Sahl, *Mol. Microbiol.* **1998**, 30, 317.
- [23] B. De Kruijff, V. van Dam, E. Breukink, *Prostaglandins Leukot. Essent. Fatty Acids* **2008**, 79, 117.
- [24] H. E. Hasper, N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff, E. Breukink, *Science* **2006**, 313, 1636.
- [25] L. Smith, H. Hasper, E. Breukink, J. Novak, J. Cerkasov, J. D. Hillman, S. Wilson-Stanford, R. S. Orugunty, *Biochemistry* **2008**, 47, 3308.
- [26] D. Hughes, *Nat. Rev. Genet.* **2003**, 4, 432.
- [27] S. Chen, S. Wilson-Stanford, W. Cromwell, J. D. Hillman, A. Guerrero, C. A. Allen, J. A. Sorg, L. Smith, *Appl. Environ. Microbiol.* **2013**, 79, 4015.
- [28] M. Geng, L. Smith, *Appl. Environ. Microbiol.* **2018**, 84, e00830-18.
- [29] P. J. Knerr, W. A. van der Donk, *Annu. Rev. Biochem.* **2012**, 81, 479.
- [30] F. L. Macrina, R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, K. R. Jones, *Gene* **1983**, 25(1), 145.
- [31] I. Biswas, L. Drake, S. Johnson, D. Thielen, *Biotechniques* **2007**, 42, 487.
- [32] Raman M, Martin K. *Nat. Methods* **11** (2014). Published online 28 August 2014.
- [33] L. Snyder, W. Champness, J. Peters, T. M. Henkin, T. Tina Henken, *Molecular Genetics of Bacteria*. 4th ed., ASM Press, London, UK **2013**.
- [34] J. D. Hillman, K. P. Johnson, B. I. Yaphe, *Infect. Immun.* **1984**, 44(1), 141.
- [35] D. Paul, H. D. Slade, *Infect. Immun.* **1975**, 12, 1375.
- [36] P. Fredericq, *Annu. Rev. Microbiol.* **1957**, 11, 7.
- [37] J. A. Kers, R. E. Sharp, A. W. DeFusco, J. H. Park, J. Xu, M. E. Pulse, W. J. Weiss, M. Handfield, *Front. Microbiol.* **2018**, 9, 415.
- [38] J. Kers, A. W. Defusco, J. H. Park, J. Xu, M. E. Pulse, W. J. Weiss, M. Handfield, *PLoS ONE* **2018**, 13, e0197467.
- [39] A. Bachmair, D. Finley, A. Varshavsky, *Science* **1986**, 234, 179.
- [40] A. Varshavsky, *Proc. Natl Acad. Sci. USA* **1996**, 93, 12142.
- [41] J. Escano, B. Stauffer, J. Brennan, M. Bullock, L. Smith, *J. Bacteriol.* **2015**, 197, 1173.
- [42] R. Rink, J. Wierenga, A. Kuipers, L. D. Kluskens, A. J. Driessen, O. P. Kuipers, G. N. Moll, *Appl. Environ. Microbiol.* **2007**, 73, 5809.
- [43] A. Chugunov, D. Pyrkova, D. Nolde, A. Polyansky, V. Pentkovsky, R. Efremov, *Sci. Rep.* **2013**, 3, 1678.
- [44] D. Field, P. D. Cotter, C. Hill, R. P. Ross, *Front. Microbiol.* **2015**, 6, 1363.
- [45] F. X. Zhou, H. J. Merianos, A. T. Brunger, D. M. Engelman, *Proc Natl Acad Sci U S A.* **2001**, 98, 2250.

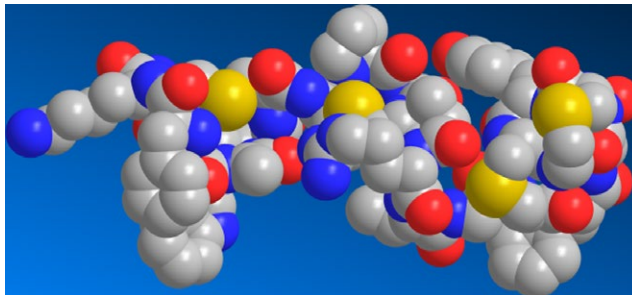
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Graphical Abstract

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Lantibiotics represent a large untapped pipeline of attractive scaffolds for the development of novel antibiotics. Saturation mutagenesis was employed to substitute every amino acid of the core peptide of a lantibiotic called mutacin 1140, creating an unbiased expression library of 418 variants that was used to study the structure–activity relationship of key residues. Blueprints are proposed for directing further development of therapeutic lantibiotics, which enable the rational design, development, manufacture, and formulation of an entirely new class of anti-infectives.