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Ring A of Nukacin ISK-1: A Lipid II-Binding Motif for Type-A(II) Lantibiotic

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Supporting Information

ABSTRACT: Ring A of nukacin ISK-1, which is also present in different type-A(II) lantibiotics, resembles a lipid II-binding motif (TxS/TxD/EC, x denotes undefined residues) similar to that present in mersacidin (type-B lantibiotics), which suggests that nukacin ISK-1 binds to lipid II as a docking molecule. Results from our experiments on peptidoglycan precursor (UDP-MurNAc-pp) accumulation and peptide antagonism assays clearly indicated that nukacin ISK-1 inhibits cell-wall biosynthesis, accumulating lipid II precursor inside the cell, and the peptide activity can be repressed by lipid I and lipid II. Interaction analysis of nukacin ISK-1 and different ring A variants with lipid II revealed that nukacin ISK-1 and nukacin D13E (a more active variant) have a high affinity (K_D = 0.17 and 0.19 μM, respectively) for lipid II, whereas nukacin D13A (a less active variant) showed a lower affinity, and nukacin C14S (a negative variant lacking the ring A structure) exhibited no interaction. Therefore, on the basis of the structural similarity and positional significance of the amino acids in this region, we concluded that nukacin ISK-1 binds lipid II via its ring A region and may lead to the inhibition of cell-wall biosynthesis.

Lantibiotics are ribosomally synthesized peptide antibiotics that contain unusual amino acids, lanthionine, 3-methyllanthionine, and dehydroamino acids.[1] These peptides have traditionally been subdivided into two major groups: type-A and type-B lantibiotics, which comprise peptides with straight-chain and globular structures.[2] Type-A lantibiotics are further divided into two subtypes, namely, type-A(I) and type-A(II), based on their biosynthetic enzymes. In addition, a third class, two-component lantibiotics, is now gaining recognition, wherein two structurally different peptides act synergistically to kill the target bacteria. Nukacin ISK-1 is a type-A(II) lantibiotic with 27 amino acids, including four unusual residues, and is produced by *Staphylococcus warneri* ISK-1.[3]

The modes of action of different lantibiotics have been extensively studied for a long time. The prototype lantibiotic nisin from the type-A(I) group targets the membrane-bound cell-wall precursor lipid II, resulting in the inhibition of peptidoglycan biosynthesis[4] and pore formation.[5] It has also been shown that the pores formed by nisin in the membranes containing lipid II are much more stable than those formed in the absence of the receptor.[6] The binding motif of nisin to lipid II involves only the first 10 N-terminal amino acids, containing the lanthionine rings A and B, termed the "pyrophosphate cage". A similar binding motif was also observed for other type-A(I) lantibiotics such as subtilin, epidermin,[7] and gallidermin.[7] Another lipid II-binding motif for mersacidin and other lantibiotics has been described. The ability of mersacidin to specifically bind lipid II is well documented.[8] Mersacidin and similar type-B lantibiotics possess a conserved sequence (CTLT/SHEC, underlined residues are the ones modified during their maturation) comprising residues 12−18, suggesting that these residues may form the core lipid II-binding site.[9] A sequence identical to that of mersacidin is found in plantaricin C,[10] LtnA1 of lacticin 3147,[11] Halα of haloduracin,[12] and Lchα of lichenicidin,[13] which are predicted to be involved in lipid II binding.

The mode of action of type-A(II) lantibiotics, comprising a large number of peptide members, is still unknown. Specifically, their interaction with lipid II has not been investigated, though they possess TxS/TxD/EC (x denotes undefined residues) sequence in their ring A structure, which suggests that they can bind lipid II as a docking molecule (Figure 1; Figure S1).

Here we focused on a potential lipid II-binding motif in nukacin ISK-1, with particular emphasis on the similar ring A structure, by using different variants in this region. We investigated their interaction with different peptidoglycan precursors.

The positional significance of the individual residues in the nukacin ISK-1 ring A region has been previously investigated using site saturation systematic mutagenesis (NNK scanning), and different ring A variants have been generated.[14] In this study, we purified the ring A variants, namely, H12A, D13A, D13E, and C14S, and determined their minimum inhibitory concentrations (MICs) against different indicator strains (Table 1). As anticipated, H12A and D13A variants showed

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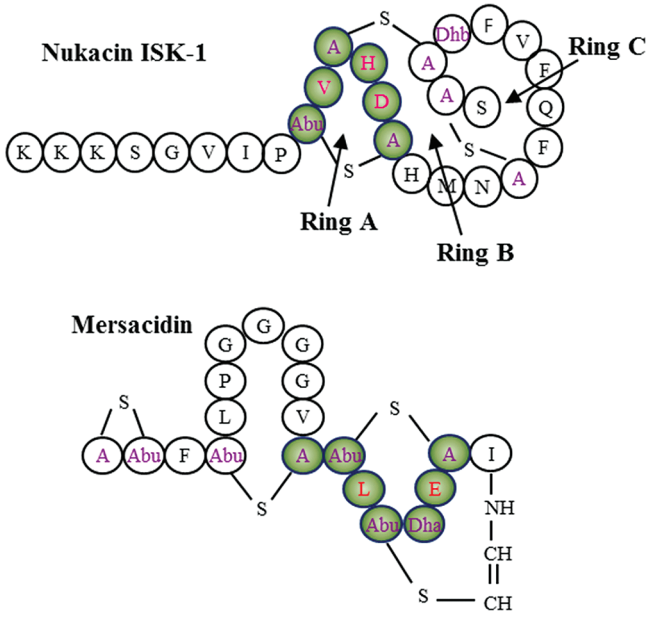

Figure 1. Primary structure of nukacin ISK-1 and mersacidin. The lipid II-binding motif of mersacidin and similar amino acids in nukacin ISK-1 is highlighted in green shad. Different rings of nukacin ISK-1 are indicated by arrows. Unusual amino acids are as follows: Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, 3-methyllanthionine.

Table 1. Specific Activities (MIC, μM)[a] of Nukacin ISK-1 Ring A Variants

	peptide[c]				
indicator strain[b]	ISK-1	H12A	D13A	D13E	C14S
Lb. sakei	0.62	80.0	80.0	0.12	>120.0
L. lactis	10.00	>80.0	>80.0	0.23	>120.0
B. coagulans	1.25	80.0	>80.0	0.31	>120.0
L. lactis HP	2.50	>80.0	>80.0	0.62	>120.0
E. faecalis	20.00	>80.0	>80.0	5.00	>120.0
S. simulans 22	5.00	>80.0	>80.0	0.62	>120.0

[a]Specific activity was determined twice for each peptide and the average value is shown. [b]*Lactobacillus sakei* JCM 1157^T, *Lactococcus lactis* NZ9000, *Bacillus coagulans* JCM 2257^T, *Lactococcus lactis* HP, *Enterococcus faecalis* JCM 5803^T, *Staphylococcus simulans* 22. [c]ISK-1, nukacin ISK-1; H12A, nukacin H12A; D13A, nukacin D13A; D13E, nukacin D13E; C14S, nukacin C14S.

significantly reduced antimicrobial activity, while the ring-disrupted C14S variant was found completely inactive at the concentrations used. Replacement of H12 and D13 with Ala might obstruct in ring A formation, as Ala is not preferable at the N-terminal of the ring closing C14 residue.[15] The D13E variant showed markedly higher activity (4-fold to 40-fold) than the wild type, as assessed by observing the inhibition of the growth of the indicator in the liquid medium by using different peptide concentrations, as described by Wiedemann et al.[11] This result indicates that the ring A structure of nukacin ISK-1 is critically important for maintaining the structural integrity and effectiveness of this peptide. This result also correlates with the LtnA1 of lacticin 3147[16] and mersacidin,[17] where a negatively charged Asp/Glu residue in this region was found to be essential.

The mechanism underlying the antimicrobial activity of nukacin ISK-1 has not been extensively studied. Recently, Asaduzzaman et al. showed that nukacin ISK-1 significantly reduced cell-wall thickness, and the treated cells did not show complete septum formation in *Bacillus subtilis*.[18] These data suggest that nukacin ISK-1 might inhibit cell-wall biosynthesis by targeting the cell-wall components. Antibiotics such as vancomycin or bacitracin, which interfere with the membrane-bound stages of peptidoglycan synthesis, trigger the accumulation of the final soluble peptidoglycan precursor, UDP-*N*-acetylmuramyl pentapeptide (UDP-MurNAc-pp), in the cytoplasm (Figure S2).[19] Therefore, in this study, we determined the cytoplasmic levels of the lipid II precursor, UDP-MurNAc-pp, in *Staphylococcus aureus* SG511 by using nukacin ISK-1 and 2 nukacin D13 variants (D13E and D13A). Both nukacin ISK-1- and nukacin D13E-treated cells showed substantial precursor accumulation, similar to vancomycin (Figure 2). The less active variant, namely, nukacin D13A,

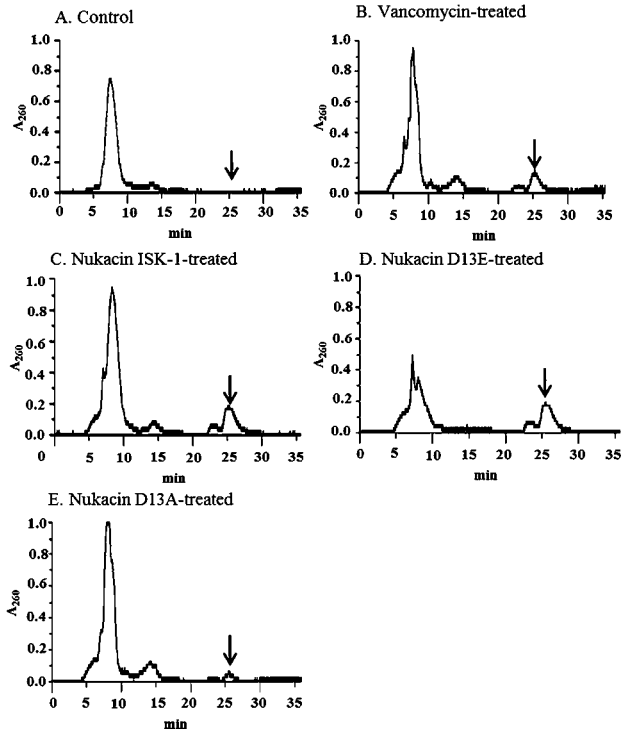

Figure 2. Intracellular accumulation of the soluble cell-wall precursor UDP-*N*-acetylmuramyl pentapeptide (UDP-MurNAc-pp) in *Staphylococcus aureus* SG511. Cells were treated with 10 × MIC of the respective antibiotic compound, incubated for 30 min, and subsequently extracted with boiling water. Nukacin D13A was added at 30 × MIC of nukacin ISK-1. The intracellular nucleotide pool was analyzed by applying standardized aliquots to reverse-phase HPLC. UDP-MurNAc-pp was eluted at 25−26 min and is indicated by the arrow. (A) Control cells, untreated, (B) vancomycin-treated, (C) nukacin ISK-1-treated, (D) nukacin D13E-treated, and (E) nukacin D13A-treated cells.

did not lead to significant accumulation of the precursor. This result suggests that nukacin ISK-1 and nukacin D13E might interfere with one of the later membrane-associated or extracellular processes of peptidoglycan biosynthesis (Figure S2).

To investigate the possibility of nukacin ISK-1 interacting with lipid II, an antagonization assay was carried out with nukacin ISK-1 and the highly potent variant nukacin D13E in the presence of different peptidoglycan precursors (C_{55}-P, lipid

I, and lipid II). Nisin and mersacidin, which are known to tightly bind lipid II, were used as positive controls. Thus, strong inhibition of nukacin ISK-1 and nukacin D13E activities was observed in the presence of lipid I and lipid II, and the growth of the indicator strain was similar to that observed in the positive control (Table 2). This experiment presents the first indication that nukacin ISK-1 could specifically bind to lipid I and lipid II.

Table 2. Antagonization of Lantibiotic Activity by Peptidoglycan Precursors

	growth with antagonists[a]		
lantibiotic	C_{55}-P	lipid I	lipid II
nisin Z	−	+	+
mersacidin	−	+	+
nukacin ISK-1	−	+	+
nukacin D13E	−	+	+

[a]*Lactococcus lactis* HP was incubated with 4 × MIC of the corresponding peptides. Antagonists were added in 8-fold higher molar concentration of individual peptide MIC. Growth with precursors: +, lantibiotic activity antagonized; −, lantibiotic activity unaffected.

Direct interaction of nukacin ISK-1 and different ring A variants with lipid II was analyzed by isothermal titration calorimetry (VP-ITC). To investigate whether lipid II has an impact on the ITC traces of nukacin ISK-1 as well as its variants, we loaded 150 μM lipid II solutions in the syringe that was titrated into the peptide solutions in the reaction cell. As indicated in the titration profiles of nukacin ISK-1 (3.4 μM) and nukacin D13E (3.4 μM), the exothermic heat flow decreased with an increase in the number of injections, and free peptide concentration in the reaction cell decreased simultaneously (Figure 3A,B). The binding appeared to be very strong for both peptides, as observed in the ITC chromatograms. For nukacin ISK-1 and nukacin D13E, the saturation of heats was achieved after 14 and 10 injections, respectively (Figure 3A,B). The early saturation of heats for nukacin D13E might be because of a stronger affinity for lipid II, although the binding constant did not significantly differ. The apparent dissociation constants (K_D) for nukacin ISK-1 and nukacin D13E for lipid II were estimated to be 0.17 and 0.19 μM, respectively. The binding affinity of nukacin D13A at 3.4 μM for lipid II was significantly lower (Figure 3C), whereas at a higher concentration (7.1 μM) of the peptide, the K_D was measured to be 3.6 μM (data not shown). These results suggest that the decreased potency of nukacin D13A could be because of a decreased affinity for lipid II. Nukacin C14S (5.6 μM) did not show any interaction with lipid II (Figure 3D). The affinity of nukacin ISK-1 for lipid II is similar to that for other lipid II-binding lantibiotic peptides, like nisin,[20] bovicin HC5,[21] and clausin.[22] The thermodynamic parameters of the interaction between lipid II and different peptides are provided in Table S1. The binding of nukacin ISK-1 and its variants (nukacin D13E and nukacin D13A at 7.1 μM) to lipid II is predominantly enthalpy-driven, as observed from the parameters. In summary, ITC data clearly indicate that nukacin ISK-1 plausibly interacts with lipid II, and the interaction is mainly facilitated by the ring A structure where Asp13 plays a critical role.

In conclusion, the results presented here show that nukacin ISK-1 acts on the cell wall by initially interacting with lipid II as the docking molecule, and then inhibits cell-wall biosynthesis, which might lead to the cessation of cell growth. This is the first report discussing the interaction of type-A(II) lantibiotics with lipid II, and it presents the first evidence that type-A(II) lantibiotics possess the lipid II-binding motif in their ring A structure. Therefore, our present findings will contribute to our understanding of the mode of action of this group of lantibiotics and their application to lantibiotic bioengineering.

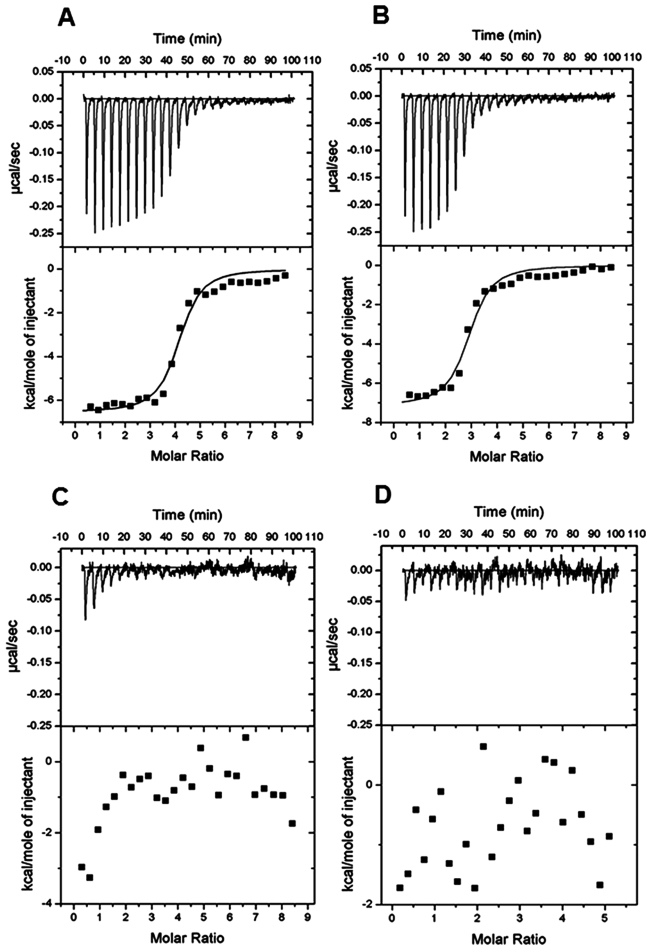

Figure 3. Calorimetric titration of nukacin ISK-1 and different ring A variants with lipid II. The titration cell was loaded with peptides, and 25 injections of 10 μL of lipid II (150 μM) were administered at 240-s intervals. The temperature was adjusted to 25 °C. The top graph shows the heat peaks after each injection. The bottom graph displays the integrated heat per injection, normalized to the injected amount of moles of lipid II, which is displayed against the molar ratio of lipid II versus the different peptides used. The corresponding chromatograms are (A) nukacin ISK-1 (3.4 μM), (B) nukacin D13E (3.4 μM), (C) nukacin D13A (3.4 μM), and (D) nukacin C14S (5.6 μM). The results were analyzed using the Origin software ver. 7 provided by MicroCal Inc.

■ ASSOCIATED CONTENT

Supporting Information

Detail experimental procedures, Figures S1 and S2, and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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Supporting online material for

Ring A of nukacin ISK-1: a lipid II-binding motif for type-A(II) lantibiotic

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This PDF file includes:

Supporting online material

Experimental procedures:

Bacterial strains and growth condition:

Lactococcus lactis was grown in M17 medium supplemented with 0.5% glucose (GM17) at 30°C. *Lactobacillus sakei* subsp. *sakei* JCM 1157^T and *Enterococcus faecalis* JCM 5803^T were grown in MRS medium at 30°C. *Bacillus coagulans* JCM 2257^T was cultured in Luria-Bertani (LB) broth at 37°C. *Staphylococcus aureus* SG511 and *Staphylococcus simulans* 22 were grown in half-concentrated Mueller-Hinton (M-H) broth (Oxoid, Basingstoke, United Kingdom) at 37ºC. *Micrococcus luteus* DSM1790 and the nukacin ISK-1-producer strain *Staphylococcus warneri* ISK-1 were grown in trypticase soy broth (TSB) medium (Difco Laboratories, MI, USA) at 37°C.

Purification of lantibiotics:

Nukacin ISK-1 and derivative peptides were purified from the respective culture supernatants as described by Aso *et al.*[1] The nukacin ISK-1-producer strain *S. warneri* ISK-1 was grown in TSB for the purification of nukacin ISK-1, and nukacin derivatives were purified from *L. lactis* NZ9000 recombinant strains constructed in a previous study by Islam *et al.*[2] Commercial nisin A (Sigma-Aldrich, MO, USA) was purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Nisin Z was purified from the culture supernatant of *L. lactis* NIZO22186 as described previously.[3]

Purification of peptidoglycan precursor:

Synthesis and purification were performed according to the protocol described by Brötz *et al*.[4] with modifications by Schneider T. *et al.*[5]. Briefly, lipid II was synthesized *in vitro* using membrane preparations of *M. luteus* DSM1790. The membranes were isolated from lysozyme-treated cells by centrifugation (35,000 *g*), washed twice in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5, and stored at -80^{0}C until use. Substrate UDP-*N*-acetylmuramic acid pentapeptide (UDP-MurNAc-pp) was purified from *S. simulans* 22 as previously described by Kohlrausch and Höltje.[6] An analytical assay was performed to optimize the suitable conditions by using different

volumes of the membrane fraction and substrate purified as mentioned above. A total volume of 50 µL containing different volumes of the membrane protein, 5 nmol of C_{55}-P, different volumes of UDP-MurNAc-pp, 10 mM of UDP-GlcNAc in 1 M Tris-HCl, 1 M $MgCl_2$, pH 8, and 0.5% (w/v) Triton X-100 was used. For purifying higher quantities of lipid II, the analytical procedure was scaled up by a factor of 200. The reaction mixtures were incubated for 2 h at 30°C, and the lipids were extracted with the same volume of *n*-butanol/6 M pyridine-acetate, pH 4.2. Lipid I was synthesized by the methods described for lipid II with the omission of UDP-GlcNAc from the synthesis reaction. Lipid I/lipid II was purified using Sephadex G-25 column (HiTrap Desalting; GE Healthcare, Uppsala, Sweden) and eluted in a linear gradient from chloroform: methanol: water (2:3:1) to chloroform: methanol: ammonium bicarbonate (300 mM) (2:3:1). Lipid I- and lipid II-containing fractions were identified by TLC (silica gel 60 A, Whatman) using chloroform: methanol: water: ammonia (88:48:10:1) as the solvent.[7] Spots were visualized by PMA staining reagent. The concentration of purified lipids was determined as the amount of inorganic phosphates present after treatment with perchloric acid.[8]

MIC and antagonization tests:

MIC values were determined in microtiter plates as described by Wiedemann *et al.*[9] Respective indicator strains were grown in the appropriate culture broth. Serial 2-fold dilutions of the peptides were prepared in the appropriate growth medium with 0.1% Tween 80. Bacteria were added to a final inoculum of 10^5 CFU/ml in a volume of 0.2 mL. The microtiter plates were incubated at the temperature appropriate for each indicator strain. The MIC was considered as the lowest peptide concentration causing inhibition of visible growth; determinations were carried out at least twice.

For the antagonization test, cell wall precursors were mixed with 4×MIC of lantibiotics at a molar ratio of 2:1 (precursor-lantibiotic). After overnight incubation at 30°C, the antagonization potency was determined by observing the growth of *L. lactis* HP and comparing it to the control.

Intracellular accumulation of UDP-MurNAc-pp:

For the analysis of the cytoplasmic nucleotide pool, we followed the method of Sass *et al.*[10] In short *S. aureus* SG511 was grown in M-H broth to an OD_{600} of 0.5 and supplemented with 130 µg/mL of chloramphenicol. Chloramphenicol is necessary to prevent the induction of autolytic

processes as well as the *de novo* synthesis of the enzymes hydrolyzing the nucleotide-activated sugars interfering with the determination of the soluble precursor. After 15 min, peptides were added at 10×MIC, and the mixture was incubated for 30 min. Cells were harvested and extracted with boiling water. The suspension was then centrifuged (48,000×g, 30 min), and the supernatant was lyophilized. Nucleotide-linked cell wall precursors were analyzed by HPLC.

ITC measurements of peptide binding:

Isothermal titration calorimetry (ITC) experiments were carried out with a VP-ITC from MicroCal (Northampton, MA, USA). The device was electrically calibrated. Purified lipid II was solubilized in 10 mM Tris-Cl and 150 mM NaCl (pH 7.5) and subjected to repeated extrusion for 15 times through 0.1 µm polycarbonate filters. Peptides were also dissolved in the same buffer after complete lyophilization of HPLC purified samples. Prior to use, all the samples were degassed and equilibrated to the appropriate temperature before measurement. We selected 25°C as the experimental temperature. The reference power was set to 10 µal/s by using a syringe-stirring speed of 300 rpm. Titrations were performed by 25 injections of 10 µL each of lipid II solution (150 µM) into the sample cell containing 1.443 mL of the peptide at concentrations varying from 3.4 µM to 7.1 µM. The change in the heat rate during the titration steps was registered in real time. Raw data were processed using the Origin® 7 software provided with the instrument. In control experiments, the corresponding peptide solution (or lipid II solution) was injected into the buffer without lipid II (or without peptide). Heats of dilution were significantly lower and were subtracted from the actual measurements. Under the assumption of the one-site binding model with the legend in the sample cell, the equilibrium binding constant (K_D), and the thermodynamic parameters of enthalpy (ΔH), free energy binding (ΔG), and entropy change ($T\Delta S$) were calculated.

Supporting figure legends:

Figure S1:

Alignment of the unmodified propeptide sequence of different lantibiotics possessing mersacidin like lipid II-binding motif. Ring A of type-A(II) and additional lantibiotics showing similar sequence to lipid II-binding of mersacindin are shaded in gray. Underlined residues are the ones

modified during their maturation. A consensus sequence is drawn, in which ‘x' denotes undefined residues.

Figure S2:

Reactions in membrane-associated cell wall biosynthesis in *Staphylococcus aureus*. UDP-MurNAc-pp is synthesized on the cytoplasmic side and translocates to the inner face of the cytoplasmic membrane. MraY, MurG, and FemXAB enzymes catalyze the reactions to produce the membrane-bound precursor molecule lipid II with a pentaglycine chain. Lipid II translocates across the membrane by an unknown mechanism, and PBP converts lipid II into peptidoglycan. When this cycle is inhibited by an antibiotic at any stage, the soluble precursor UDP-MurNAc-pp accumulates in the cytoplasm.

Table S1: Thermodynamic parameters of peptides and lipid II interaction as determined by Origin® 7 software

Peptide/ Parameters[a]	$C_p°$ (µM)	$C_L°$ (µM)	ΔH (kcal/mol)	-TΔS (kcal/mol)	ΔG (kcal/mol)
Nukacin ISK-1	3.4	150	-6.6	2.6	-9.2
Nukacin D13E	3.4	150	-7.1	2.1	-9.2
Nukacin D13A	3.4	150	-	-	-
Nukacin D13A	7.1	150	-18.1	-10.7	-8.6
Nukacin C14S	5.6	150	-	-	-

[a] $C_p°$, initial peptide concentration; $C_L°$, initial lipid II concentration; ΔH, enthalpy change; -TΔS, entropy change; ΔG, free energy change

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Figure S1: Sequence alignment of unmodified propeptide of different lantibiotics containing mersacidin like lipid II-binding motif.

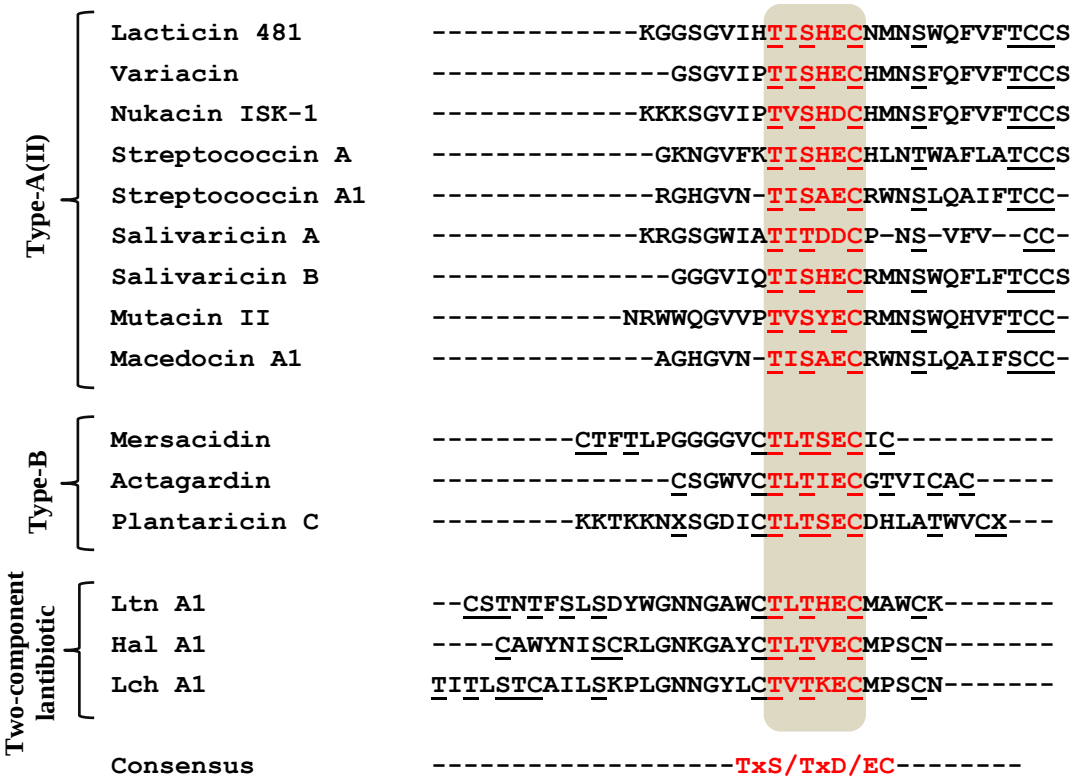

Figure S2: Reactions in membrane-associated cell wall biosynthesis in *Staphylococcus aureus*