Supporting Information

NMR structure of the S-linked glycopeptide Sublancin 168

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Ramachandran Plot

Glycine residues are shown as triangles all other residues as squares

Plot Statistics

89.3% Residues in most favored regions (Regions A, B, L shown in **RED**)

0.0% Residues in additional allowed regions (Regions a, b, l, p shown in **YELLOW**)

10.7% Residues in generously allowed regions (Regions ~a, ~b, ~l, ~p shown in **LIGHT YELLOW**)

0.0% Residues in disallowed regions

Figure S1. Ramachandran plot. The quality of the NMR structure composed of 15 conformers was evaluated using PROCHECK.

Comparison with other loop structures

Figure S2. Comparison of the interhelical loop of sublancin 168 with a similarly structured loop in MutM. A sequence alignment of the two loops is also shown.

Backbone and Sugar Flexibility for Sublancin

Figure S3. Three-dimensional solution structure of sublancin 168. Ribbon diagram of the final ensemble of 15 conformers. Helices colored in cyan, the loop regions, and N- and C-termini are colored pink, the sugar is colored in orange, and cysteine residues in beige.

a)

b)

Figure S4. Sublancin backbone ribbon diagram of the 15 lowest energy conformers calculated without hydrogen bond restraints. The figure also displays predicted hydrogen bonds (cyan) obtained using the Chimera software package.

Figure S5. Sublancin surface model representation. A small hydrophobic cleft is formed by Gly1, Gln6, Leu10, Ile20, Gly21, Gly23, Val27, Ala28, Asn31 and Phe35 might be a site for interaction with a target.

Figure S6. Sublancin surface model representation. The sulfur atoms of Cys7, Cys29, and Cys36, involved in disulfide bridges, are partially solvent exposed. Exposed sulfur atoms are shown in yellow.

Table S1. Sublancin Chemical Shift Assignments

Residue	CA	CB	HN	N	HA	HB	HG	HD	HE	Other
Gly 1	45.204		8.334	108.203	3.931, 3.868					
Leu 2	55.001	43.955	8.344	121.139	4.416	1.636	1.514	0.714		
Gly 3	44.497		8.344	108.32	4.200, 4.028					
Lys 4	54.781	41.252	8.494	119.456	3.988	1.89	1.585, 1.474	1.722, 1.29	3.03	HZ 7.385
Ala 5	55.360	18.172	8.748	121.755	4.264	1.461				
Gln 6	59.309	28.519	8.148	119.671	4.217	2.29, 2.012	2.555, 2.456			
Cys 7	60.891	40.744	8.623	119.391	4.636	3.173				
Ala 8	55.588	17.749	8.498	124.035	4.404	1.602				
Ala 9	55.352	18.019	7.724	120.921	4.277	1.583				
Leu 10	57.752	43.619	8.184	119.961	4.244	2.395, 2.02	1.85	1.126, 1.041		
Trp 11	60.555	28.132	8.788	120.212	3.701	3.697, 3.28		6.902	9.976, 6.27	HZ 7.352, NE 128.588
Leu 12	58.041	41.819	7.733	117.556	3.803	1.864, 1.685	1.849	0.959		
Gln 13	58.688	29.016	7.738	118.567	3.947	2.153, 1.99	2.517, 2.303		7.597, 6.943	NE 112.561
Cys 14	57.758	38.714	8.213	118.351	4.335	2.564				
Ala 15	54.049	17.783	8.173	121.693	3.766	0.717				
Ser 16	59.453	63.733	7.607	111.866	4.337	3.918				
Gly 17	45.606		7.841	109.738	3.997					
Gly 18	45.800		8.158	108.279	4.054, 3.944					
Thr 19	62.334	69.872	8.064	113.347	4.359	4.261	1.221			
Ile 20	61.909	38.328	8.061	122.333	4.145	1.883	1.512, 1.206	0.919		
Gly 21	45.302		8.423	112.495	4.061, 3.899					
Cys 22	61.077	33.832	8.284	120.376	4.673	3.274, 3.035				
Gly 23	45.985		8.656	112.782	4.075, 3.957					
Gly 24	45.478		8.371	108.819	3.986					
Gly 25	46.140		8.397	109.405	3.907					
Ala 26	54.815	18.82	8.064	123.138	4.161	1.478				
Val 27	65.836	31.908	8.102	119.932	3.775	2.059	1.07, 0.965			
Ala 28	55.751	18.36	8.032	122.939	4.249	1.517				
Cys 29	56.997	38.88	8.278	114.339	4.452	3.211, 3.082				
Gln 30	59.203	28.233	8.118	121.5	4.086	2.208	2.417		7.416, 6.851	NE 111.492
Asn 31	59.417	38.533	8.724	119.134	4.532	3.008, 2.874		7.417, 7.088		ND 111.448
Tyr 32	62.984	38.643	8.869	122.198	3.895	3.362, 3.203		7.27	6.886	
Arg 33	59.299	30.135	8.068	117.088	3.904	2.031, 1.956	1.707	3.301, 3.229		
Gln 34	58.242	29.327	7.705	115.563	3.947	1.688,	1.822		7.284,	NE

						1.299			6.766	110.644
Phe 35	58.275	41.424	7.993	113.257	4.986	3.339, 2.879				
Cys 36	55.826	43.065	8.095	117.360	4.857	2.622, 2.162				
Arg 37	57.35		7.524	125.329	4.186	1.893, 1.762	1.603, 1.531			
	H1, C1	H2, C2	H3, C3	H4, C4	H5, C5	H6, C6				
GLC	4.60, 88.3	3.31, 75.2	3.52, 80.2	3.43, 72.6	3.53, 82.8	3.93, 3.72, 64.0				

Mass spectrum of natural abundance sublancin

Figure S7. MALDI-TOF mass spectrum of purified natural abundance sublancin that was produced in parallel with the isotopic labeling preparations except using natural abundance culture growth medium. Expected [M+H]: 3876.74, observed: 3880.76.

Mass spectrum of ^{15}N -labeled sublancin

Figure S8. MALDI-TOF mass spectrum of purified, uniformly ^{15}N -labeled sublancin. Expected $[\text{M}+\text{H}]$: 3926.31, observed: 3926.89.

Mass spectrum of ^{13}C -labeled sublancin

Figure S9. MALDI-TOF mass spectrum of purified uniformly ^{13}C -labeled sublancin. Expected $[\text{M}+\text{H}]$: 4040.38, observed: 4038.96.

Mass spectrum of $^{13}\text{C}/^{15}\text{N}$ -labeled sublancin

Figure S10. MALDI-TOF mass spectrum of purified uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled sublancin. Expected $[M+H]^+$: 4090.38, observed: 4086.41.

Table S2 Hydrogen Bond Identification by Deuterium Exchange

Time	# Signals	HN signals observed
7 min	27	G3, Q6, C7, A8, A9, L10, W11, L12, Q13, C14, A15, S16, G17, G18, I20, A26, V27, A28, C29, Q30, N31, Y32, R33, Q34, F35, C36, R37
14 min	20	C7, A8, A9, L10, W11, L12, Q13, C14, A15, S16, A28, C29, Q30, N31, Y32, R33, Q34, F35, C36, R37
30 min	15	A9, L10, W11, L12, Q13, C14, A15, C29, Q30, N31, Y32, R33, Q34, F35, C36
60 min	12	A9, L10, W11, L12, Q13, C14, A15, Y32, R33, Q34, F35, C36
45 h	8	L10, W11, L12, Q13, C14, Y32, F35, C36

Production of isotopically labeled sublancin and NMR analysis

Four samples of sublancin were prepared for NMR analysis. For a sample in 100% D₂O, lyophilized sublancin was dissolved in 100% D₂O (Cambridge Isotope Laboratories) to exchange amide protons and simplify the NMR spectrum in the down field region. The sample was lyophilized, the procedure was repeated twice, and the final sample was dissolved in D₂O to a final concentration of approximately 2.5 mM. For a sample in 90% H₂O/10% D₂O, lyophilized sublancin was dissolved in 90% H₂O/10% D₂O to a final concentration of approximately 2.5 mM.

A ¹³C-labeled sublancin sample was obtained by growing *B. subtilis* 168 on M9 minimal medium containing uniformly ¹³C-labeled glucose. A culture of *B. subtilis* 168 was grown in LB medium for 15 h with aeration. This culture was used to inoculate a 500 mL volume of minimal media in a 2 L flask. The minimal media contained the following components per 500 mL total volume: 25 mL of 20% ¹³C-glucose, 100 mL of 5X M9 salts, 5 mL of 4 mg mL⁻¹ leucine, 500 µL of 20 mg mL⁻¹ CaCl₂, 500 µL of 120 mg mL⁻¹ MgSO₄, 100 µL of 5 mg mL⁻¹ D-biotin, 100 µL of 5 mg mL⁻¹ thiamine-HCl, 500 µL of 1000X heavy metal stock solution, and 368.3 mL of millipore water to bring the total volume to 500 mL. The 5X M9 salts solution consisted of 64 g Na₂HPO₄ 7H₂O, 15 g KH₂PO₄, 2.5 g NaCl, 5 g NH₄Cl, and 1 L millipore water. The 1000X heavy metal stock solution was prepared by combining the following components in 1 M HCl, stirring overnight at 25° C, and sterilizing by filtration to remove insoluble material.

Component	Amount
MoNa ₂ SO ₄ •2H ₂ O	500 mg
CoCl ₂	250 mg
CuSO ₄ •5H ₂ O	175 mg
MnSO ₄ •H ₂ O	1 g
MgSO ₄ •7H ₂ O	8.75 g
ZnSO ₄ •7H ₂ O	1.25 g
FeCl ₂ •4H ₂ O	1.25 g
CaCl ₂ •2H ₂ O	2.5 g
H ₃ BO ₃	1 g
1 M HCl	to 1 L

A ¹⁵N-labeled sublancin sample was obtained by growing *B. subtilis* 168 on M9 minimal medium containing glucose. A culture of *B. subtilis* 168 was grown in LB medium for 15 h with aeration. This culture was used to inoculate a 500 mL volume of minimal media in a 2 L flask. The minimal media contained the following components per 500 mL total volume: 25 mL of 20% glucose, 100 mL of 5X M9 salts, 5 mL of 4 mg mL⁻¹ leucine, 500 µL of 20 mg mL⁻¹ CaCl₂, 500 µL of 120 mg mL⁻¹ MgSO₄, 100 µL of 5 mg mL⁻¹ D-biotin, 100 µL of 5 mg mL⁻¹ thiamine-HCl, 500 µL of 1000X heavy metal stock solution, and 368.3 mL of millipore water to bring the total volume to 500 mL. The 5X M9 salts solution consisted of 64 g Na₂HPO₄ 7H₂O, 15 g KH₂PO₄, 2.5 g NaCl, 5 g ¹⁵NH₄Cl, and 1 L millipore water. The 1000X heavy metal stock solution was prepared as described above.

A $^{13}\text{C}/^{15}\text{N}$ -labeled sublancin sample was obtained by growing *B. subtilis* 168 on M9 minimal medium containing uniformly ^{13}C -labeled glucose and $^{15}\text{NH}_4\text{Cl}$. The minimal media contained the following components per 500 mL total volume: 25 mL of 20% ^{13}C -glucose, 100 mL of 5X M9 salts, 5 mL of 4 mg mL $^{-1}$ leucine, 500 μL of 20 mg mL $^{-1}$ CaCl_2 , 500 μL of 120 mg mL $^{-1}$ MgSO_4 , 100 μL of 5 mg mL $^{-1}$ D-biotin, 100 μL of 5 mg mL $^{-1}$ thiamine-HCl, 500 μL of 1000X heavy metal stock solution, and 368.3 mL of millipore water to bring the total volume to 500 mL. The 5X M9 salts solution consisted of 64 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 15 g KH_2PO_4 , 2.5 g NaCl, 5 g $^{15}\text{NH}_4\text{Cl}$, and 1 L millipore water. The 1000X heavy metal stock solution was prepared as described above.

A culture of *B. subtilis* 168 was grown in LB medium for 15 h with aeration. This culture was used to inoculate a 500 mL volume of minimal media in a 2 L flask and was grown at 37° C with aeration. The production of labeled sublancin was monitored by periodic sampling of the growing culture and analysis via MALDI-TOF mass spectrometry. Briefly, a 1 mL aliquot of *B. subtilis* 168 minimal media culture was acidified to pH 2 with concentrated phosphoric acid (85% in water). The sample was centrifuged to remove cells and the supernatant was ZipTip $_{\text{C18}}$ desalted, and analyzed by mass spectrometry. Typically, isolation and purification process was started 24 h after the first detection of labeled product. Labeled sublancin was generally observed within 48-72 h post-inoculation. Isotopically labeled sublancin was purified using identical conditions as described above for natural abundance sample (acidification, cell harvest, ammonium sulfate precipitation, and preparative HPLC). A natural abundance sublancin sample was prepared during the preparation of the labeled peptides as a control.