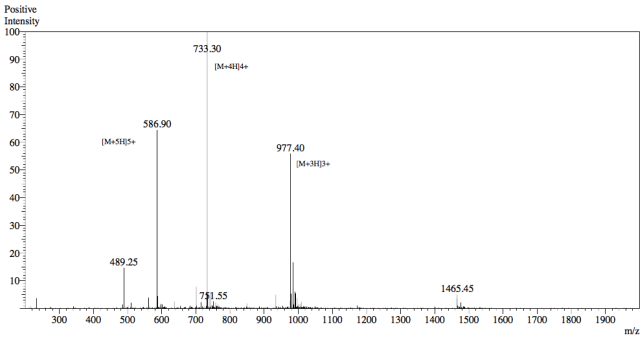
Fig S1

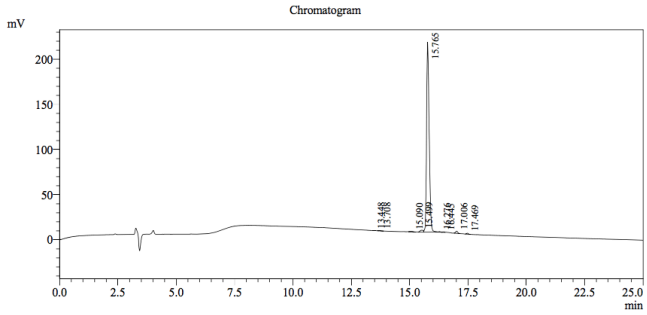
A.

Syn-safencin (library parent)
AGKETIRQYLKNEIKKKGRKAVIAW

Sample Name : As48C3-1
Sample ID : U9981BF140-351
Theoretical MW : 2929.48
Observed MW : 2929.20

Test Items	Specifications	Results
Molecular Weight	Theoretical MW: 2929.48	Consistent
HPLC purity	≥95.0%	97.1%
Appearance	White lyophilized powder	Conforms
Quantity	1-4 mg	4.9mg



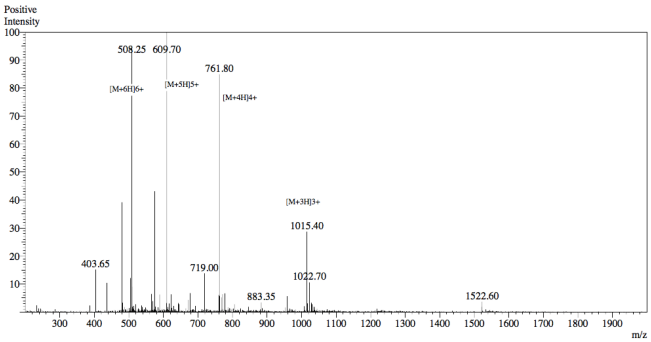


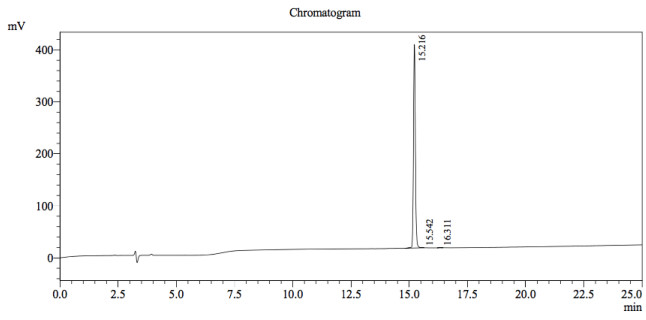
B.

Syn-safencin_11
WGKETIRQYLKNKIKKKGRKAVIAW

Sample Name : As48C3-11
Sample ID : U9981BF140-361
Theoretical MW : 3043.67
Observed MW : 3043.50

Test Items	Specifications	Results
Molecular Weight	Theoretical MW: 3043.67	Consistent
HPLC purity	≥95.0%	99.6%
Appearance	White lyophilized powder	Conforms
Quantity	1-4 mg	4.2mg



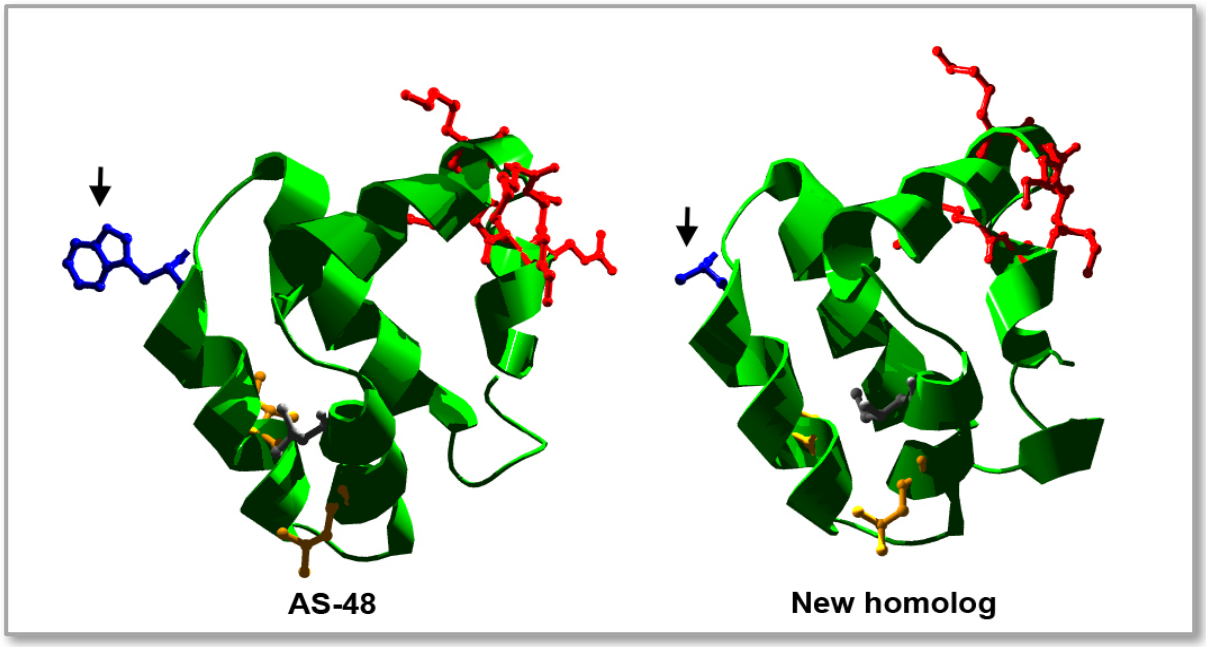


Supplementary Figure 1: Example mass spectrometry and HPLC verification of peptide purity and primary structure of **a.** syn-safencin library parent peptide and **b.** syn-safencin library peptide 11 provided by Genscript. Residues in red indicate differences between the peptides sequences.

Fig S2

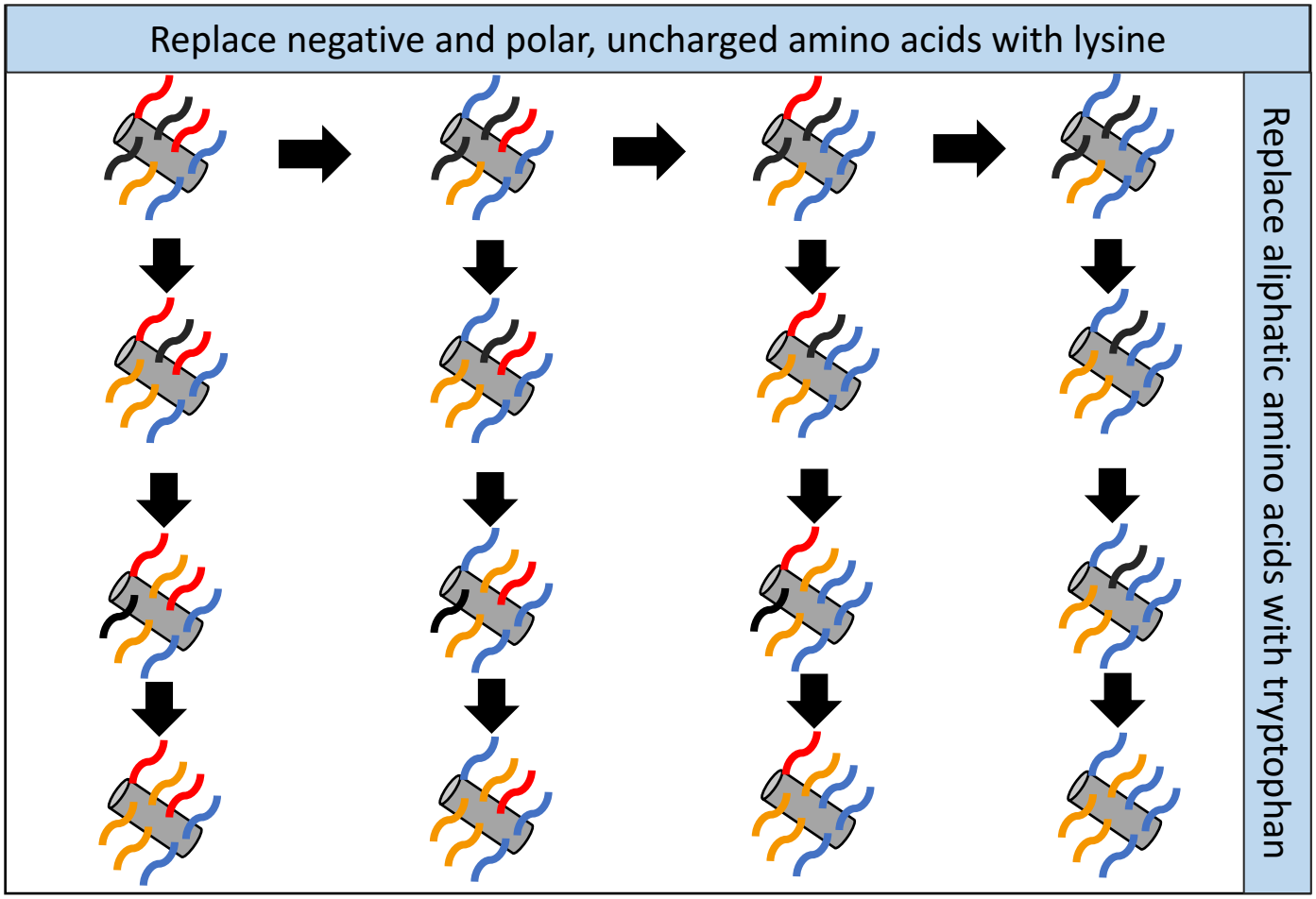
A.

B.



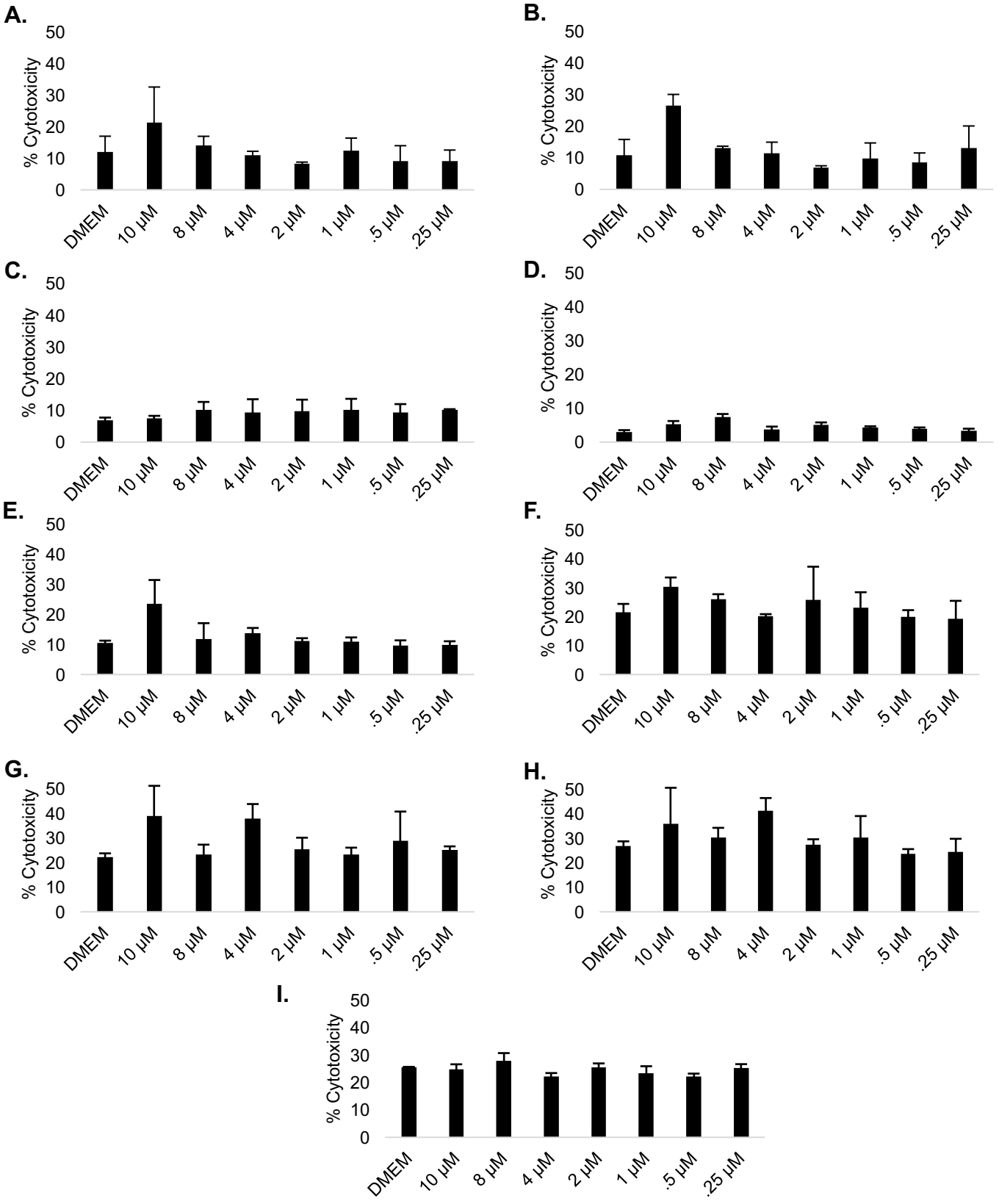
Supplementary Figure 2: Safencin AS-48 is a homologue of enterocin AS-48. **a.** Sequence alignment of AS-48 to safencin AS-48 (AS_48_hom). Boxes represent notable changes or conserved areas of hydrophobicity (blue, yellow, and gray box) or positive charge (red). **b.** Secondary structure comparison of AS-48 to safencin AS-48 (new homologue). Colored residues correspond the colored boxes in part **a**.

Fig S3



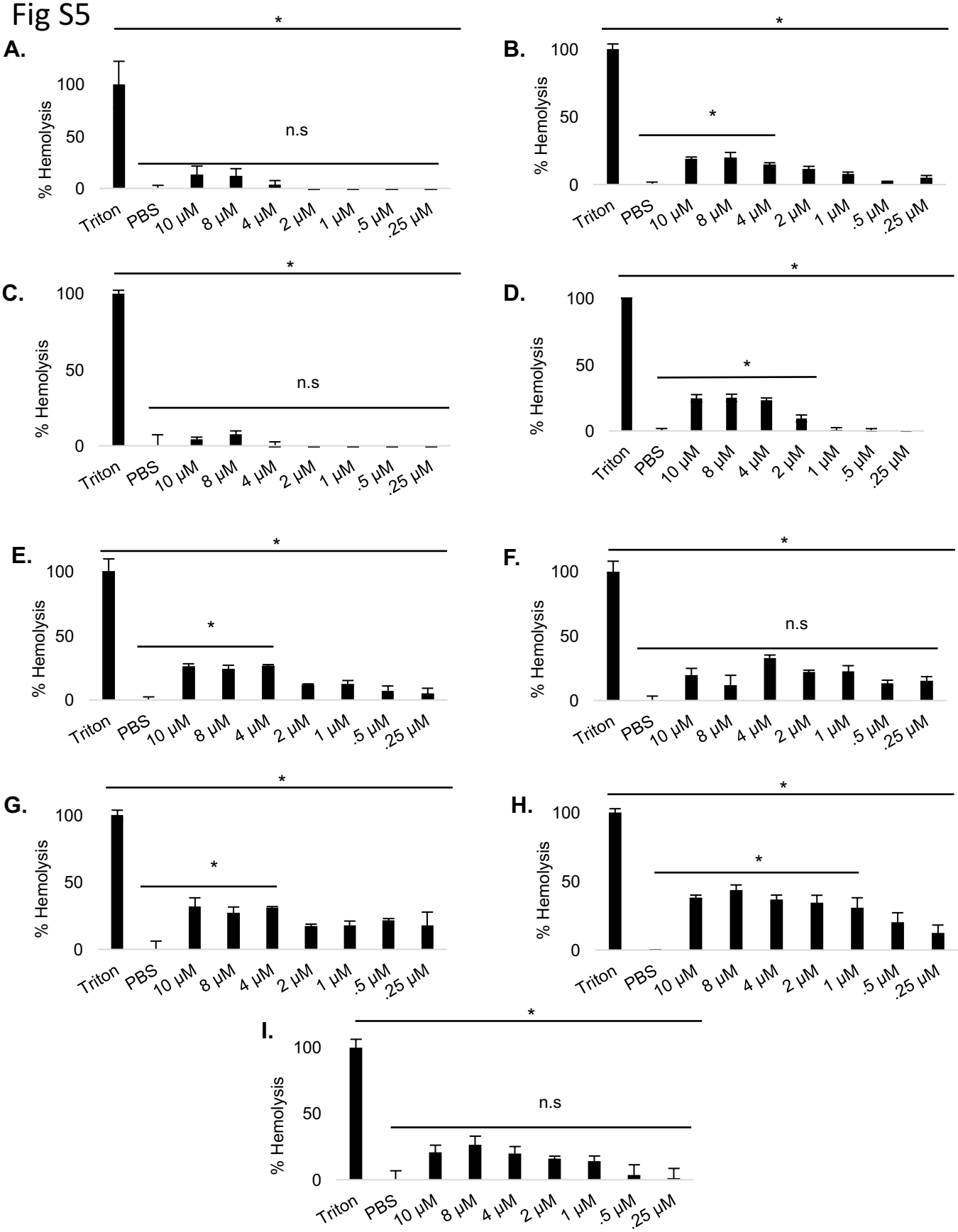
Supplementary Figure 3: Peptide library design strategy for the optimization of syn-safencin**.** For peptide library optimization, a 25 amino acid variant (top left) of syn-safencin was used as the template for the design of an optimized library consisting of 96 peptides. From this parent peptide, 7 additional peptides were created by replacing the anionic and polar, uncharged residues with lysine. These 8 (7 modified peptides and the parent peptide) were further modified by replacing the aliphatic residues with tryptophan to create an additional 88 peptides. Aliphatic residues are black, basic residues are blue, acidic residues are red, and hydrophobic/aromatic residues are in orange.

Fig S4



Supplemental Figure 4: Ethidium homodimer assays of library peptides on HaCat cells. **a.** Peptide 20 **b.** Peptide 52 **c.** Peptide 60 **d.** Peptide 90 **e.** Peptide 91 **f.** Peptide 92 **g.** Peptide 93 **h.** Peptide 94 **i.** Peptide 96. Data is representative of three technical replicates. Error bars represent standard deviation.

Fig S5



Supplemental Figure 5: Hemolysis assays of library peptides on sheep RBCs. **a.** Peptide 20 **b.** Peptide 52 **c.** Peptide 60 **d.** Peptide 90 **e.** Peptide 91 **f.** Peptide 92 **g.** Peptide 93 **h.** Peptide 94 **i.** Peptide 96. Data is representative of three biological replicates (n=3). Error bars represent the standard error of the mean. P-values were determined via one-way ANOVA and Tukey post hoc analysis. * indicates a $p < .05$. ns indicates a $p > .05$.

Table S1

Peptide Library Screen Results						
Peptide #	Gram (-)				Gram (+)	
	E. coli	*P. aeruginosa*	*X. axonopodis*	*P. syringae*	*S. pyogenes*	*S. aureus*
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	+	-	-	-
4	-	-	+	+	-	-
5	-	-	+	+	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	+	-	-	-
9	-	-	+	-	-	-
10	-	-	+	-	-	-
11	-	-	+	+	-	-
12	-	-	+	+	-	-
13	-	-	+	+	-	-
14	-	-	+	-	-	-
15	-	-	+	-	-	-
16	-	-	+	+	-	-
17	-	-	+	-	-	-
18	+	-	+	+	-	-
19	-	-	+	+	-	-
20	+	+	+	+	+	-
21	-	-	+	+	-	-
22	+	-	+	+	-	-
23	-	-	+	-	-	-
24	-	-	+	+	-	-
25	-	-	-	-	-	-
26	-	-	+	-	-	-
27	-	-	+	-	-	-
28	-	-	+	+	-	-
29	-	-	+	-	-	-
30	-	-	+	-	-	-
31	-	-	+	-	-	-
32	-	-	+	+	-	-
33	-	-	-	-	-	-
34	-	-	+	-	-	-
35	-	-	+	-	-	-
36	-	-	+	+	-	-
37	-	-	+	-	-	-
38	-	-	+	-	-	-
39	-	-	-	-	-	-
40	-	-	+	-	-	-
41	-	-	+	-	-	-
42	-	-	+	+	-	-
43	-	-	+	+	-	-
44	-	-	+	+	-	-
45	-	-	+	-	-	-
46	-	-	+	-	-	-
47	-	-	+	-	-	-
48	-	-	+	-	-	-
49	-	-	+	+	-	-
50	-	-	+	+	-	-
51	-	-	+	+	-	-
52	+	+	+	+	-	-
53	-	-	+	+	-	-
54	-	-	+	+	-	-
55	-	-	+	+	-	-
56	-	-	+	+	-	-
57	-	-	+	+	-	-
58	-	-	+	+	-	-
59	-	-	+	+	-	-
60	+	-	+	+	+	-
61	-	-	+	+	-	-
62	-	-	+	-	-	-
63	-	-	+	-	-	-
64	-	-	+	+	-	-
65	-	-	+	-	-	-
66	-	-	+	+	-	-
67	-	-	+	+	-	-
68	-	-	+	+	-	-
69	-	-	+	-	-	-
70	-	-	+	-	-	-
71	-	-	+	-	-	-
72	-	-	+	+	-	-
73	-	-	+	-	-	-
74	-	-	+	+	-	-
75	-	-	+	-	-	-
76	-	-	+	+	-	-
77	-	-	+	-	-	-
78	-	-	+	-	-	-
79	-	-	+	+	-	-
80	-	-	+	+	-	-
81	-	-	+	-	-	-
82	-	-	+	+	-	-
83	-	-	+	+	-	-
84	+	-	+	+	-	-
85	-	-	+	-	-	-
86	-	-	+	-	-	-
87	-	-	+	-	-	-
88	-	-	+	+	-	-
89	+	-	+	+	-	-
90	+	-	+	+	+	-
91	+	-	+	+	+	-
92	+	+	+	+	+	-
93	+	-	+	+	+	-
94	+	-	+	+	+	-
95	+	-	+	+	-	-
96	+	+	+	+	+	-

Supplementary Table 1: Peptide library screen results. A (+) indicates that the peptide inhibits growth of indicated bacterial strain. A (-) indicates that the peptide was unable to inhibit growth of tested bacterial strain. Growth was assessed at 16 hours for all organisms except *E. coli* which was assessed at 8 hours. All organisms were screened with 8 μM peptide except *X. axonopodis* which was screened with 4 μM peptide.