**SUPPLEMENTARY DATA FOR**

# Mechanism of bacterial membrane permeabilization of crotalicidin (Ctn) and its fragment Ctn[15-34], antimicrobial peptides from rattlesnake venom

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**FIGURE S1. Analytical reverse-phase HPLC chromatograms and ESI-MS spectra of peptides used in this study. a)** Analytical reverse-phase HPLC chromatograms before (dotted line) and after (continuous line) purification of synthetic peptides. Specific linear gradients of solvent B (0.036 % TFA in ACN) into A (0.045 % TFA in $H_2O$) used for each peptide are shown over each chromatogram. **b)** ESI-MS spectra of pure peptides.

**FIGURE S2. Bacterial cells viability and membrane permeabilization of *E. coli* upon treatment with RhB-Ctn and RhB-Ctn[15-34].** $5x10^5$ CFU/mL *E. coli* suspensions were incubated with increasing peptide concentrations of RhB-Ctn (red, top) or RhB-Ctn[15-34] (blue, bottom). Negative (live bacteria) and positive (dead bacteria) controls without peptide or incubated with 70 % (v/v) isopropanol are represented in black and grey, respectively. **a)** Histograms on the SYTOX® Green channel detected by flow cytometry. **b)** Histograms on the Rhodamine B channel detected by flow cytometry. **c)** Percentage of permeabilized bacteria (solid lines) and percentage of peptide uptake (discontinuous lines). Data correspond to mean ± SD of three independent experiments.

**FIGURE S3. Distribution of residuals over time.** The deviation of the experimental data on Figures 4c and S3c from the two-state model is represented as residuals plots. **a)** Residuals plots of bacteria permeabilization for unlabeled Ctn (red) and Ctn[15-34] (blue). **b)** Residuals plots of bacteria permeabilization for RhB-Ctn (red) and RhB-Ctn[15-34] (blue).

**FIGURE S4. Time-resolved bacterial membrane permeabilization and peptide uptake.** RhB-Ctn (red) or RhB-Ctn[15-34] (blue) at their MBC were added to $10^7$ CFU/mL *E. coli* suspensions. Changes on the SYTOX® Green and RhB gates were monitorized during 90 min immediately after the peptides addition. **a)** Flow cytometry correlograms at different time points in the presence of RhB-labeled peptides. $P_n$ and $U_n$ are the percentages of permeabilized bacteria and peptide uptake at each time interval (n) (seg). **b)** Flow cytometry dot plots for the negative (live bacteria) and positive (dead bacteria) controls. **c)** Percentages of permeabilized bacteria (dark colour) and percentages of peptide uptake (light colour). Percentages of permeabilized bacteria were fitted to the two-state model (1) (solid line) as described in the experimental section.

**FIGURE S5. Confocal point scanning microscopy images of *E. coli* upon treatment with Ctn and Ctn[15-34], as well as the RhB-labelled versions.** *E. coli* suspensions at $10^7$ FU /mL were incubated with peptides at their MBC. The fluorescence emission intensity of RhB (red signal) allows to detect the peptides distribution. Fluorescence emission intensity of SYTOX® Green (green signal) allows to detect bacterial membrane permeabilization. Transmitted light images are also shown.

**FIGURE S6. Effect of RhB-label in the membrane binding affinity of Ctn[15-34] as followed by SPR. a)** Dose response curves obtained with Ctn[15-34] or RhB-Ctn[15-34] over POPC or POPC/POPS (80:20) model membranes deposited onto L1 chip. **b)** Sensorgrams obtained with 64 μM of peptide injected over POPC or POPC/POPS (80:20).

**FIGURE S7. Time-dependent POPC/POPS (80:20) disruptive effect of Ctn[15-34] and RhB-Ctn[15-34].** Percentage of membrane leakage at 5, 10 and 15 min was determined by CF dequenching. LUVs composed with POPC/POPS (80:20) with total lipid concentration of 5 μM was incubated with various concentrations of peptide and measurements were acquired at specific time points.

**FIGURE S8. Time-resolved membrane permeabilization. a)** Linear calibration curve for the % of permeabilized bacteria, obtained using known percentages of live and dead bacteria. **b)** The kinetic curve for the untreated control demonstrate that bacteria remain alive during the whole acquisition and the changes observed on figure 4c are due to the peptide activity.

**MOVIE S1a.** Changes on the SYTOX® Green fluorescence intensity after Ctn addition.

**MOVIE S1b.** Changes on the SYTOX® Green fluorescence intensity after Ctn[15-34] addition.

**MOVIE S2a.** Changes on the SYTOX® Green and RhB fluorescence intensity after RhB-Ctn addition.

**MOVIE S2b.** Changes on the SYTOX® Green and RhB fluorescence intensity after RhB-Ctn[15-34] addition.

**FIGURE S1**

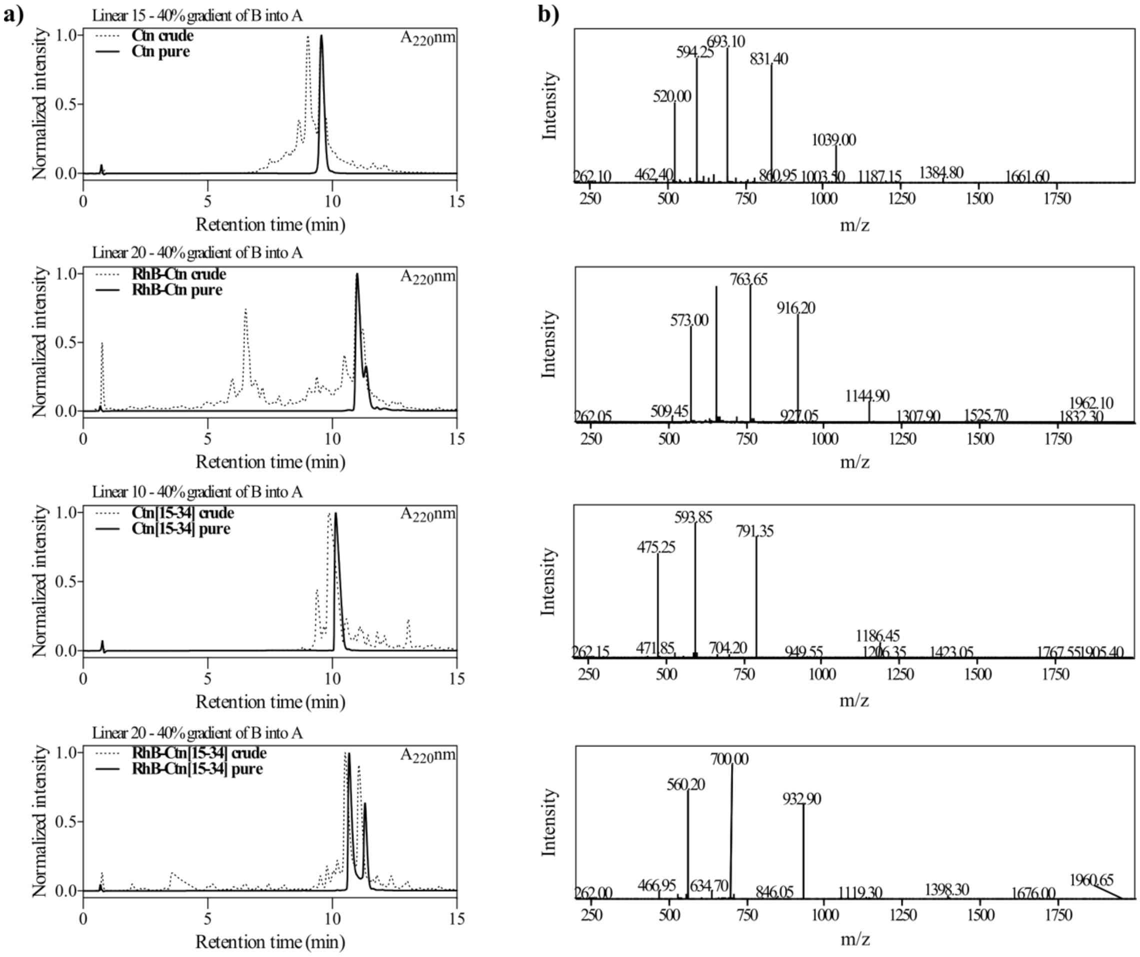
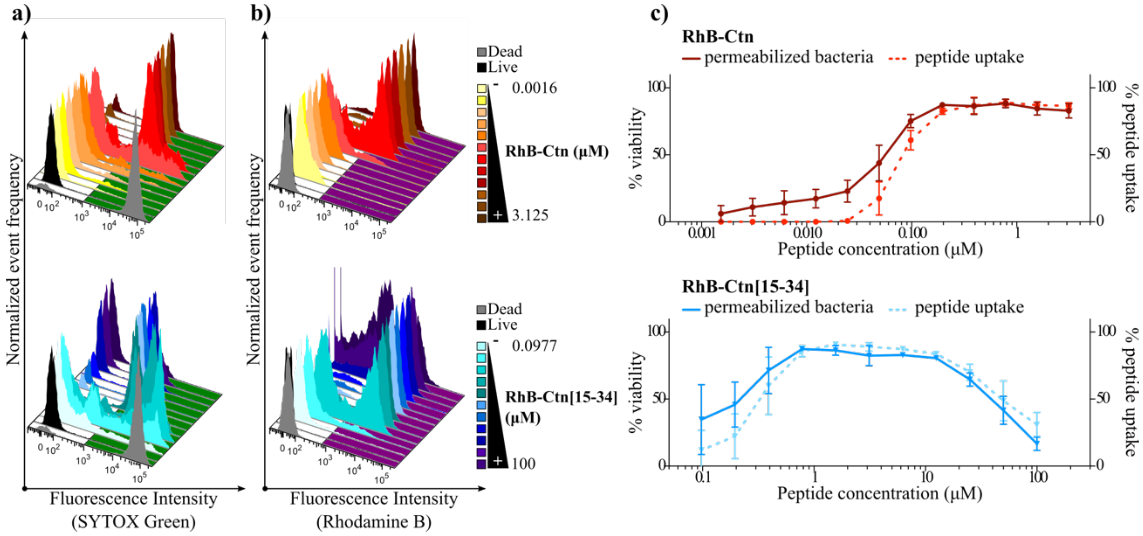

a)
Linear 15 - 40% gradient of B into A
Ctn crude
Ctn pure
A220nm
Normalized intensity
Retention time (min)
Linear 20 - 40% gradient of B into A
RhB-Ctn crude
RhB-Ctn pure
Linear 10 - 40% gradient of B into A
Ctn[15-34] crude
Ctn[15-34] pure
Linear 20 - 40% gradient of B into A
RhB-Ctn[15-34] crude
RhB-Ctn[15-34] pure
b)
Intensity
m/z
693.10
594.25
831.40
520.00
1039.00
262.10
462.40
860.95
1003.50
1187.15
1384.80
1661.60
763.65
916.20
573.00
1144.90
1962.10
262.05
509.45
927.05
1307.90
1525.70
1832.30
593.85
791.35
475.25
1186.45
262.15
471.85
704.20
949.55
1206.35
1423.05
1767.55
1905.40
700.00
560.20
932.90
1960.65
262.00
466.95
634.70
846.05
1119.30
1398.30
1676.00

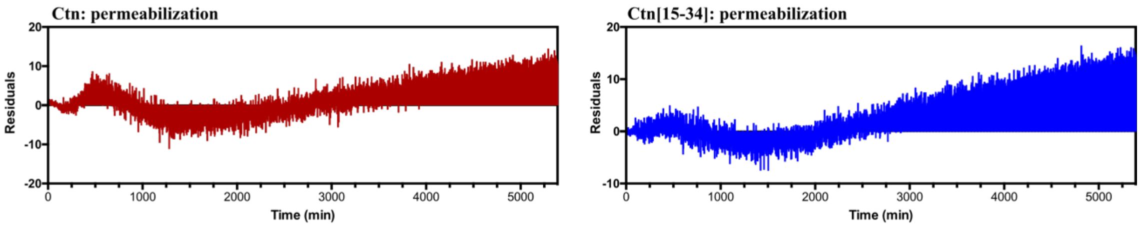
**FIGURE S2**



a)
b)
c)
Normalized event frequency
Fluorescence Intensity
(SYTOX Green)
Fluorescence Intensity
(Rhodamine B)
Dead
Live
0.0016
RhB-Ctn (µM)
3.125
0.0977
RhB-Ctn[15-34]
(µM)
100
RhB-Ctn
permeabilized bacteria
peptide uptake
% viability
% peptide uptake
Peptide concentration (µM)
RhB-Ctn[15-34]

**FIGURE S3**

**a)**


Ctn: permeabilization
Residuals
Time (min)
Ctn[15-34]: permeabilization
Residuals
Time (min)

**b)**

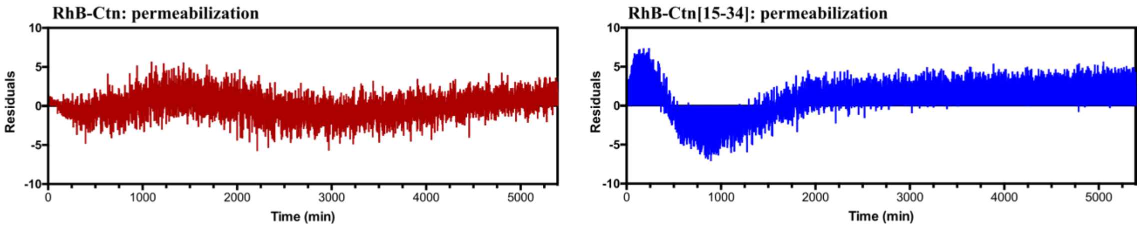
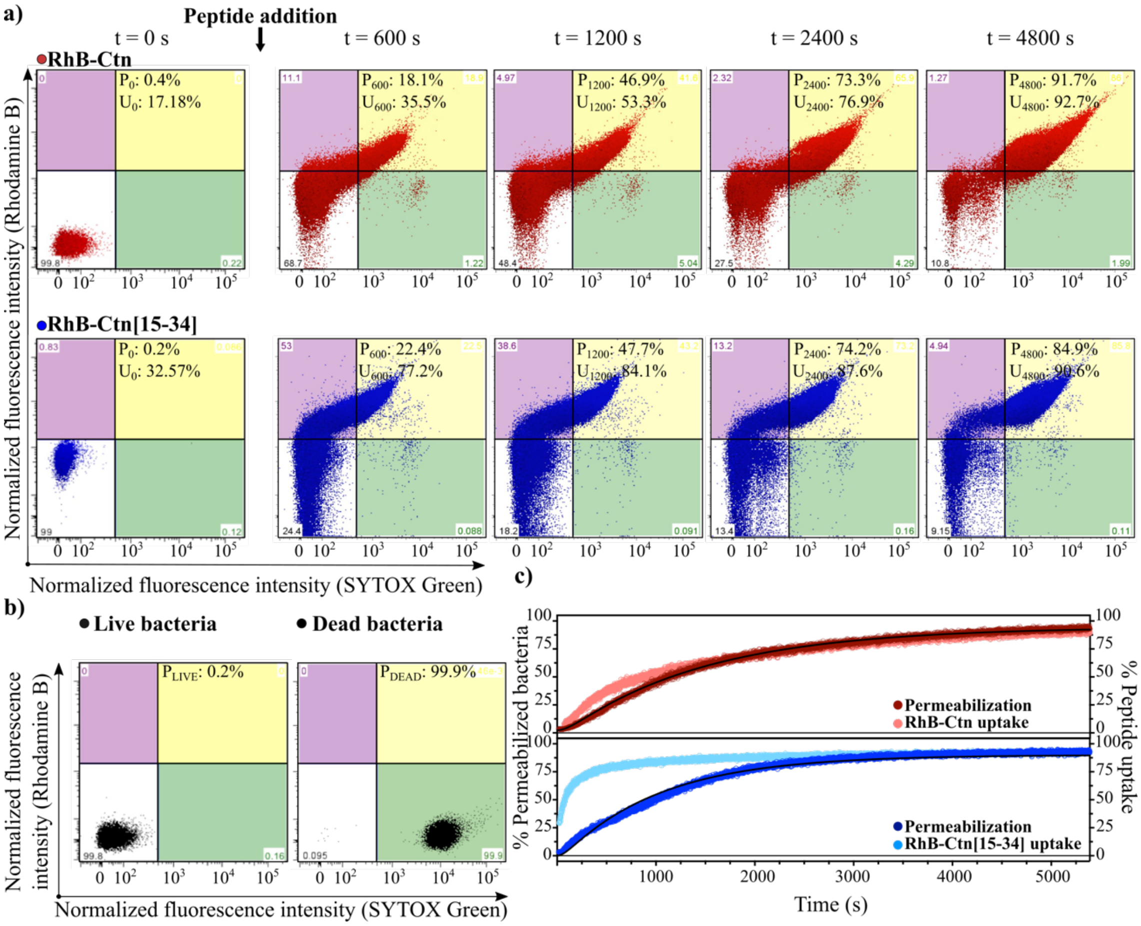

RhB-Ctn: permeabilization
Residuals
Time (min)
RhB-Ctn[15-34]: permeabilization
Residuals
Time (min)

**FIGURE S4**



a)
Peptide addition
t = 0 s
t = 600 s
t = 1200 s
t = 2400 s
t = 4800 s
RhB-Ctn
P0: 0.4%
U0: 17.18%
P600: 18.1%
U600: 35.5%
P1200: 46.9%
U1200: 53.3%
P2400: 73.3%
U2400: 76.9%
P4800: 91.7%
U4800: 92.7%
RhB-Ctn[15-34]
P0: 0.2%
U0: 32.57%
P600: 22.4%
U600: 77.2%
P1200: 47.7%
U1200: 84.1%
P2400: 74.2%
U2400: 87.6%
P4800: 84.9%
U4800: 90.6%
Normalized fluorescence intensity (Rhodamine B)
Normalized fluorescence intensity (SYTOX Green)
b)
Live bacteria
Dead bacteria
PLIVE: 0.2%
PDEAD: 99.9%
c)
% Permeabilized bacteria
% Peptide uptake
Permeabilization
RhB-Ctn uptake
RhB-Ctn[15-34] uptake
Time (s)

**FIGURE S5**

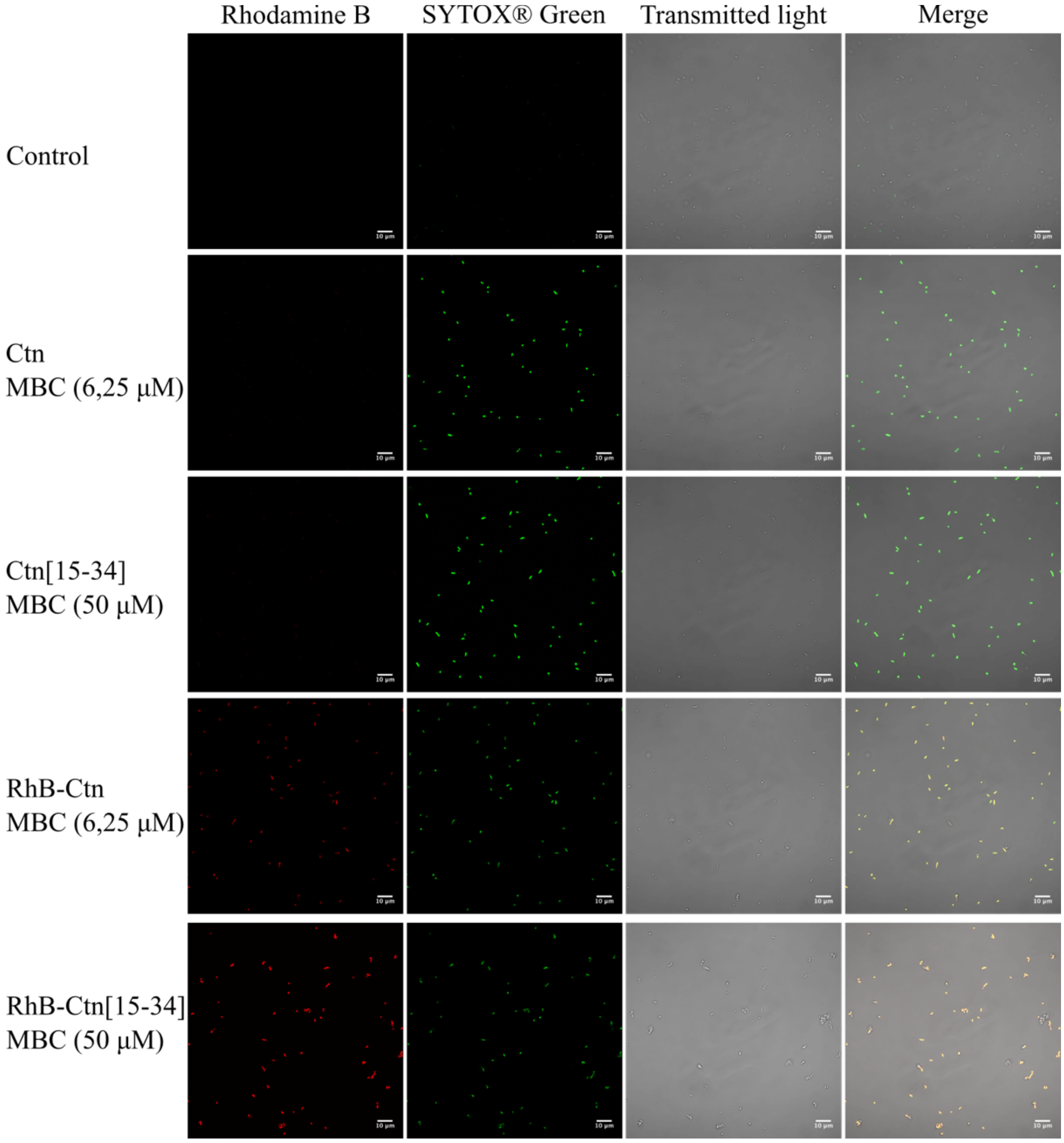
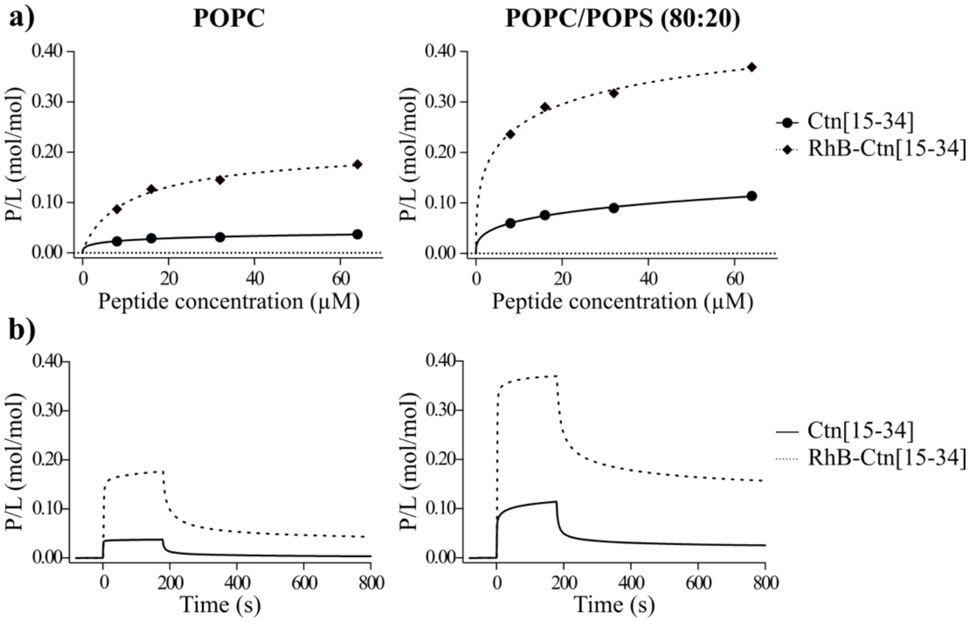

Rhodamine B
SYTOX® Green
Transmitted light
Merge
Control
Ctn
MBC (6,25 µM)
Ctn[15-34]
MBC (50 µM)
RhB-Ctn
MBC (6,25 µM)
RhB-Ctn[15-34]
MBC (50 µM)

**FIGURE S6**



a)
POPC
POPC/POPS (80:20)
P/L (mol/mol)
0.00
0.10
0.20
0.30
0.40
0
20
40
60
Peptide concentration (μM)
Ctn[15-34]
RhB-Ctn[15-34]
b)
0
200
400
600
800
Time (s)

**FIGURE S7**

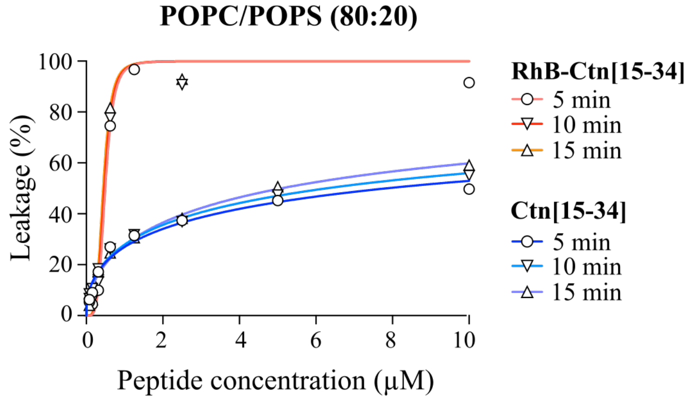
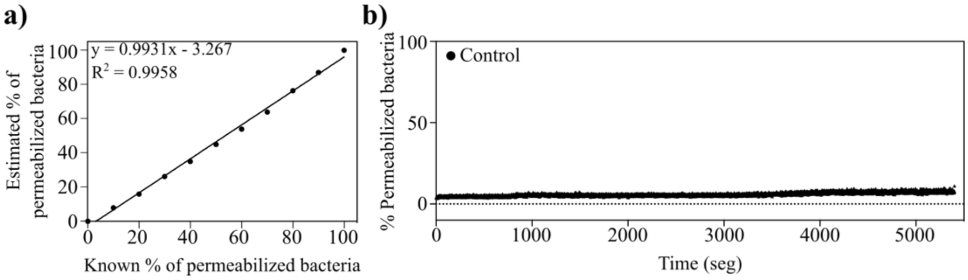

POPC/POPS (80:20)
Leakage (%)
Peptide concentration (μM)
RhB-Ctn[15-34]
5 min
10 min
15 min
Ctn[15-34]
5 min
10 min
15 min

**FIGURE S8.**



a)
y = 0.9931x - 3.267
R² = 0.9958
Estimated % of permeabilized bacteria
Known % of permeabilized bacteria
0
20
40
60
80
100
b)
Control
% Permeabilized bacteria
Time (seg)
0
50
100
1000
2000
3000
4000
5000

## REFERENCES:


1. Freire, J. M., Gaspar, D., de la Torre, B. G., Veiga, A. S., Andreu, D., and Castanho, M. A. (2015) Monitoring antibacterial permeabilization in real time using time-resolved flow cytometry. *Biochim Biophys Acta* **1848**, 554-560