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Discovery and membrane-selective bactericidal activity of Spat1, a novel cyclotide from *Spigelia anthelmia* L. (Loganiaceae)

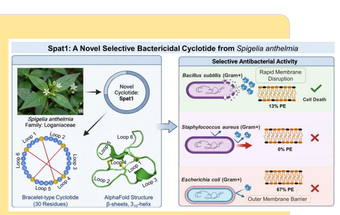
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Key finding

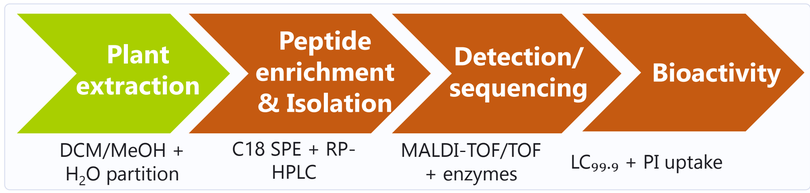
Spat1 is a new 30-residue bracelet cyclotide from Spigelia anthelmia that rapidly kills *Bacillus subtilis* by membrane permeabilization, while showing no detectable activity against *Staphylococcus aureus* or *Escherichia coli* up to 400 μM.



Background & aim

Cyclotides are plant-derived circular mini-proteins stabilized by a cyclic cystine-knot motif. Their exceptional stability and membrane-active properties make them attractive antimicrobial scaffolds. However, they exhibit a remarkably restricted phylogenetic distribution, occurring in only ~1% of angiosperm families (Rubiaceae, Violaceae, Cucurbitaceae, Fabaceae, Poaceae, and Solanaceae). This work asked whether an unexplored Loganiaceae medicinal plant contains novel cyclotides and whether these peptides possess selective bactericidal activity.

Experimental workflow



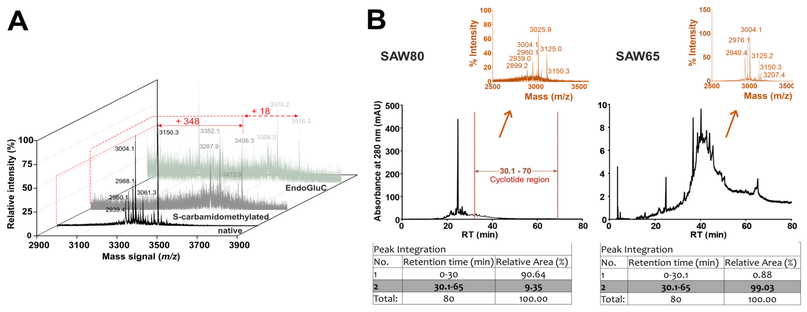
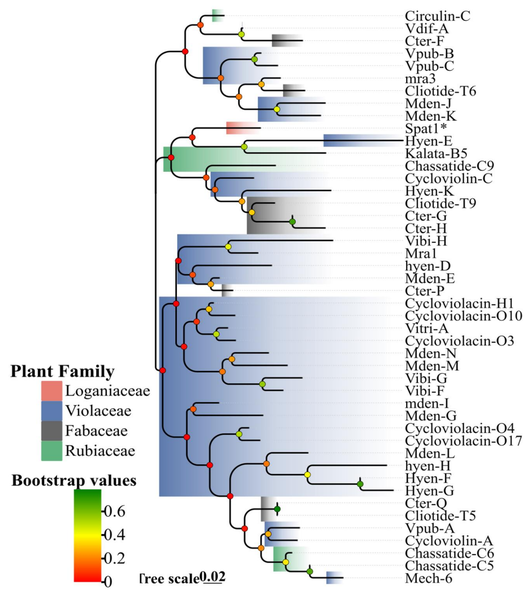


Fig. 1. MALDI-TOF detection and enrichment of the cyclotide-containing fraction from *S. anthelmia*.
Detection of cyclotides in Spigelia anthelmia whole plant. (A) MALDI MS spectra showing a +348 Da shift were observed after carbamidomethylation and an additional +18 Da shift (+366 total) following EndoGluC proteinase digestion, which implies a hydrolytic cleavage of Glutamic acid that is usually conserved in cyclotides, leading to the circular opening of the cyclotides. (B) Analytical HPLC chromatogram showing peptide enrichment to improve solid-phase peptide extraction toward large-scale extraction and isolation after a 10% buffer B wash and 80% buffer B elution, 30% buffer B wash, and 65% elution.

Spat1 sequence & Phylogenetic relationship

***cyclo*-GIPCGESCVYIPCFTSVIGCSCSNKVCYKN**

30 residues · bracelet-type cyclotide · m/z 3150.1 · net charge +1



Phylogenetic relationship of Spat1 with other RefSeq cyclotides (≤86% similarity) from the UniProt BLAST interface.

Purification-guided bactericidal activity

Bioassay-guided fractionation localized bactericidal activity to fraction G, which yielded purified Spat1.

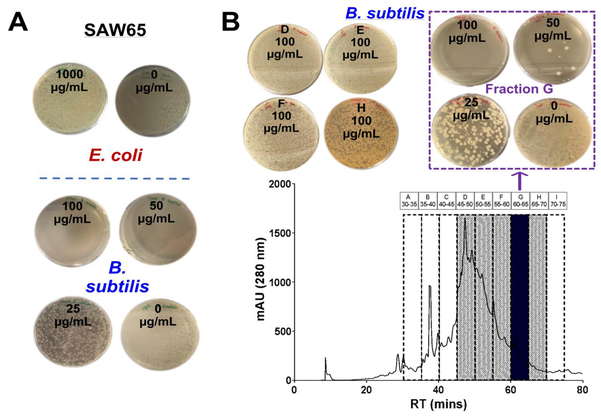


Figure 2: Bactericidal effect of SAW65 peptide extract (A) and HPLC fractions (C) on Gram-negative *E. coli* and Gram-positive bacteria *B. subtilis* after 1 h incubation. Fraction G (highlighted in lilac; B) maintained the bactericidal effect observed in the SAW65 peptide extract.

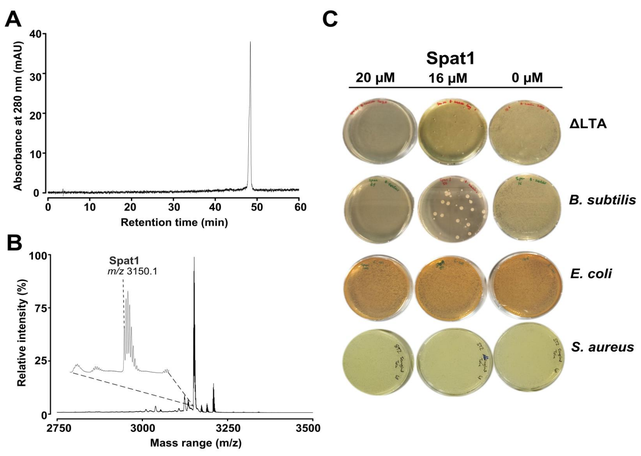


Figure 3: After 1 h incubation with bacterial cells, pure Spat1 (≥95% purity) demonstrated $LC_{99.9}$ at 20 µM against B. subtilis and LTA-deficient B. subtilis but no effect against Gram-negative E. coli and Gram-positive *S. aureus*.

Activity profile

● ***Bacillus subtilis***	$LC_{99.9}$ = 20 µM within 1 h
● **ΔLTA B. *subtilis***	$LC_{99.9}$ = 20 µM
● ***Staphylococcus aureus***	No activity up to 400 µM
● ***Escherichia coli***	No activity up to 400 µM

Interpretation: Activity does not require lipoteichoic acid and is consistent with direct engagement of cytoplasmic membrane phospholipids.

Membrane permeabilization

Propidium iodide influx occurred rapidly at bactericidal concentrations, matching the lethal concentration range.

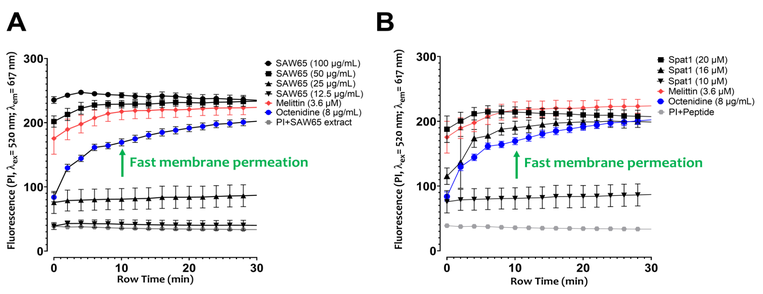


Figure 4: Permeabilization of bacterial membranes by peptide cocktails (A) and Spat1 (B).

Structural and mechanistic insight

- AlphaFold-based modelling supports a canonical cyclic cystine-knot fold with amphipathic surface topology.
- Spat1 has a low net positive charge (+1), which may limit penetration of the Gram-negative outer membrane.
- Low LTA affinity and preserved activity in ΔLTA B. subtilis support a membrane-phospholipid-driven mechanism.

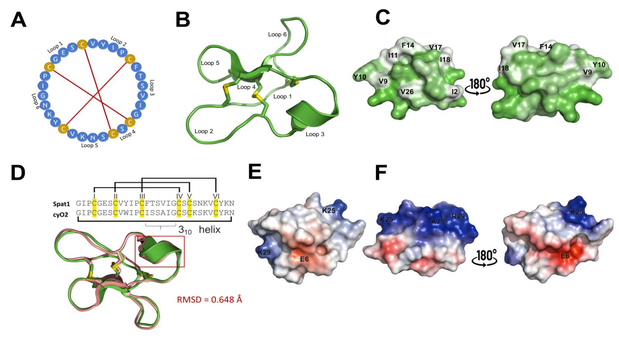


Figure 5: Primary sequence (A), cartoon (B) representations displaying the two antiparallel β-sheets, the 3_{10}-helix, and three disulfide linkages (yellow); intercysteine loops are indicated. Surface representation showing the hydrophobicity (C) of Spat1 using the Eisenberg hydrophobicity scale; hydrophobic residues are displayed in green, whereas hydrophilic residues are displayed in white. Structural alignment of Spat1 (green) and another bracelet cyclotide, cycloviolacin O2 (salmon), at RMSD = 0.648 Å (D); Electrostatic potential of Spat1 (E) and cyO2 (F), outlining negatively charged residues in red and positively charged residues in blue.

De novo sequencing evidence

Reduction/alkylation, EndoGluC, trypsin, and chymotrypsin fragments supported full sequence assignment.

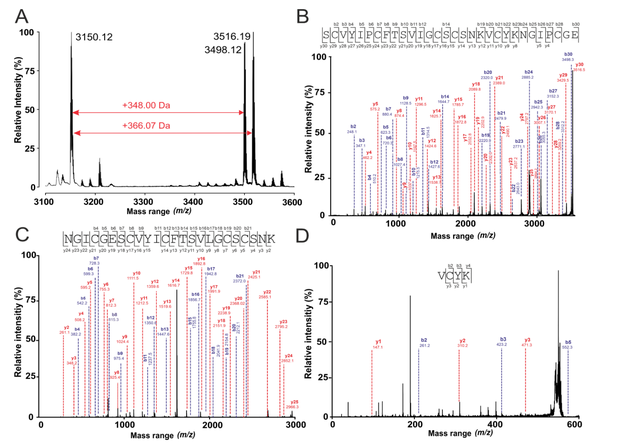


Figure 6: MALDI-TOF MS/MS de novo annotation. (A) Ring opening of the native peptide m/z 3150.12 to a linear product m/z 3516.19 was observed in a digestion experiment with endopeptidase GluC. The m/z 3496.12 corresponds to the cyclic reduced and S-acetamidated intermediate. (B). The peptide m/z 3516.19 was used for an MS/MS fragmentation experiment, and the b- and y-ions of the resulting fragmentation spectrum were de novo annotated, providing the full-length amino acid sequence of Spat1. Similarly, a trypsin digestion experiment was carried out, and two tryptic fragments, m/z 2966.3 (C) and m/z 570.3 (D), were observed.

Conclusions

- Spat1 expands the known phylogenetic distribution of cyclotides to Loganiaceae.
- It shows narrow-spectrum, rapid bactericidal activity against B. subtilis.
- Membrane permeabilization is the dominant measurable killing event.
- Net charge and surface topology appear to tune bacterial specificity and should guide SAR design.

Future direction

Engineer Spat1 analogues with tuned basic residues and hydrophobic patch orientation to test whether Gram-negative penetration and spectrum can be rationally improved without losing selectivity.

Proposed membrane-selective model

1 Spat1 scaffold — Cyclic cystine-knot peptide; 30 aa · bracelet type · +1 charge

→ **2 Selective engagement** — PE-containing Bacillus membrane; minimal dependence on LTA

→ **3 Rapid permeabilization** — PI influx within minutes; $LC_{99.9}$ = 20 µM in 1 h

→ **4 Narrow spectrum** — No detectable killing of *S. aureus* or *E. coli* up to 400 µM

Overall interpretation: Spat1 activity is best explained by direct engagement with the PE-containing cytoplasmic membrane rather than by teichoic acid binding. Species specificity likely reflects a combination of membrane lipid composition, outer-membrane barrier effects, net charge, and hydrophobic surface topology.

Selected references

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