Synergistic activity of the tyrocidines, antimicrobial cyclodecapeptides from *Bacillus aneurinolyticus*, with amphotericin B and caspofungin towards *Candida albicans* biofilms

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Supplementary data

Purification and analysis of peptides in this study

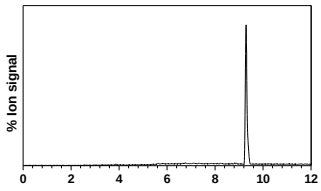
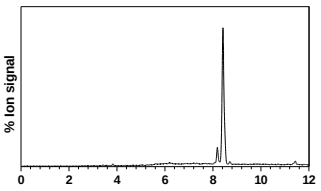
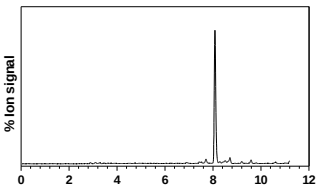
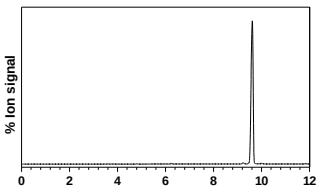
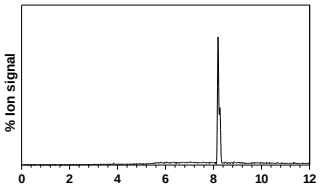
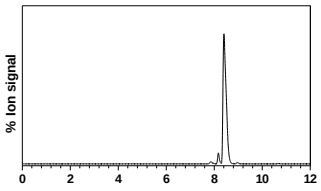
The tyrocidine peptide complex (Trc mixture) was isolated from a commercial tyrothricin complex from *B. aneurinolyticus* using a modified organic extraction method (1, 2). The tyrocidines A, B and C, phenycidine A and tryptocidine C (Table 1) were purified from the Trc mixture as described by Rautenbach *et al.* (3). The composition of the Trc mixture extracted from tyrothricin and the identity and purity of the individual peptides were determined using high resolution time-of-flight electrospray mass spectrometry (TOF-ESMS) and ultra-performance liquid chromatography (UPLC) (Table S1). Direct injection TOF-ESMS analyses were performed on a Waters Q-TOF Ultima mass spectrometer fitted with an electrospray ionisation source. The peptide samples (3 µL of 200 µg/mL in acetonitrile/water, 1:1, *v*/*v*) was injected into the ESMS and subjected to a capillary voltage of 3.0 kV. The source voltage was 15 V and the temperature 120˚C. Data was collected in the positive mode by scanning over an *m/z* range of 300-2000. For UPLC 2 µL peptide sample in water was chromatographed on an Acquity UPLC® BEH C_{18} column at a flow rate of 0.450 mL/min, using a 1 % formic acid (A) to acetonitrile (B) gradient (100% A from 0 to 0.5 minutes, 0 to 58% B from 0.5 to 12 minutes and then 58 to 90% B from 12 to 13 minutes. In-line ESMS analysis of the analytes separated via UPLC was done with same settings as for the direct injection of peptide samples. Also refer to Eyéghé-Bickong (4), Spathelf (5) and Tang *et al.* (6) for more detail on the analysis and purification of the tyrocidines.

Membrane permeability, ROS and activity assays

Refer to main text for details on methods. Comparison of dose response curves of PI induction and growth inhibition by the tyrocidines is given in Figure S1. The concentration dependent induction of ROS is given in Figure S2.

Table S1

Summary of TOF-ESMS and LC-MS analysis data of the purified peptides in this study. Details of the peptide structures are given in Table 1

Tyrocidine A		1269.6546	1269.6515 1291.6343	TrcA TrcA + $2Na^+$	9.29	98
Tyrocidine B		1308.6655	1308.6610 1322.6772 1384.7028	TrcB $TrcB_1$ $TpcC_1$	8.41	90
Tyrocidine C		1347.6764	1347.6713 1369.6488	TrcC TrcC + $2Na^+$	8.07	91
Phenycidine A		1253.6597	1253.6625 1267.6758 1275.6411 1297.6293	PhcA $PhcA_1$ PhcA + $2Na^+$ PhcA + $4Na^+$	9.61	98
Tryptocidine C		1370.6924	1370.6866 1371.6887 1392.6675	TpcC TpcC + H^+ TpcC + $2Na^+$	8.19	95
Gramicidin S		1140.7059	1126.7009 1140.7067 1141.7164 1154.7256	unknown GS GS + H^+ GS + $2H^+$,Na^+	8.40	93

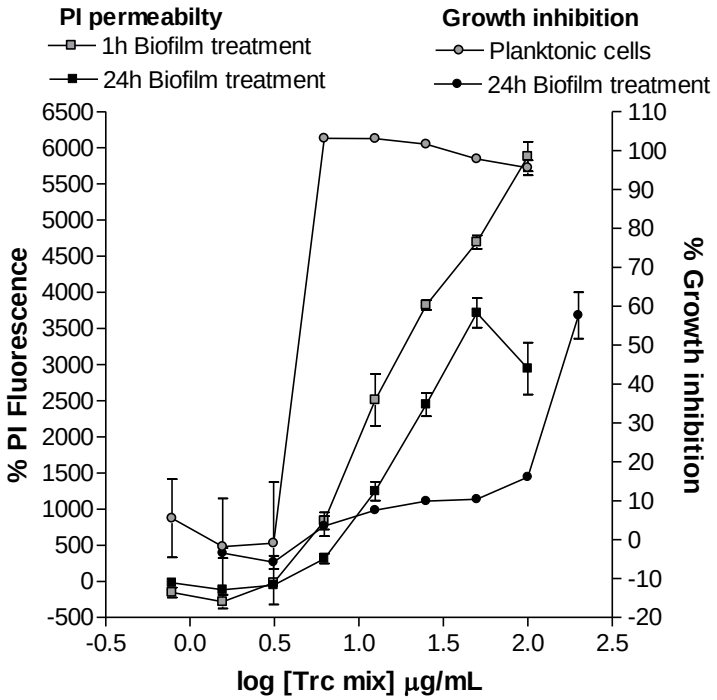


Figure S1: Comparison of dose response curves of PI induction and growth inhibition by the tyrocidines. Twenty-four hour old *C. albicans* biofilms were treated with the Trc mixture either for 1 hour or 24 hours (PI fluorescence on left hand y-axis). Plotted on the right hand axis is the growth inhibition of planktonic and 24 hour old biofilm *C. albicans* cells. Each data point represents the mean of triplicate biological repeats ± SEM, with triplicate technical repeats per assay.

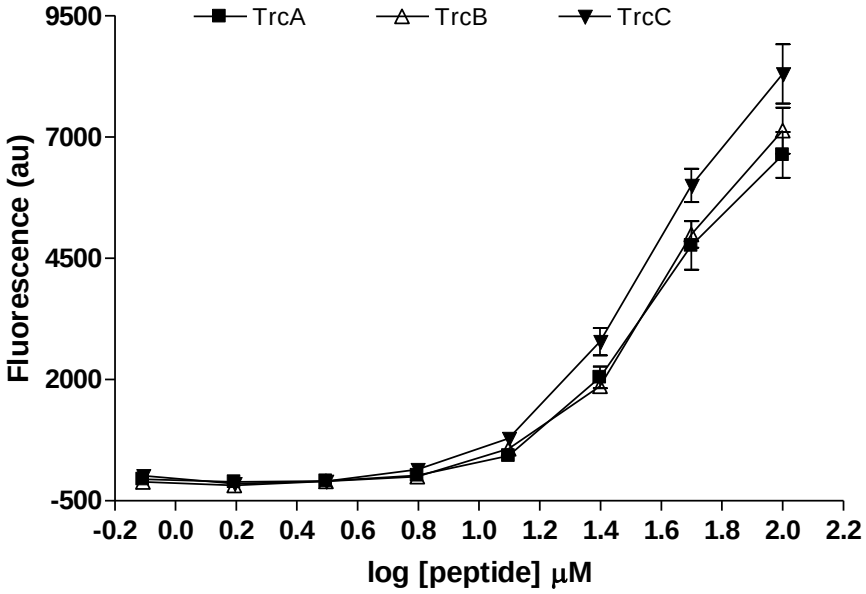


Figure S2: Dose response curves of ROS induction in 24 hour old *C. albicans* biofilms treated with peptides in relation to untreated cells. Biofilms were incubated for 24 hours with TrcA, TrcB or TrcC prior to staining with H_2DCFDA. Each data point represents the mean of triplicate biological repeats ± SEM, with triplicate technical repeats per assay.

Supplementary references

1. **Hotchkiss RD, Dubos RJ.** 1941. The isolation of bactericidal substances from cultures of *Bacillus brevis*. J. Biol. Chem. **141:**155-162.

2. **Spathelf BM, Rautenbach M.** 2009. Anti-listerial activity and structure–activity relationships of the six major tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*. Bioorg. Med. Chem. **17:**5541-5548.

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4. **Eyéghé-Bickong HA.** 2011. Role of surfactin from *Bacillus subtilis* in protection against antimicrobial peptides produced by other *Bacillus* species. PhD Thesis, Department of Biochemistry, University of Stellenbosch, http://scholar.sun.ac.za/handle/10019/6773.

5. **Spathelf BM.** 2010. Qualitative structure-actvity relationships of the major tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*. PhD Thesis, Department of Biochemistry, University of Stellenbosch, http://scholar.sun.ac.za/handle/10019.1/4001.

6. **Tang X-J, Thibault P, Boyd RK.** 1992. Characterisation of the tyrocidine and gramicidin fractions of the tyrothricin complex from *Bacillus brevis* using liquid chromatography and mass spectrometry. Int. J. Mass Spectrom. Ion Processes. **122:**153-179.