antibiotics

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*SUPPLEMENTARY DATA*

# The Influence of Cellulose-Type Formulants on Anti-*Candida* Activity of the Tyrocidines

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## Peptide purification and characterization

Tyrocidine mixture (Trc mix) was extracted from commercial tyrothricin using an optimised diethyl ether (DEE) and acetone precipitation protocol [42] and subjected to RP-HPLC (Figure S1). The insolubility of tyrocidine analogues (Trcs) and solubility of the hydrophobic linear gramicidins (Grms) in DEE-acetone, due to their different hydrophobic properties, allowed for the separation of the two groups of peptides from the tyrothrcin mixture. However, is has been observed that the DEE-acetone wash results in the loss of some Trcs analogues. Therefore, each complex (tyrothricin and the Trcs fraction) were subjected to UPLC-ESMS to compare the abundance of different peptides analogues present within the preparation (Figure S1). The UPLC-ESMS was performed based on an optimised method [42].

The DEE-acetone wash resulted in complete loss of minor analogues and significant loss of some of the major analogues present within the fraction despite the overall increase in purity with the removal of the Grms (Table S2).

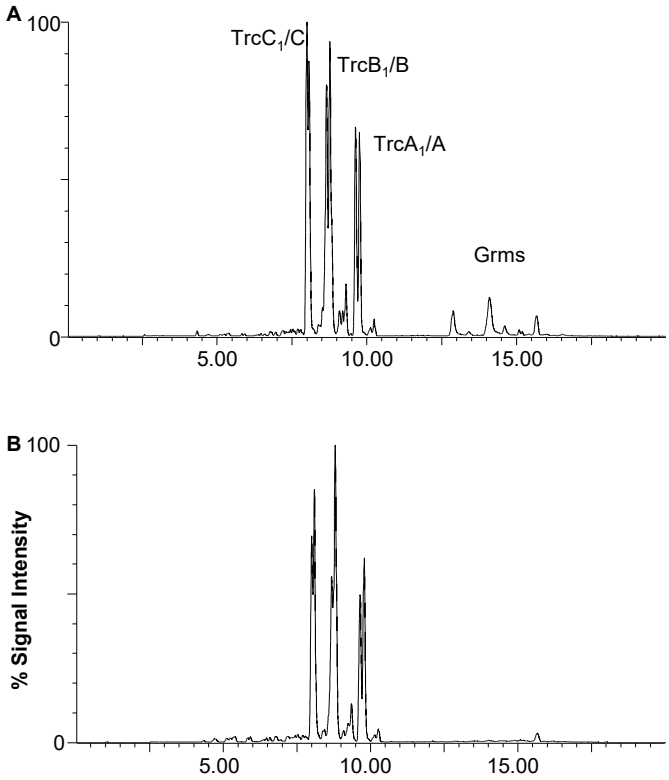


**Figure S1:** UPLC chromatograms of commercial tyrothricin complex with (**a)** the untreated tyrothricin, (**b**) the precipitate of the DEE: acetone wash containing the Trc mix.

**Table S1:** Percentage abundance and theoretical mass (mg) yield of the major Trc analogues present within the commercial tyrothricin complex before and after the wash with DEE: Acetone.

| | %Abundance [a] | | Theoretical mass (mg) yield of the major analogue [c] | | %abundance Trc mix | |
|---|---|---|---|---|---|---|
| | Tyrothricin complex | Extracted Trc mix | Tyrothricin complex | Extracted Trc mix | Vosloo [20] | Troskie *et al.* [6] |
| $TrcC_1$ | 14.2 | 12.4 | 2.13 | 1.27 | 1.6 | 12.5 |
| TrcC | 13.6 | 15.0 | 2.04 | 1.53 | 10.7 | 14.8 |
| $TpcC_1$ | 1.1 | 1.2 | 0.17 | 0.12 | 0.3 | - |
| TpcC | 1.5 | 2.9 | 0.23 | 0.3 | 2.5 | 1.7 |
| $TrcB_1$ | 14.4 | 9.3 | 2.16 | 0.95 | 3.5 | 19.2 |
| TrcB | 15.2 | 22.9 | 2.28 | 2.34 | 9.4 | 18.2 |
| TrcB′ | 3.9 | - | 0.59 | - | 8.5 | 4.0 |
| TpcB | 2.7 | 3.8 | 0.41 | 0.27 | 6.2 | 1.0 |
| $TrcA_1$ | 9.9 | 10.4 | 1.49 | 1.06 | 4.4 | 15.8 |
| TrcA | 9.6 | 13.0 | 1.44 | 1.32 | 21.1 | 12.9 |
| TpcA | 0.7 | 1.4 | 0.11 | 0.1 | 6.3 | Trace |
| IGB | 2.2 | - | 0.33 | - | 0.6 | - |
| VGA | 4.1 | - | 0.62 | - | 6.1 | - |
| VGB | 1.8 | - | 0.27 | - | 0.6 | - |
| %Trcs [b] | 86.8% | 92.1% | 13.05 mg | 9.26 mg | 81.8% | 100.1% |

[a] % Abundance was calculated by expressing the peak area of each peptide as a percentage of the sum of the peak areas of all peptides present in the extract. It was assumed that the response factors of all peptides are similar due to their analogue structure.

[b] % Trcs was determined by the sum of the peak areas of all the Trcs present in the tyrothricin complex and each of the extracts.

[c] The mass (mg) of the predominant analogues present in the tyrothricin complex and each of the extracts was calculated by multiplying of % abundance by the total mass (mg) of in the tyrothricin complex and each of the extracts.

## Activity of Trc mix and commercial antifungal compounds against *Candida albicans*

**Table S2**: Comparison of $IC_{50}$ and MIC values of selected antifungal compounds against planktonic cultures of *C. albicans* CAB1653. Tabulated $IC_{50}$ and MIC values represents the mean of 3–4 biological repeats and 12–32 technical repeats with SEM.

| Drug or peptide | $IC_{50}$ (μM) | MIC (μM) |
|---|---|---|
| Trc mix | 11 ± 1.3 | 12.5–25 |
| Caspofungin | 4.8 ± 0.7 | 8.6 ± 1.1 |
| Amphotericin B | 13 ± 2.9 | 23 ± 4.2 |
| Fluconazole | >325 | >325 |

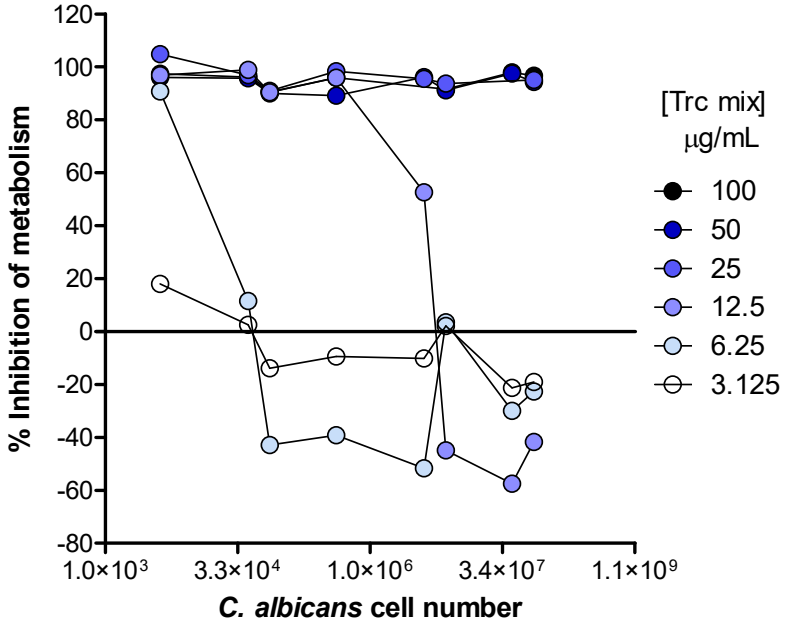


**Figure S2:** Correlation between metabolic inhibition of a range of *C. albicans* cell concentrations versus Trc mix concentrations. The values below 0% inhibition indicate higher conversion of the metabolic dye versus the control cultures. Each data point is the average determined for two cultures.

## Fluorescence analysis of Trc mix and its formulations

**Table S3:** Summary of % relative fluorescence unit (RFU) loss measured for each of the eight formulants of Trc mix 1:1 (*m*/*m*) from 1 to 4 h and 1 to 20 h of maturation. The relative loss is also indicated as a heat map with blue the lowest lost and red the highest loss.

| | Mean of % RFU loss from 1 hour | |
|---|---|---|
| **Cellulose derivatives** | **4 hours of maturation** | **20 hours of maturation** |
| Control | 23 | 45 |
| A4M | 22 | 32 |
| E4M | 20 | 25 |
| E10M | 24 | 31 |
| KLUE | 19 | 26 |
| KLUL | 22 | 30 |
| K15M | 20 | 27 |

**Table S4:** Student t-test statistical comparison of Trp fluorescence between different Trc mix of fresh (1 hour) and matured (20-hour) preparations. Tabulated Trp fluorescence represents the mean 12 preparations with SD. Unpaired Student t-test was done on each of the analysed pairs.

| Formulant | Fresh sample (1 hour) mean ± SD | Matured sample (20hours) mean ± SD | P-value | % Change 1 h to 20 h |
|---|---|---|---|---|
| Trc mix: cellulose derivatives 1:1 (*m*/*m*) | | | | |
| A4M | 5844 ± 72.0 | 3839 ± 1557 | 0.0010 | 34 |
| E4M | 6087 ± 249.7 | 4443 ± 1573 | 0.0044 | 27 |
| E10M | 5244 ± 282.5 | 3511 ± 1173 | 0.0007 | 33 |
| K15M | 7130 ± 260.7 | 5133 ± 1129 | <0.0001 | 28 |
| Trc mix: cellulose derivatives 1:4 (*m*/*m*) | | | | |
| A4M | 5910 ± 623.3 | 5381 ± 646.8 | ns | 9 |
| E4M | 6850 ± 501.1 | 6326 ± 516.9 | ns | 8 |
| E10M | 7120 ± 537.2 | 6443 ± 518.0 | ns | 10 |
| K15M | 7335 ± 401.6 | 6392 ± 373.6 | ns | 13 |
| Control | 5539 ± 4481 | 2345 ± 1584 | 0.0277 | 58 |

**Table S5:** One-way Anova statistical comparison Trp fluorescence between different formulations of Trc mix at 20 hours of maturation. Analysed Trp fluorescence represents the mean of 12 preparations with SD (refer to Table S3). One-way Anova with Bonferroni correlation test was done between the selected data sets.

| | | | 20 hours maturation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1:4 | | | | 1:1 | | | | |
| | | | K15M | E10M | E4M | A4M | K15M | E10M | E4M | A4M | Control |
| 20 hours of maturation | 1:4 | K15M | | ns | ns | ns | ns | 0.001 | ns | <0.01 | 0.001 |
| | | E10M | | | ns | ns | ns | 0.001 | ns | 0.001 | 0.001 |
| | | E4M | | | | ns | ns | 0.001 | <0.05 | <0.01 | 0.001 |
| | | A4M | | | | | ns | ns | 0.001 | ns | 0.001 |
| | 1:1 | K15M | | | | | | ns | ns | ns | 0.001 |
| | | E10M | | | | | | | ns | ns | ns |
| | | E4M | | | | | | | | ns | <0.05 |
| | | A4M | | | | | | | | | ns |

## Statistical analysis of activity data

**Table S6:** Student t-test statistical comparison between different Trc mix of fresh (1 h) and matured (20 h) formulations of the observed inhibition parameters against planktonic cultures of *C. albicans* CAB1653. Tabulated $IC_{50}$ and MIC values (μg/mL) represents the mean of 3–4 biological repeats and 12–30 technical repeats with SEM. Unpaired Student t-test was done on each of the analysed pairs and only those with significant differences are shown.

| Formulation at 1-hour vs 20-hours | $IC_{50}$ ± SEM (μg/mL) 1 hour | $IC_{50}$± SEM (μg/mL) 20 hours | P value |
|---|---|---|---|
| Control | 11.4 ± 1.3 | 5.3 ± 0.4 | 0.0003 |
| Trc mix:A4M (1:2) | 8.9 ± 0.6 | 4.9 ± 0.2 | <0.0001 |
| Trc mix:A4M (1:4) | 9.6 ± 1.3 | 5.6 ± 0.5 | 0.0196 |
| Trc mix:E4M (1:2) | 10.3 ± 0.8 | 6.8 ± 1.2 | 0.0143 |
| Trc mix:E10M (1:2) | 9.2 ± 0.3 | 6.2 ± 0.5 | <0.0001 |
| Trc mix:E10M (1:4) | 7.0 ± 0.8 | 2.3 ± 0.7 | 0.0002 |
| Trc mix:K15M (1:2) | 8.9 ± 0.9 | 5.1 ± 0.3 | 0.0017 |
| Formulation at 1-hour vs 20-hours | MIC ± SEM (μg/mL) 1 hour | MIC± SEM (μg/mL) 20 hours | P value |
| Control | 14.9 ± 1.5 | 7.5 ± 0.5 | 0.007 |
| Trc mix:A4M (1:1) | 14.0 ± 2.4 | 6.7 ± 0.5 | 0.0203 |
| Trc mix:A4M (1:4) | 14.8 ± 1.6 | 8.8 ± 0.9 | 0.0065 |
| Trc mix:E10M (1:4) | 10.1 ± 1.2 | 4.6 ± 0.8 | 0.0023 |

**Table S7:** One way Anova statistical comparison of IC50 (μg/mL) value correlation between the different preparations of Trc mix (1 h vs 20 h). The analysed $IC_{50}$ values were the mean of 3–4 biological repeats and 12–30 technical repeats with SEM. One-way Anova with Bonferroni correlation test was done between each of the selected data sets.

| | | | 20 hours of maturation | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1:4 | | | | 1:2 | | | | 1:1 | | | | |
| | | | K15M | E10M | E4M | A4M | K15M | E10M | E4M | A4M | K15M | E10M | E4M | A4M | Control |
| 1 hour of maturation | 1:4 | K15M | ns | <0.01 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | E10M | ns | <0.001 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | E4M | ns | <0.001 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | A4M | ns | <0.001 | ns | ns | ns | ns | ns | <0.05 | ns | ns | ns | <0.05 | ns |
| | 1:2 | K15M | ns | <0.001 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | E10M | ns | <0.001 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | E4M | ns | <0.001 | ns | ns | ns | ns | ns | <0.05 | ns | <0.05 | <0.05 | <0.05 | <0.05 |
| | | A4M | ns | <0.001 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1:1 | K15M | ns | <0.001 | ns | ns | ns | ns | ns | ns | ns | <0.05 | ns | ns | ns |
| | | E10M | ns | <0.01 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | E4M | ns | <0.01 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | A4M | ns | <0.001 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | Control | ns | <0.001 | ns | <0.01 | <0.01 | <0.001 | ns | <0.01 | ns | <0.01 | <0.01 | <0.001 | <0.001 |

**Table S8:** One-way Anova statistical comparison of $IC_{50}$ (μg/mL) between different preparations of Trc mix after 20 hours of maturation. The analysed $IC_{50}$ values were the mean of 3–4 biological repeats and 12–30 technical repeats with SEM. One-way Anova with Bonferroni correlation test was done between each of the selected data sets.

| | | | 20 hours of maturation | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1:4 | | | | 1:2 | | | | 1:1 | | | | |
| | | | K15M | E10M | E4M | A4M | K15M | E10M | E4M | A4M | K15M | E10M | E4M | A4M | Control |
| 20 hour of maturation | 1:4 | K15M | | <0.01 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | E10M | | | <0.01 | <0.001 | <0.001 | <0.001 | <0.01 | <0.01 | <0.001 | <0.001 | ns | <0.05 | ns |
| | | E4M | | | | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | | A4M | | | | | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1:2 | K15M | | | | | | ns | ns | ns | ns | ns | ns | ns | ns |
| | | E10M | | | | | | | ns | ns | ns | ns | ns | ns | ns |
| | | E4M | | | | | | | | ns | ns | ns | ns | ns | ns |
| | | A4M | | | | | | | | | ns | ns | ns | ns | ns |
| | 1:1 | K15M | | | | | | | | | | ns | ns | ns | ns |
| | | E10M | | | | | | | | | | | ns | ns | ns |
| | | E4M | | | | | | | | | | | | ns | ns |
| | | A4M | | | | | | | | | | | | | ns |