Tyrocidines are a family of cyclic decapeptides produced by the soil bacterium, *Brevibacillus parabrevis*. These antibiotic peptides can be used to prevent infections in agriculture and food industry but also to prepare antimicrobial lozenges, creams, and dressings for medical applications. It has been observed that the tyrocidines interact with saccharides such as cellulose from their soil environment, as well as sugars in culture media and glycans in fungal cell walls. Here, we investigated the interactions of tyrocidines with glucose, sucrose, and cellotetraose (as cellulose model) in a quantitative fashion utilising CD and NMR spectroscopy. The CD and NMR spectra of tyrocidine A (TrcA) were analysed as a function of solvent composition, and the spectral properties agree with the formation of oligomeric structures that are governed by β-sheet secondary structures once the acetonitrile content of the solvent is increased. Saccharides seem to also induce TrcA spectral changes reverting those induced by organic solvents. The CD spectral changes of TrcA in the presence of glucose agree with new ordered H-bonding, possibly β-sheet structures. The amides involved in intramolecular H-bonding remained largely unaffected by the environmental changes. In contrast, amides exposed to the exterior and/or involved in TrcA intermolecular association show the largest 1H chemical shift changes. CD and NMR spectroscopic investigations correlated well with TrcA-glucose interactions characterized by a dissociation constant around 200 μM. Interestingly, the association of cellotetraose corresponds closely to the additive effect from four glucose moieties, while a much higher dissociation constant was observed for sucrose. Similar trends to TrcA for binding to the three saccharides were observed for the analogous tyrocidines, tyrocidine B, and tyrocidine C. These results therefore indicate that the tyrocidine interactions with the glucose monosaccharide unit are fairly specific and reversible.

**KEYWORDS**
antimicrobial coating, antimicrobial peptide, cell wall interactions, cyclodecapeptide, dressings, glucose, gramicidin S, tyrothricin
1 | INTRODUCTION

Tyrothricin, a nonribosomally produced antimicrobial peptide (AMP) complex produced by the soil bacterium *Brevibacillus parabrevis*, was the first antibiotic preparation to be used in clinical practices (topical applications) but was soon replaced by penicillin. 1 With pathogens displaying resistance to many of the existing drugs, AMPs and specifically nonribosomally produced AMPs are now reconsidered as potential antibiotics because of their broad spectrum of activity against bacteria, fungi, parasites, and certain viruses. 2-4 Furthermore, the rapid antimicrobial action of AMPs and their ability to affect multiple targets decrease the likelihood of resistance developing against them. 5,6

Tyrothricin contains the gramicidins and a group of cationic cyclodecapeptides, the tyrocidines (Figure 1). The tyrocidines are conserved in their amino acid sequence and share 50% identity with the constrained β-turn/β-sheet structure. 7,8 The tyroidine cyclodecapeptide structure is highly conserved and rich in aromatic amino acids with 4 out of 10 residues being either Phe, Trp, or Tyr leading to most of the variability of the tyrocidines and analogues. 9 The primary structure of one of the major analogues, tyrocidine A (TrcA), is given in Figure 1.

Tyrocidines have a broad potential for application, not only in the clinic but also outside. 10 They are active against various bacterial species such as the food pathogen *Listeria monocytogenes*, 11,12 as well as a broad range of filamentous fungi. 13 The tyrocidines also inhibit planktonic *Candida albicans* and its biofilms, as well as synergise with caspofungin. 14 The tyrocidines are biodegradable with potential applications) but was soon replaced by penicillin. 1 With pathogens displaying resistance to many of the existing drugs, AMPs and specifically nonribosomally produced AMPs are now reconsidered as potential antibiotics because of their broad spectrum of activity against bacteria, fungi, parasites, and certain viruses. 2-4 Furthermore, the rapid antimicrobial action of AMPs and their ability to affect multiple targets decrease the likelihood of resistance developing against them. 5,6

Tyrothricin contains the gramicidins and a group of cationic cyclodecapeptides, the tyrocidines (Figure 1). The tyrocidines are conserved in their amino acid sequence and share 50% identity with the constrained β-turn/β-sheet structure. 7,8 The tyroidine cyclodecapeptide structure is highly conserved and rich in aromatic amino acids with 4 out of 10 residues being either Phe, Trp, or Tyr leading to most of the variability of the tyrocidines and analogues. 9 The primary structure of one of the major analogues, tyrocidine A (TrcA), is given in Figure 1.

Tyrocidines have a broad potential for application, not only in the clinic but also outside. 10 They are active against various bacterial species such as the food pathogen *Listeria monocytogenes*, 11,12 as well as a broad range of filamentous fungi. 13 The tyrocidines also inhibit planktonic *Candida albicans* and its biofilms, as well as synergise with caspofungin. 14 The tyrocidines are biodegradable with potential agricultural application as they show limited activity against bees 15 and nematodes. 14

Recently, it has been shown that the activity of the tyrocidines is more complex than merely the result of membranolytic activity because it was observed that membrane proteins involved in peptidoglycan synthesis are influenced by tyrocidine action. 16 Evidence has also been accumulating that the tyrocidines interact with a variety of saccharides, potentiating their activity. Glucose is ubiquitously present, not only in the environments of the agricultural and food industries where many tyrocidine-sensitive microbial pathogens exist; it also forms part of the target cell structures. The cell wall of fungi comprised predominantly of glycoproteins and polysaccharides of mainly glucan and chitin whose monomeric subunits are derived from glucose. 17 The importance of the fungal cell wall on the antifungal activity of the tyrocidines has previously been demonstrated by Rautenbach et al. 13,18 Furthermore, Rautenbach and Van Rensburg 10 have shown that the tyrocidines are readily adsorbed onto cellulose matrices where they maintain potent bactericidal activity. The tyrocidines have been used as part of the tyrothricin complex in throat lozenges (eg, Tyrozets) containing sucrose and are safe for oral consumption. 19 Moreover, glucose is present at 0.25% to 1.0% (m/v) in the growth media used to culture the microbes with the reported activity of the tyrocidines. Sugars/saccharides may thus have a profound influence on the tyrocidine structure, particularly on the dimeric structure that is proposed to be the membrane active moiety of the tyrocidines. 7,8

Therefore, here, we elucidate the interactions of one of the major tyrocidines, TrcA, with glucose, sucrose, and saccharides containing the β(1 → 4) linked D-glucose units, such as cellotetraose (as cellulose model) (Figure 1), utilising circular dichroism (CD) and nuclear magnetic resonance spectroscopy (NMR). On one hand, CD spectroscopy provides a global view on the secondary structure, H-bonding interactions, membrane topology, and conformation of polypeptides, 20,21 where spectral changes have been used to follow conformational equilibria. 22,23 On the other hand NMR spectroscopy is well established to provide structural and dynamic information on an atomic scale where distinct approaches are used to investigate polypeptides in solution 24 or in larger complexes such as membranes, amyloid fibres, polymers, or solid surfaces. 25-30

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** The chemical structure of the nonribosomally produced cyclodecapeptide tyrocidine A, as well as the saccharides used in this study. For the tyrocidine structure, the amino acid residues are abbreviated by the standard three-letter abbreviations, except Orn that was used for ornithine. In brackets are alternative substitutions at residue positions 3, 4, 7, and 9. Tyrocidine B, contains a Trp3-D-Phe4, and tyrocidine C, contains a Trp3-D-Trp4 in the aromatic dipeptide moiety depicted in the shaded area. In the upper right, a representation of the threedimensional X-ray structure of TrcA7 is shown including the intramolecular H-bonds (hatched lines). The amide H with large changes in the chemical shift upon glucose titration are shown in magenta, those with little changes in light blue (cf. Figures 5 and 6)
2 | MATERIALS AND METHODS

2.1 | Purification of tyrocidines

The chromatographic purification of TrcA, as well as the B and C analogues, was performed according to the methods described in Rautenbach et al\(^\text{31}\) using commercial tyrothricin (Sigma-Aldrich, Steinheim, Germany) or Br. parabrevis culture extracts, prepared as described by Vosloo et al\(^\text{32}\) as source materials. The respective purified peptide preparations were analysed utilising ultraperformance liquid chromatography linked to mass spectrometry (UPLC-MS) to determine the purity of the isolated peptides (refer to the detailed description below and supplementary data Figure S1). Only peptides with a single peptide purity of >90% according to UPLC-MS and >98% tyrocidine peptide purity were used in the analyses (Figures S1, S2).

2.2 | Mass spectrometry analysis of tyrothricin extracts and purified peptides

For electrospray mass spectrometry (ESMS), the peptide sample solutions (2-5 \(\mu\)L) were introduced by a Waters Acquity UPLC into a Waters Quadrupole Time-of-Flight Synapt G2 mass spectrometer with a Z-spray electrospray ionisation source in positive mode (Milford, USA). Peptide extracts were subjected to a capillary voltage of 3.0 kV and cone voltages of 15 or 25 V at a temperature of 120°C at the source, desolvation gas of 650 L/hour, and desolvation temperature of 275°C. Data acquisition was performed by scanning over a mass over charge ratio (\(m/z\)) range of 300 to 2000 in continuum mode at a rate of 0.2 scans per second.

Ultra-performance liquid chromatography (UPLC) of samples on an Acquity UPLC BEH C\(_{18}\) column (Waters, Milford, USA) via the Waters Acquity UPLC chromatographic system was achieved at a column temperature of 60°C and flow rate of 0.300 mL/min, using a 1% (v/v) formic acid in analytical grade water (A) to acetonitrile (ACN, B) gradient: 100% A from 0 to 0.5 minutes for loading, from 0 to 30% B over 0.5 to 1 minute, then 30% to 60% B from 1 to 10 minutes, 60% to 80% B from 10 to 15 minutes. The column was done from 80% to 0% B from 15 to 15.1 minutes and then 100% A from 15.1 to 18 minutes. Refer to supplementary data (Figures S1, S2) for the analysis of the purified peptides.

2.3 | Circular dichroism of TrcA

Dried aliquots of analytically weighed pure TrcA were dissolved in 60% (v/v) ACN in water and diluted to a concentration of 250 \(\mu\)M (pH \(\approx\) 7), varying ACN from 15% to 85% (v/v). Alternatively, TrcA was dissolved in 30% ACN containing a range of glucose (Glc) concentrations (0.025-6.25 mM final [Glc]). Comparative spectra were acquired with TrcA in trifluoroethanol:ACN:water 50:30:20 (v/v/v). Samples were analysed at ambient temperature (24 \(\pm\) 1°C) on a Chirascan Plus CD spectropolarimeter (Applied Photophysics, UK). CD scans were performed twice in triplicate between 185 to 300 nm at a bandwidth of 0.5 nm using a quartz cuvette with a path length of 0.5 mm. CD and ultraviolet (UV) absorption spectra were collected simultaneously with data collection set over 0.2 second per step of 0.5 nm (average of 8000 data points per nm).

From the CD titration data, the apparent \(K_d\) values were calculated from the change from 185 to 240 nm using first, a one-site binding hyperbolic equation:

\[
\% \text{Change in } \theta = \frac{\% \text{Change}_{\max} x \text{Glc}}{K_d + \text{[Glc]}} \quad (1)
\]

Or, second a two-site binding hyperbolic equation:

\[
\% \text{Change in } \theta = \frac{\% \text{Change}_{\max 1} x \text{Glc}}{K_{d1} + \text{[Glc]}} + \frac{\% \text{Change}_{\max 2} x \text{Glc}}{K_{d2} + \text{[Glc]}} \quad (2)
\]

where \(\theta\) is taken as the normalised total molar ellipticity from 185 to 240 nm with TrcA in TFE normalised to 0 and TrcA in 6.25 mM Glc set as 100.

2.4 | Liquid-state NMR analysis of TrcA and analogues

For all conditions described here, the tyrocidines were dissolved 16 hours beforehand at a concentration of 250 \(\mu\)M. At first, the peptide was dissolved in ACN: H\(_2\)O with the ACN concentration varying from 2% to 50% (v/v) in steps of 10%. Sugar titrations were performed in ACN: H\(_2\)O:D\(_2\)O a 30:60:10 (v/v/v) throughout the experiments. Stepwise glucose and sucrose titrations were performed ensuring sugar-to-TrcA ratios ranging from 0 to 25. For cellotetraose, titrations were performed up to a five-fold excess of cellotetraose.

Whereas most titration experiments were achieved by conventional one-dimensional \(^1\)H NMR spectroscopy, an additional two-dimensional experiment allowed assignment of the peak position. \(^1\)H-\(^1\)H TOCSY spectra of TrcA in 30% ACN were recorded on a Bruker spectrometer at a 500-MHz proton field. The experimental parameters were as follows: 16 scans averaged for each \(t_2\) value, spectral width of 10 ppm, 160 \(t_1\) values, a selective water presaturation during the 1-second recycle delay, and a mixing time of 120 ms.

The chemical shift of a number of amides changes with addition of glucose (\(\Delta CS\)), thereby allowing the determination of an apparent dissociation constant \(K_d\) following the relation:

\[
\Delta CS = \Delta CS_{\max} \frac{R}{K_d + \frac{R}{TrcA}} \quad (3)
\]

where R is the Glucose-to-Trc A ratio.
3 | RESULTS

3.1 Solvent dependence of TrcA structure and oligomerisation

Tyrocidines are known to assemble as oligomers in an aqueous solution, and they are generally prone to oligomerisation and aggregation\(^8,33-36\) (also refer to Supplementary data, Figure S2). Here, we use CD and NMR spectroscopy to gain deeper insight in the equilibria that govern structure, oligomerisation, and stability of the peptide in a solution and the influence of different saccharides and amino acid replacements.

Studies of TrcA at different ACN concentrations show that the organic solvent environment has a major influence on the CD spectra indicating a change in the ordered hydrogen-bonded structures. The total molar ellipticity of TrcA over 185 to 240 nm increased with the addition of organic solvent (Figure 2, Table 1). This has been taken as an indication of more ordered hydrogen-bonded structures with an asymmetric character.\(^37,38\) All the spectra with TrcA in ≥30% ACN displayed minima at 189 ± 1 nm, 207 ± 1 nm, and 217 ± 1 nm, as well as a maximum at 196 ± 1 nm (Figure 2). Due to the presence of D-amino acids, the CD spectra of TrcA are distorted, resembling those of proteins with \(\alpha\)-helices. However, both the X-ray\(^7\) and the solution NMR structures of TrcA\(^39-42\) showed that this peptide and its dimer are dominated by \(\beta\)-turns and \(\beta\)-sheets. Therefore, for TrcA and its analogues, including gramicidin S, such distorted CD spectra are the result of the hydrogen-bonded \(\beta\)-structures.\(^33,43-46\)

The ellipticity at 206 to 218 nm is due to the \(\pi \rightarrow \pi^*\) transition of 2p unpaired electrons of the carbonyl oxygen and is highly influenced by hydrogen bonds.\(^47\) An increase in the intensity of the minima over 206 to 218 nm is therefore associated with more ordered \(\beta\)-turn and \(\beta\)-sheet structures.\(^38,47-50\) Increasing the ACN concentration from 15% to 60% had a major influence on these minima (Figure 2, Table 1). At 15% ACN, there was only a broad shallow minimum between 209 and 211 nm, but the spectrum is probably dominated by adsorption flattening and/or scattering artefacts due to peptide aggregation.\(^51\) This minimum deepened and blue shifted to 206 nm upon addition of ACN (Figure 2, Table 1), indicating an increase in \(\beta\)-turn-type hydrogen-bonded structures.\(^38,49,50\) The minimum expected at 217 ± 1 nm deepened with the increased proportion of the organic solvent. A change in the ratio of the intensities at the two ellipticity minima \((\theta_{206} \pm 1\text{nm}/\theta_{217} \pm 1\text{nm})\) is associated with a change in the backbone conformation.\(^43,48\) An increase in %ACN or the addition of TFE led to a higher ratio, indicating a change in the backbone structure and/or interactions involving the peptide bonds (Table 1). TFE is known to support H-bonding and has been used in many protein and peptide structural studies as membrane mimic.\(^52\) It was used to get a maximum CD response for hydrogen-bonded and structured TrcA.

The ellipticity at 187 to 198 nm is due to the \(\pi \rightarrow \pi^*\) transition of carbonyl group p-electrons.\(^37,47\) Increasing the %ACN or addition of TFE led to a decrease in the maximum at 197 ± 1 nm. The minimum at 188 ± 1 nm deepened significantly with the addition of ACN, approaching the intensity of TrcA in 50% TFE (Figure 2), as well as displaying a 2-nm red shift to 190 nm for TrcA in 60% ACN indicating an increase in hydrogen bonding (Table 1).\(^37,47\)

In order to gain complementary information on an atomistic scale and to optimise the NMR conditions for sugar interaction studies, we also evaluated the \(^1\)H NMR spectra of 250-\(\mu\)M Trc at increasing ACN concentrations in water. At 2% (v/v) ACN, broad amide peaks were observed indicative of chemical exchange and/or slow tumbling due to oligomer formation (Figure 3). With increasing ACN concentrations, most amide peaks appear and sharpen. The Gln6 and Asn5 side chain resonances are also apparent as singulets broadened by exchange. In 30% ACN (v/v), the \(^1\)H amide resonances of eight residues can be seen and could be assigned to individual amino acids using 2D \(^1\)H-\(^1\)H spectroscopy\(^44\) in agreement with prior work.\(^8\)

In addition to the sharpening of the peaks with increasing ACN concentration, a gradual shift of all of the amide resonance positions was observed, albeit the effects were differential with the most pronounced changes for the D-Phe\(^1\), D-Phe\(^4\), Asn\(^5\), Val\(^6\), and Orn\(^9\) amide protons (by up to 0.10 ppm, Table 2).

The spectra at 30% ACN were characterized by well-resolved amide resonances which allowed us to measure the \(^3\)J\(_{HN-HA}\) scalar
couplings which were within <1 Hz of the published values for the NMR structure in 50% ACN. During the ACN titration, the coupling constants and the CαH chemical shifts remained constant indicating a conserved secondary structure in the solvents tested.

Interestingly, we observed a significant sharpening of the amide peaks at 15% ACN during 24 hours of incubation (Figure S3). In particular, the Asn5 side chain and D-Phe1 amide resonances undergo profound changes during this incubation period in 15% (v/v) acetonitrile. This shows that the structures and/or oligomers giving rise to the broad peaks can be converted into more mobile units also at lower ACN concentrations even though reaching the equilibrium is a slow process. Notably, the D-Phe1 amide and the Asn5 side chain exhibit strong changes in appearance and chemical shift upon ACN addition. They become only visible in the presence of 30% ACN and exhibit a change of chemical shift of about 0.065 ppm when the concentration of organic solvent is further increased to 50%. In the X-ray structure, these have an intermolecular H-bond to Tyr7 carbonyl and the Val8 amide, respectively. In comparison, the Asn5 backbone amide first appears at 10% ACN and shifts by about 0.07 ppm when ACN reaches 50%.

### 3.2 TrcA interaction with saccharides

To better understand the binding of tyrocidines to saccharides containing the β(1 → 4) linked D-glucose, such as cellulose, we first studied the interaction between TrcA and a single glucose moiety in 30% ACN/water (v/v). Following the influence of glucose on the CD spectra of TrcA, it is interesting that even low Glc concentrations have an influence on the TrcA CD spectrum (Figure 4A, Table 1). The transition that is most influenced by glucose was the π→π* of p-electrons of C=O (197 ± 1 nm, 188 ± 1 nm), which could indicate changes in the hydrogen-bonded structures, specifically the participation of glucose in a hydrogen-bonded network.

| Medium          | Total CD molar θ ×10^6 ± SD (185-240), nm | Minima, nm | Maxima, nm | θ 206 ± 1 nm | θ 217 ± 1 nm |
|-----------------|------------------------------------------|------------|------------|--------------|--------------|
| 50% TFEb        | -18.53 ± 0.88                            | 188, 206, 217 | 196        | 1.10         |
| 15% ACN         | -8.93 ± 0.36                              | 187, 209-211, 218 | 196        | 0.83         |
| 30% ACN         | -9.87 ± 0.20                              | 188, 207, 218 | 196        | 1.03         |
| 45% ACN         | -14.95 ± 0.35                             | 189, 206, 218 | 197        | 1.05         |
| 60% ACN         | -19.07 ± 0.93                             | 190, 206, 218 | 197        | 1.06         |
| 0.025 mM glucoseb | -9.54 ± 0.28                             | 188, 207, 218 | 196        | 1.01         |
| 0.25 mM glucoseb | -10.99 ± 0.37                             | 189, 207, 217 | 196        | 1.01         |
| 2.5 mM glucoseb | -12.60 ± 0.14                             | 207, 217     | 196        | 1.02         |

*aBroad shallow minimum.

*bSolvent system contains 30% (v/v) ACN.

![Figure 3](image-url) 1H NMR spectra of the amide region 250-μM tyrocidine A in ACN/water with the ACN concentration shown next to the spectra (v/v). The spectra were recorded at 500 MHz at ambient temperature.
Glucose led to significant deepening of the 188 ± 1 nm minimum and decrease of the 197 ± 1 nm maximum, compared with TrcA alone in 30% ACN (Figure 4A). At the highest Glc concentration (6.25 mM) or 1:25 Trc:Glc molar mixture, Glc led to spectral changes that approached the influence that TFE has on the TrcA CD spectrum (Figure 4, Table 1). The minima between 206 and 218 nm were less influenced, but some gain in intensity with the increase in Glc concentration over the whole range was observed. The $\theta_{206 \pm 1\text{nm}} / \theta_{217 \pm 1\text{nm}}$ ratio remained relatively stable and correlated with that of TrcA in >30% ACN (Table 1). Using the global influence of Glc on the CD spectrum over 185 to 240 nm of TrcA, we were able to calculate the apparent $K_d$ for the interaction of Glc with TrcA. With the one-site binding model (Equation 1) we calculated an apparent $K_d$ of 264 ± 96 µM, and with a slightly better fitting curve using the two-site binding model (Equation 2) a $K_{d1}$ of 45 µM and $K_{d2}$ of 20 mM (Figure 4B).

In order to better understand the changes due to the presence of Glc observed by CD spectroscopy, additional experiments were performed. Following the amide protons by $^1$H-NMR spectroscopy, we detected interesting differences among the residues in their response to increasing glucose concentrations (Figures 5, 6, and S4). Residue D-F1 showed a significantly sharper peak at high glucose concentrations and a slight change in chemical shift. Noteworthy, the resonances that change position due to Glc addition, all move down-field whereas the increased ACN concentrations induced up-field changes in peak position.

The largest shifts were observed for D-Phe$^6$ and Orn$^9$ followed by D-Phe$^4$, Val$^8$, Asn$^5$ and Tyr$^7$ in decreasing order ($\leq 0.039$ ppm). The

### TABLE 2
Comparison of H-bonding pattern in the X-ray structure of the TrcA dimer$^7$ and the $^1$H amide chemical shift changes upon addition of ACN to water or of glucose to TrcA in 30% ACN

| H-Bond (Amide to Carbonyl)$^a$ | Max Change in Amide Chemical Shift, ppm (−1000x) +%ACN | Max Change in Amide Chemical Shift, ppm (1000x) + Glucose | $K_d$ (µM) Glucose | $K_d$ (µM) Sucrose |
|---------------------------------|------------------------------------------------------|-------------------------------------------------------|-------------------|-------------------|
| F3—L10                          | 30 (20%-50%)                                         | 2                                                     | 197               | 174               |
| N5—V8                           | 70 (10%-50%)                                         | 9                                                     | 205               | 428               |
| L10—F3                          | 30 (10%-50%)                                         | 1                                                     | 106               | n.d.              |
| V8—N5 side chain                | 80 (10%-50%)                                         | 17                                                    | 232               | 343               |
| D-F1—Y7; D-F1′—Y7               | 70 (30%-50%)                                         | 23                                                    | 695               | 368               |
| O9—O9′; O9′—O9                 | 100 (20%-50%)                                         | 31                                                    | 221               | 474               |
| D-F4 (no H-bond)                | 80 (20%-50%)                                         | 39                                                    | 230               | 392               |
| Y7$^b$                          | 20 (20%-50%)                                         | 9                                                     | 187               | 348               |
| N5 s.c. (7.663 ppm)             | 60 (30%-50%)                                         | 20                                                    |                   |                   |
| Q6 s.c. (6.623 ppm)             | 60 (30%-50%)                                         | 20                                                    |                   |                   |

$^a$The first column indicates the H-bonding from NH of the first residue indicated to the carbonyls of the second residues as observed in the XR structure by Loll et al.$^7$ In NMR, the amide proton of the first residues is monitored. No chemical shift data are available for Pro$^2$ and Gln$^6$ (cf text for discussion). Y7′ and F1′ are the interactions partners on the second monomer of the symmetric dimer.

$^b$Possible H-bond to the N5 side chain.

![FIGURE 4 Influence of glucose concentration (0.025-6.25 mM) in the tyrocidine A solution on the CD spectra of 250-µM tyrocidine A in 30% ACN (v/v). Each spectrum depicts in A, an average of six acquired spectra, with background correction and smoothing over nine neighbours. Molar ellipticity considered only the concentration of tyrocidine A to eliminate the molar ellipticity dilution effect of glucose in the spectra. The graph in B, depicts the “one-site” and “two-site” hyperbolic binding curves fitted to the global changes in the TrcA CD spectrum from 185 to 240 nm as a function of glucose concentration](image)
FIGURE 5  $^1$H NMR spectra of the amide region 250-µM tyrocidine A in ACN/water 30/70 (v/v) in the presence of increasing amounts of glucose. The Glc-to-TrcA ratio is shown next to the spectra. The spectra were recorded at 500 MHz at ambient temperature.

FIGURE 6  The chemical shift alterations of TrcA amide resonances as a function of Glc/TrcA ratio (cf. Figure 5). The solid line represents the fits that provided the dissociation constants listed in Tables 2 and 3.
amide protons of Leu\(^{10}\) and Phe\(^{2}\) were not affected by the increasing glucose concentration (Figure 6) and were not considered for the quantitative analysis of binding. We plotted the shift against the glucose concentration for all residues and fitted the data towards a standard binding curve (Table 2, Figure 6). Similar dissociation constants (K\(_d\)) were extracted with a mean of 215 ± 35 µM. This K\(_d\) value correlates well with the K\(_d\) = 263 ± 96 µM observed with the one-site binding hyperbolic model used for the CD data (Figure 4). Based on the previously published structures for TrcA,\(^7,8\) the amide protons that are mostly affected by glucose are all surface exposed, whereas those involved in intramolecular hydrogen bonding hardly changed. In combination with the weak affinity, the differences in response suggest that the sugar is loosely bound to the surface of the peptide. We evaluated the generality of the glucose binding by including both tyrocidine B and tyrocidine C, which differ in one or both aromatic amino acids in position 3 or 3 and 4, respectively (Figures 1, S6, S7, S9, and S10). As for TrcA, the biggest effects were observed at position 4, 9, and 1, and the affinities were similar. When sucrose, a glucose-fructose disaccharide, was investigated (Figures 1 and S5), a slight decrease of the affinity is observed (K\(_d\) = 397 µM), but the affected amino acid residues were the same.

When performing the same experiment with cellotetraose, a cellulose oligomer containing four glucose moieties (Figures 1 and S8), we obtained a K\(_d\) of 50 µM. This value is in good agreement with a model containing four identical independent binding sites, each with an affinity that has been determined for the glucose monosaccharide towards TrcA. The pattern of affected amino acid residues was identical to that observed for glucose, confirming a similar mechanism of association (Table 3).

### 4 DISCUSSION

Because tyrocidines have been observed to interact with saccharides in the cell walls\(^{13}\) of fungal pathogens that are important in agriculture,\(^{13}\) and to provide potent antibiotic coverage for cellulose-based dressings, our goal was to investigate the interactions of saccharides with these cyclic AMPs. Initial structures have already been obtained from the peptide dissolved in 50% ACN for NMR studies,\(^8\) or from crystals made from methanol solution.\(^7\) In a first series of experiments, we screened the CD and \(^1\)H NMR spectra of Trc A as a function of solvent mixture in the solvents with varying ACN (Figures 2 and 3).

Increasing the ACN content drastically increased the CD of TrcA, leading to the deepening of minima from 207 to 220 nm and maintaining the 197-nm maximum at higher %ACN. At the same time, the \(^1\)H NMR spectral lines improved, both in intensity and resolution with the increase in %ACN of the TrcA solution. The CD data indicate that ACN caused an increase in the hydrogen bonding, possibly dissolving and rearranging larger aggregates which depend on aromatic stacking and other hydrophobic interactions. Increased hydrogen bonding interactions would, eg, result from more extended intermolecular \(\beta\)-sheet oligomers (Figure 2, Table 1), small enough for well-resolved NMR spectra (Figure 3).

Because many \(^1\)H NMR peaks start to appear and sharpen at 30% ACN, further experiments were performed in this mixture where most chemical shifts suggest that the fold of the peptide is close to the one in the aqueous solvent. At the same time, the good overall quality of the spectra is suggestive of small structures that tumble fast in the solution and that line broadening by chemical exchange with \(^1\)H of the aqueous solution or by conformational exchange is reduced. At this concentration of ACN, the peptide is close to a transition from broad to sharp resonances and the NMR spectra should be particularly sensitive to changes in the chemical environment that arise from interactions with the sugars, changes in solvation, or oligomerisation. Chemical shift assignments and J-couplings in the range of 9 Hz are in agreement with previous NMR structural investigations in 50% ACN and thereby the \(\beta\)-sheet and \(\beta\)-turn conformations found in prior NMR and X-ray crystallographic investigations.\(^7,6\) Notably, during the titrations, the J-couplings and \(\delta\)\(\_\)H chemical shifts remained unchanged indicating that the secondary structure of this circular peptide encompassing stable intramolecular H-bonds remained largely unaffected.

Whereas previously the Gln\(^6\) amide resonance was observed at 8.34 ppm when investigated at 50% ACN\(^6\), here we did not detect this NMR signal. Notably, Gln\(^6\) is in one of the loops and occupies an analogous position as Phe\(^2\) in the opposite loop region of the peptide. Therefore, we suspect that even at higher ACN concentrations, there is considerable line broadening of the Gln\(^6\) amide signal due to chemical exchange. Such a line broadening effect is clearly visible for

### TABLE 3  The chemical shift alterations and resulting apparent dissociation constants upon addition of glucose to various thyrocidin analogues or of various saccharides to TrcA

| Peptide               | Mean \(K_d\), µM | Maximum Chemical Shift Change, ppm | Most Shifting Amides          | Least Shifting Amides          |
|-----------------------|------------------|-----------------------------------|--------------------------------|--------------------------------|
| TrcA (F3, D-F4)       | 215              | 0.036                             | D-F4 > O9 > D-F1              | L10 < F3                       |
| TrcB (W3, D-F4)       | 286              | 0.035                             | D-F4 > O9 > D-F1              | L10 × Y7 ≈ N5                  |
| TrcC (W3, D-W4)       | 146              | 0.053                             | D-W4 > O9 > D-F1              | N5 < W3                        |
| Saccharide            |                  |                                   |                                |                                |
| Glucose               | 215              | 0.036                             | D-F4 > O9 > D-F1              | L10 < F3                       |
| Sucrose               | 397              | 0.038                             | D-F4 > O9 > D-F1              | L10 < F3                       |
| Cellotetraose         | 50               | 0.056                             | D-F4 > D-F1 > O9             | L10 < F3                       |
| Cellotetraose treated as 4 glucose | 200 |                                   |                                |                                |
The largest chemical shift changes due to the change of solvent or the addition of glucose occur for Phe⁴ and the Asn⁵-Gln⁶ side chains. Except for Asn⁵ in the crystal structure, these have not been found involved in intermolecular or intramolecular H-bonding interactions and should therefore be most exposed to the environment. However, large changes also occur with amides ¹H that are involved in intermolecular H-bonding capacity that help stabilise the dimer as well as potentially higher oligomeric assemblies (cf. Figure S2). The apparent dissociation constants between Trc A and all the sugars investigated are in the 10⁻⁴ M range and follow a stoichiometry that parallels the number of glucose moieties in the compound (four glucose moieties per peptide). These observations in combination are suggestive of a certain specificity in the interaction of this saccharide with tyrocidines. Interestingly, when compared with glucose, the cellotetraose exhibits an additive effect with regard to stoichiometry and dissociation constant suggesting that the glucose moieties interact with the peptide in a relatively independent fashion where each sugar can form H-bonds to a few sites at the peptide surface.

The CD data obtained when glucose is titrated into a TrcA solution in 30% ACN correlate with the NMR studies. Up to the highest Trc:Glc ratio of 1:25, the sugars result in increased CD, suggesting that they further enhance the H-bonding interactions (Figure 4). This could be due to glucose interfering with aromatic stacking, hydrophobic interactions, and H-bonding interactions that are responsible for the formation of larger aggregates and thereby favouring the formation of structured and better defined β-sheet oligomers and/or the CD spectra reflect additional H-bonding interactions between Glc and TrcA. One may speculate that at the higher concentrations the glucose molecules not only compete with aggregates but also with the formation of extended oligomers through intermolecular β-sheets.

Structural models of the glucose pyranose ring indicate that the associated hydroxyl groups are approximately 3 Å apart which matches the distance between the backbone NH and CO hydrogen donor/acceptors of successive amino acids in a pleated β-sheet. Therefore, four glucose molecules are needed to saturate the sites of the β-sheet. These could also be located at the dimer interface which in the X-ray structure is made up of four H-bonding donors/acceptors. Notably, the multiple binding sites for glucose on the peptide surface seem largely independent from each other when glucose and cellotetraose association are compared with each other (Table 3).

5 CONCLUSIONS

The CD and NMR spectroscopic investigations presented in this paper represent a first step to delineate the interactions of the cyclic tyrocidine decapeptides with the solvent and with saccharides. Thereby, the study provides a valuable insight in how the peptides can maintain their high antimicrobial efficiencies when interacting with bacterial cell surfaces, sugars that occur in agricultural environments or the food industry, as well as cellulose with antimicrobial properties that can
be used in a variety of applications. The data are suggestive that a more hydrophobic solvent results in the dissociation of large tyrocidine aggregates and the formation of oligomeric structures that are governed by β-sheet secondary structures. Upon addition of glucose, sucrose, or cellobiose, some of the NMR spectral changes from the addition of acetonitrile are reverted suggesting an increase in polarity and/or H-bonding interactions, in agreement with the CD spectral changes. The least affected amide $^1$H are those involved in intramolecular H-bonding, whereas the most affected are those exposed to the environment and/or involved in hydrogen bonding interactions of the TrcA dimers. The glucose-TrcA interactions in 30% ACN are characterized by a dissociation constant with glucose in the 10$^{-4}$ M range making the interactions with the monosaccharide somewhat specific when keeping association reversible. The association of cellobiose corresponds closely to the addition from four glucose moieties. An affinity in the 10$^{-4}$ M range represents a stable but reversible association and may be important when the peptides interact with the cell wall of target cells or are used to coat solid cellulose for antimicrobial dressings and materials.

ACKNOWLEDGEMENTS
We kindly acknowledge the help by Bruno Vincent from the NMR service, Chemistry, Strasbourg FR2010 and Marietjie Stander from the LCMS-CAF (Stellenbosch University). The financial contributions of the Hubert Curien PROTEA grant (Campus France 33948) to B.B. and South Africa/France PROTEA grant and South African National Research Foundation CSUR Grant to M.R., which have allowed to deepen the international collaboration between the teams involved in this project, the Région Grand-Est (previously Alsace) for funding a PhD position to X.B. together with the RTRA International Center of Frontier Research in Chemistry, the Agence Nationale de la Recherche (projects membraneDNP 12-BSV5-0012, MemPepSyn 14-CE34-0001-01, InMembrane 15-CE11-0017-01, Biosupramol 17-CE18-0033-3, and the LabEx Chemistry of Complex Systems 10-LABX-0026_CSC), the University of Strasbourg, and the CNRS are gratefully acknowledged. B.B. is grateful to the Institut Universitaire de France for providing additional time to be dedicated to research.

ORCID
Wilma van Rensburg https://orcid.org/0000-0001-7533-9138
J. Arnold Vosloo https://orcid.org/0000-0002-1304-6150
Marina Rautenbach https://orcid.org/0000-0001-7198-9213
Burkhard Bechinger https://orcid.org/0000-0001-5719-6073

REFERENCES
1. Dubos RJ, Hetchkiss RD. The production of bactericidal substances by aerobic sporulating bacilli. J Exp Med. 1941;73(5):629-640.
2. Rautenbach M, Troskie AM, Vosloo JA. Antifungal peptides: to be or not to be membrane active. Biochimie. 2016;130:132-145.
3. Mangoni ML, McDermott AM, Zasloff M. Antimicrobial peptides and wound healing: biological and therapeutic considerations. Exp Dermatol. 2016;25(3):167-173.
4. Dostert M, Belanger CR, Hancock REW. Design and assessment of anti-biofilm peptides: steps toward clinical application. J Innate Immun. 2018;1:1-12.
5. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002;415(6870):389-395.
6. Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol. 2011;29(9):464-472.
7. Loll PJ, Upton EC, Nahoum V, Economou NJ, Cocklin S. The high resolution structure of tyrocidine a reveals an amphipathic dimer. Biochim Biophys Acta. 1838;2014:1199-1207.
8. Munyuki G, Jackson GE, Venter GA, et al. Beta-sheet structures and dimer models of the two major tyrocidines, antimicrobial peptides from Bacillus aneurinolyticus. Biochemistry. 2013;52(44):7798-7806.
9. Tang XJ, Thibault P, Boyd RK. Characterization of the Tyrocidine and gramicidin fractions of the Tyrothricin complex from Bacillus-brevis using liquid chromatography and mass-spectrometry. Int J Mass Spectrom. 1992;122:153-179.
10. Rautenbach M, van Rensburg W, inventors; Method for preventing or treating microbial growth on a manufactured product. WO Patent 2015; PCT/IB2015/054166, WO2015186058A1. 2015.
11. Leussa AN, Rautenbach M. Detailed SAR and PCA of the tyrocidines and analogues towards leucocin A-sensitive and leucocin A-resistant listeria monocytogenes. Chem Biol Drug Des. 2014;84(5):543-557.
12. Spathelf BM, Rautenbach M. Anti-listerial activity and structure-activity relationships of the six major tyrocidines, cyclic decapeptides from Bacillus aneurinolyticus. Bioorg Med Chem. 2009;17(15):5541-5548.
13. Troskie AM, de Beer A, Vosloo JA, Jacobs K, Rautenbach M. Inhibition of agronomically relevant fungal phytopathogens by tyrocidines, cyclic antimicrobial peptides isolated from Bacillus aneurinolyticus. Microbiol. 2014;160(Pt_9):2089-2101.
14. Troskie AM, Rautenbach M, Delattin N, et al. Synergistic activity of the tyrocidines, antimicrobial cyclodecapeptides from Bacillus aneurinolyticus, with amphotericin B and caspofungin against Candida albicans biofilms. Antimicrob Agents Chemother. 2014;58(7):3697-3707.
15. Vosloo AJ, Beims H, Allsopp MH, et al. Tolerance of honey bee adults and larvae toward tyrothricin peptides derived from Brevibacillus parabrevis. Apidologie. 2017;48(6):833-844.
16. Wenzel M, Rautenbach M, Vosloo JA, et al. The multifaceted antibacterial mechanisms of the pioneering peptide antibiotics Tyrocidine and gramicidin S. MBio. 2018;9(5).
17. Cabib E, Arroyo J. How carbohydrates sculpt cells: chemical control of morphogenesis in the yeast cell wall. Nat Rev Microbiol. 2013;11(9):648-655.
18. Rautenbach M, Troskie AM, Vosloo JA, Dathe ME. Antifungal membranolytic activity of the tyrocidines against filamentous plant fungi. Biochimie. 2016;130:122-131.
19. Staus-Grabo M, Aïty S, Le T, Kretschmar M. Decade-long use of the antimicrobial peptide combination tyrothricin does not pose a major risk of acquired resistance with gram-positive bacteria and Candida spp. Pharmazie. 2014;69(11):838-841.
20. Miles AJ, Wallace BA. Circular dichroism spectroscopy of membrane proteins. Chem Soc Rev. 2016;45(18):4859-4872.
21. Perrone B, Miles AJ, Saltikov ES, Wallace B, Bechinger B. Lipid-interactions of the LAH4, a peptide with antimicrobial and nucleic transfection activities. Eur Biophys J. 2014;43(10-11):499-507.
22. Volevoda N, Schulthes T, Bechinger B, Seeig J. Thermodynamic and biophysical analysis of the membrane-association of a histidine-rich peptide with efficient antimicrobial and transfection activities. J Phys Chem B. 2015;119(30):9678-9687.
23. Michalek M, Salnikov ES, Werten S, Bechinger B. Membrane interactions of the amphipathic amino-terminus of huntingtin. Biochemistry. 2013;52(5):847-858.

24. Wüthrich K. NMR of Proteins and Nucleic Acids. New York: John Wiley & Sons; 1986;17(1):11-13.

25. Lesage A, Lelli M, Gajan D, et al. Surface enhanced NMR spectroscopy by dynamic nuclear polarization. J Am Chem Soc. 2010;132(44):15459-15461.

26. Gopinath T, Nelson SED, Veglia G. (1)H-detected MAS solid-state NMR experiments enable the simultaneous mapping of rigid and dynamic domains of membrane proteins. J Magn Reson. 2017;285:101-107.

27. Michalek M, Salnikov ES, Werten S, Bechinger B. Membrane interactions of the amphipathic amino-terminus of huntingtin. Biochemistry. 2013;52(5):847-858.

28. Meier BH, Riek R, Bockmann A. Emerging structural understanding of amyloid fibrils by solid-state NMR. Trends Biochem Sci. 2017;42(10):777-787.

29. Bechinger B. DNP solid-state NMR spectroscopy on membranes. eMagRes 2018:emrstm1558.

30. Bechinger B, Resende JM, Aisenbrey C. The structural and topological analysis of membrane-associated polypeptides by oriented solid-state NMR spectroscopy: established concepts and novel developments. Biophys Chem. 2011;153(2-3):115-125.

31. Rautenbach M, Vlok NM, Stander M, Hoppe HC. Inhibition of malaria parasite blood stages by tyrocidines, membrane-active cyclic peptide antibiotics from Bacillus brevis. Biochim Biophys Acta. 1862;2018:307-323.

32. Meier BH, Riek R, Bockmann A. Emerging structural understanding of amyloid fibrils by solid-state NMR. Trends Biochem Sci. 2017;42(10):777-787.

33. Laiken S, Printz M, Craig LC. Circular dichroism of the tyrocidines and gramicidin S. A. J Biol Chem. 1969;244:4454-4457.

34. Ruttenberg MA, King TP, Craig LC. The chemistry of tyrocidine. VII. Studies on association behavior and implications regarding conformation. Biochemistry. 1966;5(9):2857-2864.

35. Ruttenberg MA, King TP, Craig LC. The use of the tyrocidines for the study of conformation and aggregation behavior. J Am Chem Soc. 1965;87(18):4196-4198.

36. Paradies HH. Aggregation of tyrocidine in aqueous solutions. Biochem Bioph Res Comm. 1979;88(3):810-817.

37. Manning MC, Illangasekare M, Woody RW. Circular dichroism studies of distorted alpha-helices, twisted beta-sheets, and beta turns. Biophys Chem. 1988;31(1-2):77-86.

38. Juhl DW, van Rensburg W, Bossis X, Versloot JA, Rautenbach M, Bechinger B. Tyrocidine A interactions with saccharides investigated by CD and NMR spectroscopies. J Peptide Res. 1996;48(4):328-336.

39. Kuo MC, Gibbons WA. Determination of individual side-chain conformations, tertiary conformations, and molecular topology of tyrocidine A from scalar coupling constants and chemical shifts. Biochemistry-U. 1979;18(26):5855-5867.

40. Gibbons WA, Beyer CF, Dadok J, Sprecher RF, Wyssbrod HR. Studies of individual amino acid residues of the decapeptide tyrocidine A by proton double-resonance difference spectroscopy in the correlation mode. Biochemistry. 1975;14(2):420-429.

41. Kuo MC, Gibbons WA. Determination of individual side-chain conformations, tertiary conformations, and molecular topology of tyrocidine A from scalar coupling constants and chemical shifts. Biochemistry-U. 1979;18(26):5855-5867.

42. Juhl DW, van Rensburg W, Bossis X, Versloot JA, Rautenbach M, Bechinger B. Tyrocidine A interactions with saccharides investigated by CD and NMR spectroscopies. J Peptide Res. 1996;48(4):328-336. https://doi.org/10.1002/pse.3163

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Juhl DW, van Rensburg W, Bossis X, Versloot JA, Rautenbach M, Bechinger B. Tyrocidine A interactions with saccharides investigated by CD and NMR spectroscopies. J Peptide Res. 1996;48(4):328-336. https://doi.org/10.1002/pse.3163