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Tyrocidine A interactions with saccharides investigated by CD and NMR spectroscopies

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Tyrocidine A interactions with saccharides investigated by CD and NMR spectroscopies

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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 μ M. 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

Abbreviations: ACN, acetonitrile; AMP, antimicrobial peptide; Br. parabrevis, *Brevibacillus parabrevis*; CD, circular dichroism; ESMS, electrospray mass spectrometry; Glc, glucose; NMR, nuclear magnetic resonance spectroscopy; Orn, O, ornithine; Trc/s, tyrocidine/s; TrcA, tyrocidine A; UPLC, ultraperformance liquid chromatography; UPLC-MS, ultraperformance liquid chromatography linked to mass spectrometry; UV, ultra violet

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 gramicidin S in containing one of its pentapeptide repeats, as well as the constrained β -turn/ β -sheet structure.^{7,8} The tyrocidine 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 bees¹⁵ 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 Rensburg¹⁰ have shown that the tyrocidines are readily adsorbed onto cellulose matrixes where they maintain potent antibacterial 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 $\beta(1 \rightarrow 4)$ 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 solution²⁴ or in larger complexes such as membranes, amyloid fibres, polymers, or solid surfaces.²⁵⁻³⁰

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 three-dimensional X-ray structure of TrcA⁷ is shown including the intramolecular H-bonds (hatched lines). The amide ¹H with large changes in the chemical shift upon glucose titration are shown in magenta, those with little changes in light blue (cf. Figures 4 and 5)

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.³¹ using commercial tyrothricin (Sigma-Aldrich, Steinheim, Germany) or *Br. parabrevis* culture extracts, prepared as described by Vosloo et al.,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 µL) 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.

Ultraperformance liquid chromatography (UPLC) of samples on an Acquity UPLC BEH C₁₈ 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. Reconditioning of 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 µM (pH ≈ 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 ± 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 ultra violet (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:

$$\%Change\ in\ \Sigma\theta = \frac{\%Change_{max} \times [Glc]}{(K_d + [Glc])} \quad (1)$$

Or, second a two-site binding hyperbolic equation:

$$\%Change\ in\ \Sigma\theta = \frac{\%Change_{max1} \times [Glc]}{(K_{d1} + [Glc])} + \frac{Change_{max2} \times [Glc]}{(K_{d2} + [Glc])} \quad (2)$$

where $\Sigma\theta$ 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 µM. At first, the peptide was dissolved in ACN:H₂O with the ACN concentration varying from 2% to 50% (v/v) in steps of 10%. Sugar titrations were performed in ACN:H₂O:D₂O 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 ¹H NMR spectroscopy, additional two-dimensional experiment allowed assignment of the peak position. ¹H-¹H 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*₁ 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 (ΔCS), thereby allowing the determination of an apparent dissociation constant *K_d* following the relation:

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

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 $\geq 30\%$ ACN displayed minima at 189 ± 1 nm, 207 ± 1 nm, and 217 ± 1 nm,

FIGURE 2 Influence of % ACN in water (v/v) on the CD spectrum and structure of 250- μ M tyrocidine A. Each spectrum depicts an average of three acquisitions, with background correction and smoothing over nine neighbours. The spectrum of 250- μ M tyrocidine in 50% trifluoroethanol (TFE) containing 30% ACN and 20% water (v/v/v) is given as reference

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 α -helices. However, both the X-ray⁷ and the solution NMR structures of TrcA^{8,39-42} showed that this peptide and its dimer are dominated by β -turns and β -sheets. Therefore, for TrcA and its analogues, including gramicidin S, such distorted CD spectra are the result of the hydrogen-bonded β -structures.^{33,43-46}

The ellipticity at 206 to 218 nm is due to the $n \rightarrow \pi^*$ 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 β -turn and β -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 β -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 bond (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 increased number of hydrogen bonds (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 ^1H NMR spectra of 250- μ 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 singlets broadened by exchange. In 30% ACN (v/v), the ^1H amide resonances of eight residues can be seen and could be assigned to individual amino acids using 2D ^1H - ^1H spectroscopy²⁴ 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-Phe¹, D-Phe⁴, Asn⁵, Val⁸, and Orn⁹ 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 $^3J_{\text{HN-H}\alpha}$ scalar

TABLE 1 Comparison of CD spectra of 250- μ M TrcA in different media

Medium	Total CD molar $\theta \times 10^6 \pm$ SD (185–240), nm	Minima, nm	Maxima, nm	$\theta_{206 \pm 1 \text{ nm}}$ $\theta_{217 \pm 1 \text{ nm}}$
50% TFE ^b	-18.53 ± 0.88	188, 206, 217	196	1.10
15% ACN	-8.93 ± 0.36	187, 209–211 ^a , 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	-17.07 ± 0.93	190, 206, 218	197	1.06
0.025 mM glucose ^b	-9.54 ± 0.28	188, 207, 218	196	1.01
0.25 mM glucose ^b	-10.99 ± 0.37	189, 207, 217	196	1.01
2.5 mM glucose ^b	-12.60 ± 0.14	207, 217	196	1.02

^aBroad shallow minimum.^bSolvent system contains 30% (v/v) ACN. **FIGURE 3** ^1H NMR spectra of the amide region 250- μ M 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

couplings which were within <1 Hz of the published values for the NMR structure in 50% ACN.⁸ During the ACN titration, the coupling constants and the C_αH 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.⁷ 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 $\beta(1 \rightarrow 4)$ linked D-glucose, such as cellulose,¹⁰ 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, F4 Table 1). The transition that is most influenced by glucose was the $\pi \rightarrow \pi^*$ of p-electrons of $\text{C}=\text{O}$ (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.

TABLE 2 Comparison of H-bonding pattern in the X-ray structure of the TrcA dimer⁷ and the ¹H amide chemical shifts upon addition of glucose to TrcA in 30% ACN

H-Bond (Amide to Carbonyl) ^a	Max Change in Amide Chemical Shift (-1000×) (+%ACN)	Max Change in Amide Chemical Shift (1000×) + 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
F1–Y7'; F1'–Y7	70 (30%-50%)	23	695	368
O9–O9'; O9'–O9	100 (20%-50%)	31	221	474
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		

^aThe 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 for Pro 2 and Gln6 (cf text for discussion). Y7' and F1' are the interactions partners on the second monomer of the symmetric dimer.

^bPossible H-bond to 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

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 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 ¹H-NMR spectroscopy, we detected interesting differences among the residues in their response to increasing glucose concentrations (Figures 5, 6, and S4). Residue D-Phe¹ 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-Phe⁴ and Orn⁹ followed by D-Phe¹, Val⁸, Asn⁵ and Tyr⁷ in decreasing order (≤ 0.039 ppm). The

FIGURE 5 ^1H 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 Leu¹⁰ and Phe³ 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 $215 \pm 35 \mu\text{M}$. This K_d value

correlates well with the $K_d = 263 \pm 96 \mu\text{M}$ 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 ($K_d = 397 \mu\text{M}$), 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 $50 \mu\text{M}$. 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 walls¹⁸ 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 ¹H 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 ¹H-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 number of hydrogen bonds, 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 β -sheet oligomers (Figure 2, Table 1), small enough for well-resolved NMR spectra (Figure 3).

Because many ¹H 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 ¹H 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 β -sheet and β -turn conformations found in prior NMR and X-ray crystallographic investigations.^{7,8} Notably, during the titrations, the J-couplings and C α 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 Gln⁶ amide resonance was observed at 8.34 ppm when investigated at 50% ACN⁸, here we did not detect this NMR signal. Notably, Gln⁶ is in one of the loops and occupies an analogous position as Phe² in the opposite loop region of the peptide. Therefore, we suspect that even at higher ACN concentrations, there is considerable line broadening of the Gln⁶ amide signal due to chemical exchange. Such a line broadening effect is clearly visible for position 2 and seems even more pronounced for position 6, resulting in the annihilation of the resonance. Because the coalescence condition due to exchange varies with the resonance frequency, pH, temperature, peptide concentration, solvent, and other experimental details,

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)	193	0.036	D-F4 > O9 > D-F1	L10 < F3
TrcB (W3, D-F4)	289	0.035	D-F4 > O9 > D-F1	L10 < Y7 $\approx$ N5
TrcC (W3, W4)	178	0.053	D-W4 > O9 > D-F1	N5 < W3
Saccharide				
Glucose	193	0.036	D-F4 > O9 > D-F1	L10 < F3
Sucrose	360	0.038	D-F4 > O9 > D-F1	L10 < F3
Cellotetraose	45	0.056	D-F4 > D-F1 > O9	L10 < F3
Cellotetraose treated as 4 glucose	171			

it remains possible that in one experiment, the residue is apparent, whereas in a different setting it is missing. Whereas Munyuki et al⁸ recorded the spectra of 2 to 4 mg/mL TrcA in 50% ACN, the work presented here was performed with a 0.3 mg/mL sample at various ACN concentrations from 2% to 50% (v/v). Notably, also in our work, the Gln⁶ side chain CONH₂ protons appear at 6.64 and 7.14 ppm in 30% ACN (results not shown).

When saccharides were titrated into 250-μM Trc A in 30% ACN/water (v/v) up to concentrations of 6.25 mM, the same amides shifted both in the presence of sucrose and Glc, although the apparent association of Glc was somewhat stronger than that of sucrose. Whereas the changes in solvent composition (in the decamolar range) increased the shielding of the amides, the addition of saccharides (in the millimolar range) caused shifts of the peak positions in the opposite direction. The CD and chemical shift alterations due to the addition of Glc are of the same order of magnitude than the changes observed during the solvent exchange. At the same time, the molar amount of saccharide is orders of magnitudes lower than that of ACN; therefore, it is highly unlikely that this is a mere solvent effect. Nevertheless, the data in combination with the high H-bonding capacities of Glc and of the peptide are suggestive that H-bonding interactions and the polarity of the solvent are determinants for the amide chemical shifts. Whereas, the increase in ACN concentration reduces the number of water molecules in the proximity of the peptide, the glucose molecules associate probably involving significant H-bonding interactions with the surface exposed amides in the 10⁴ M⁻¹ range. Thereby, the sugars make the local environment more polar concomitant with chemical shift alterations that are inverse to those observed upon increase in organic solvents. A dissociation constant in the 250-μM range was consistently obtained by CD or NMR spectroscopy in 30% ACN and is probably increased in a more polar environment where the sugars find OH hydrogen binding partners from, for example, water.

The least shifting protons are from Phe³ and Leu¹⁰ (Figure 6) which in the X-ray structure form intramolecular H-bonds with each other.⁷ The H-bonding of Val⁸ which has an intramolecular H-bond to the Asn⁵ side chain shows intermediate chemical shift alterations. It is noteworthy that Phe³ in TrcA shows very little chemical shift alterations upon titration with glucose or sucrose, albeit its exchange for Trp³ in TrcB affects the apparent affinity for glucose suggesting that the exchange is not limited to the local environment but also changes some global physico-chemical features of the peptides including their tendency to oligomerise.

The largest chemical shift alterations upon addition of saccharides are observed for D-Phe¹, D-Phe⁴, and Orn⁹, which have their NH vector exposed to the exterior. The X-ray structure of the TrcA dimer indicates that D-Phe¹ and Orn⁹ are involved in intermolecular H-bonding, whereas Phe⁴ exposes the amide NH to the exterior (Figure S2 and Loll et al⁷).

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 amide ¹H that is involved in intermolecular H-bonding. Therefore, these data are suggestive that the saccharides compete with the 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.⁷ 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 cellotetraose, 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 ^1H 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 200- μM range making the interactions with the monosaccharide somewhat specific when keeping association reversible. The association of cellotetraose 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.

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