

Catherine F. Silverio  
Maryam Azad  
Frank A. Gomez

Department of Chemistry  
and Biochemistry,  
California State University,  
Los Angeles, CA, USA

## On-column derivatization of the antibiotics teicoplanin and ristocetin coupled to affinity capillary electrophoresis

Binding constants between the glycopeptides teicoplanin (Teic) and ristocetin (Rist) and their derivatives to D-Ala-D-Ala terminus peptides were determined by on-column receptor synthesis coupled to partial-filling affinity capillary electrophoresis (PFACE) or affinity capillary electrophoresis (ACE). In these techniques, the column is first partially filled with increasing concentrations of D-Ala-D-Ala terminus peptides. This is followed by plugs of buffer, antibiotic and two noninteracting standards, and acetic and/or succinic anhydride (and buffer in the case of ACE). The order of the reagent plugs containing the antibiotic and anhydride varies with the charge of the glycopeptide. Upon electrophoresis, the antibiotic reacts with the anhydride yielding a derivative of Teic or Rist. Continued electrophoresis results in the overlap of the derivatized antibiotic and the plug of D-Ala-D-Ala peptide. Analysis of the change in the relative migration time ratio (RMTR) of the new glycopeptide relative to the standards, as a function of the concentration of the D-Ala-D-Ala ligand yields a value for the binding constant  $K_b$ . The techniques described here can be used to assess how the derivatization of drugs alters their affinities for target molecules.

**Keywords:** Affinity capillary electrophoresis / Ristocetin / Teicoplanin

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### 1 Introduction

Glycopeptide antibiotics inhibit the growth of Gram-positive bacteria by hindering cell wall peptidoglycan biosynthesis [1–7]. These drugs bind to the D-Ala-D-Ala portion of peptidoglycan intermediates, thereby, inhibiting the transglycosylation reaction required for cross-linking of the cell wall. Vancomycin (Van) from *Streptomyces orientalis* is frequently called the antibiotic of last resort because it has been effective in treating infections caused by bacteria resistant to other antibiotics such as methicillin-resistant *Staphylococcus aureus*. However, bacteria have also conferred resistance to Van through the substitution of the D-Ala-D-Ala terminus of the peptidoglycan precursor by D-Ala-D-Lac [4, 8]. This has spurred interest in the design and synthesis of modified glycopeptides that may bind to other ligands on a bacteria cell wall.

**Correspondence:** Professor Frank A. Gomez, Department of Chemistry and Biochemistry, California State University, Los Angeles, 5151 State University Drive, Los Angeles, CA 90032-8202, USA

**E-mail:** fgomez2@calstatela.edu

**Fax:** + 323-343-6490

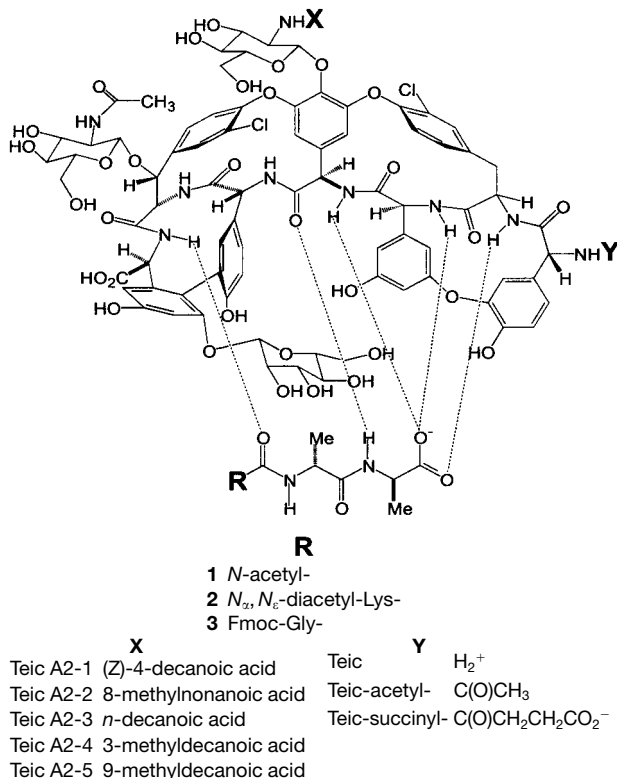
**Abbreviations:** ACE, affinity capillary electrophoresis; Fmoc, fluorenylmethoxycarbonyl; MO, mesityl oxide; PFACE, partial-filling affinity capillary electrophoresis; Rist, ristocetin; RMTR, relative migration time ratio; Teic, teicoplanin

Over the past decade, advances in molecular biology have elucidated a myriad of molecular interactions including protein-peptide, protein-DNA, and antibody-antigen. These interactions coupled to the great numbers of potential drug targets readily synthesized using combinatorial techniques have made the development of new analytical techniques for high-throughput screening of utmost importance. The ability to accurately determine the extent of interaction between a receptor and ligand is integral in rational drug design and development. Affinity capillary electrophoresis (ACE) is a versatile method that has been used to characterize a number of bimolecular noncovalent interactions [9–27]. For example, Progent *et al.* [9] used ACE to determine binding constants between anionic polydispersed polymers and peptides. Ding *et al.* [10] have examined the binding between porphyrins to human serum albumin using ACE. Finally, De Lorenzi *et al.* [11] used ACE to characterize the interaction between drugs and transthyretin. This technique uses the resolving power of CE to distinguish between the free and bound forms of a receptor as a function of the concentration of free ligand. Unlike other forms of binding assays, ACE does not require that free and bound receptor/ligand be known, hence, ACE simplifies the estimation of  $K_b$ .

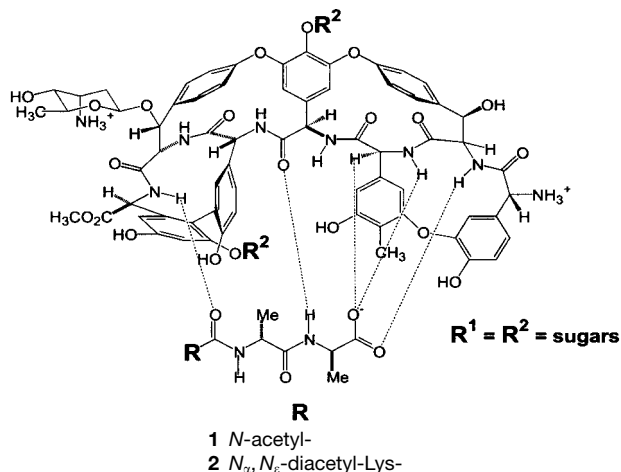
Recently, we showed that on-column derivatization could be coupled to ACE [12]. In these studies, separate plugs of glycopeptide, D-Ala-D-Ala terminus peptides, fluorenyl-

methoxycarbonyl-(Fmoc)-amino acid-*N*-hydroxysuccinimide (NHS) ester, and buffer (to initially shield the reagents from the receptor) are injected. Upon electrophoresis, a sample of newly synthesized peptide(s) and the noninteracting standard(s) are exposed to an increasing concentration of glycopeptide in a short plug causing a shift in the migration time of the ligand(s) relative to the standards. Subsequent Scatchard analysis yields a value for the binding constant(s),  $K_b$ .

Current glycopeptide research focuses on the modification of antibiotics to better understand the factors which promote and hinder binding to their target molecules. The ultimate goal is to develop drugs which can overcome the growing resistance of microorganisms to these antibiotics. Therefore, the development of efficient methods to determine how the derivatization of glycopeptides and other drugs affect their affinity for target molecules is critical. Teicoplanin (Teic) (Fig. 1) from *Actinoplanes teicomycetius* and ristocetin (Rist) (Fig. 2) from *Nocardia lurida*, like Van, inhibit cell wall synthesis by impeding the action of transglycosylases and transpeptidases [28–31]. Unlike Van, little information on the extent of binding of ligands to them is available. Hence, studies to elucidate the extent of binding are warranted and will assist in understanding the physicochemical details of glycopeptide binding behavior.



**Figure 1.** Teicoplanin complex, teicoplanin derivatives, and *D*-Ala-*D*-Ala terminus peptides 1–3 used in this study.



**Figure 2.** Ristocetin and *D*-Ala-*D*-Ala ligands 1–2 used in this study.

Herein, we combine on-column receptor synthesis and ACE to estimate the binding constants of *D*-Ala-*D*-Ala terminus peptides to the antibiotics Teic and Rist. The data described here demonstrate the advantages of using on-column receptor synthesis and ACE to estimate binding parameters of ligands for antibiotics. These methods can be further developed to evaluate a variety of on-column modified glycopeptides and drugs.

## 2 Materials and methods

### 2.1 Chemicals and reagents

All chemicals were analytical grade. Teicoplanin-HCl was purchased from Advanced Separation Technologies (Whippany, NJ, USA) and was used without further purification. Ristocetin was purchased from Bio Data Corporation (Horsham, PA, USA) and was used without further purification. Nicotinamide adenine dinucleotide (NAD), *N*-acetyl-*D*-Ala-*D*-Ala, **1**, and *N*<sub>2</sub>,*N*<sub>6</sub>-diacetyl-Lys-*D*-Ala-*D*-Ala, **2**, were purchased from Sigma Chemical Company (St. Louis, MO, USA) and were used without further purification. Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). Fmoc-Gly-*D*-Ala-*D*-Ala, **3**, was synthesized off-column based on literature procedures [13]. Stock solutions of NAD (1 mg/mL), Teic (0.4 mg/mL), and Rist (1 mg/mL), were each prepared by dissolving the samples in buffer (20 mM phosphate, pH 6.9). Stock solutions of acetic anhydride and succinic anhydride were prepared by dissolving the compounds in acetonitrile.

### 2.2 Apparatus

The capillary electrophoresis system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Phoenix,

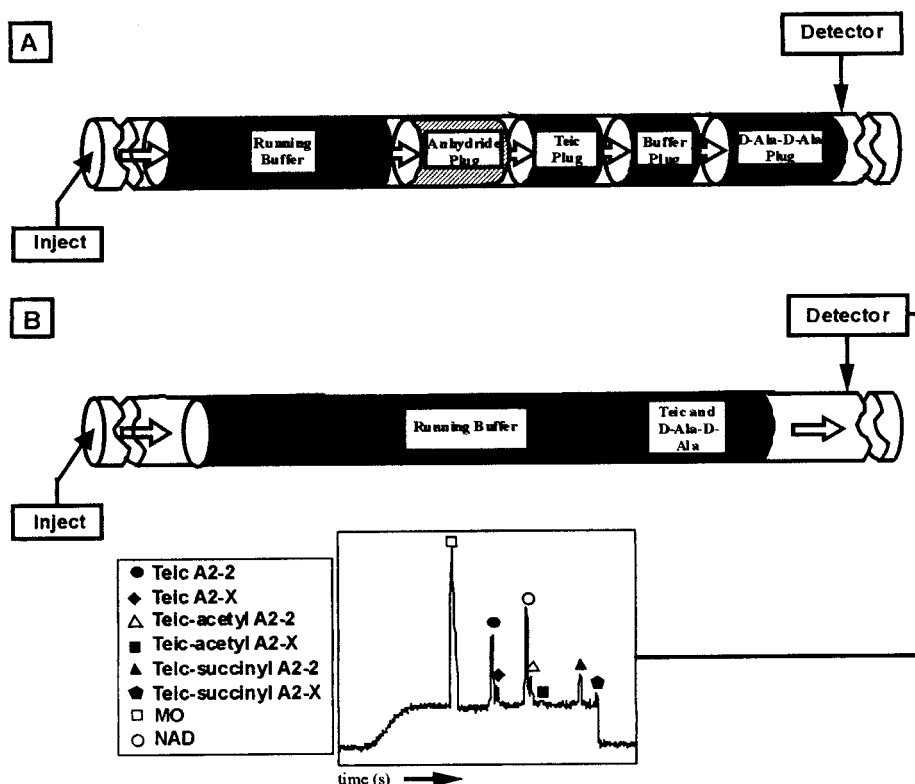
AZ, USA) was of uncoated fused silica with an internal diameter of 50  $\mu\text{m}$ , length from inlet to detector of 40.5 cm, and a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software. The conditions used in CE were as follows: voltage, 20 kV; current, 22  $\mu\text{A}$ ; detection, UV detection at 200, 214, 254 nm; temperature,  $25 \pm 0.1^\circ\text{C}$ .

### 2.3 Procedures

On-column receptor synthesis coupled to partial-filling ACE (PFACE): A sample of D-Ala-D-Ala ligand was vacuum-injected into the capillary for 8.0–12.0 s. Plugs containing buffer (3.6 nL), Teic (1.2–2.4 nL), and acetic and succinic anhydride (1.2–2.4 nL) were then introduced by vacuum injection. Electrophoresis was carried out using 20 mM phosphate buffer (pH 6.9) for 5.0 min. A similar procedure with Rist was used except that the anhydride plug preceded that of the Rist plug. On-column receptor synthesis coupled to ACE: The capillary was first equilibrated with buffer (20 mM phosphate; pH 6.9) at increasing concentrations of ligand. Plugs containing buffer (3.6 nL), Teic (1.2–2.4 nL), and acetic and succinic anhydride (1.2–2.4 nL), and buffer (3.6 nL) were then introduced by vacuum injection. Electrophoresis was carried out using increasing concentrations of the D-Ala-D-Ala ligands for 5.0 min.

### 3 Results and discussion

The Teic sample used in this experiment exists as a mixture of five compounds, Teic A2-2 (the main component) and four closely related minor compounds (Teic A2-X). These molecules differ at the *N*-acyl aliphatic chain at the  $\alpha$ -D-glucosamine at position 56. In the first series of experiments we examined the binding interaction between Teic and its derivatives to the peptide, *N*-acetyl-D-Ala-D-Ala, **1**. In these studies PFACE was used to estimate binding constants. In PFACE the capillary is partially filled with peptide. As long as a dynamic equilibrium is established between ligand and receptor prior to the point of detection a binding constant can be estimated. In the present studies a plug containing increasing concentrations of **1** was initially injected to partially fill the capillary. A buffer plug was then vacuum-injected into the capillary to separate the reagents from the ligand plug. The third injection contained Teic and the noninteracting standards MO and NAD. The final injection contained a mixture of succinic and acetic anhydride. Upon electrophoresis, the anhydrides and Teic plugs overlap and react forming new Teic derivatives. The buffer plug serves as a barrier between Teic and the D-Ala-D-Ala terminus peptides so mixing does not occur prior to ACE analysis. The Teic derivatives then migrate into the zone of **1** and a dynamic equi-

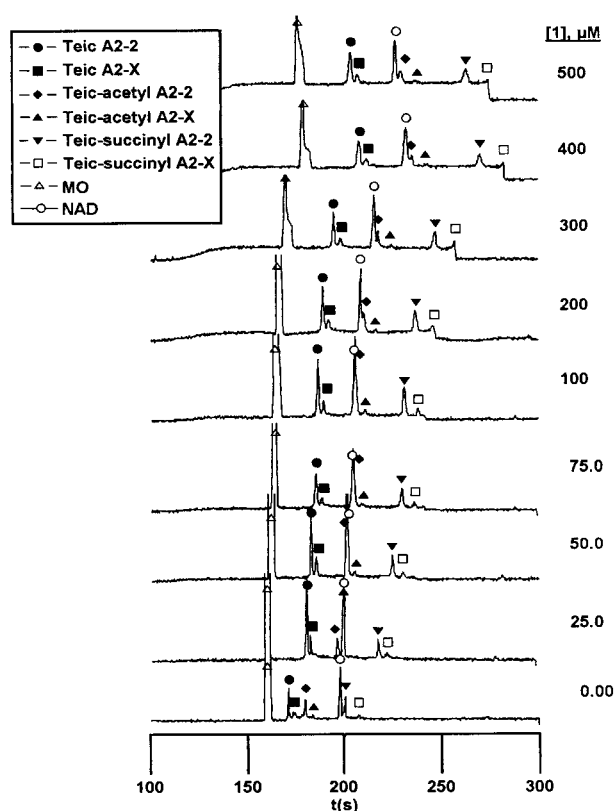


**Figure 3.** Schematic of on-column receptor synthesis coupled with PFACE experiment (A) before reaction and (B) after reaction.

brum is achieved as electrophoresis continues. Figure 3 is a representative example of on-column receptor synthesis coupled to ACE.

Figure 4 shows a representative series of electropherograms of Teic and its derivatives. The plug of **1** is observed as a box in the electropherogram which increases in height with increasing concentrations of **1** (0–500  $\mu\text{M}$ ) in the plug. The glycopeptide, its derivatives, and the D-Ala-D-Ala terminus peptides used in this study are all negatively charged at pH 6.9 and, hence, elute after the neutral marker MO. The difference in charge neutralized upon acetylation and succinylation dictates the difference in mobilities between Teic and its acetylated and succinylated derivatives. The  $\text{p}K_{\text{a}}$  of the N-terminus amine of Teic is 7.1. This equates to an approximate charge difference between Teic and its acetylated and succinylated forms of 0.4 and 1.4, respectively.

Upon addition of increasing concentrations of **1** in the running buffer the migration times of Teic and its derivatives shift to greater migration times. The complexation



**Figure 4.** A representative series of electropherograms of Teic and its derivatives in 20 mM phosphate buffer (pH 6.9) containing various concentrations of **1**, using on-column receptor synthesis coupled to PFACE. The total analysis time in each experiment was 5.0 min at 20 kV using a 46.5 cm (inlet to detector) 50  $\mu\text{m}$  ID open, uncoated quartz capillary.

between **1** and the Teic derivatives resulted in an increasing negative charge on the compounds and the complexes are detected later than the uncomplexed form. At the point of saturation, the Teic peaks no longer shift to the right despite increasing concentrations of **1** in the running buffer.

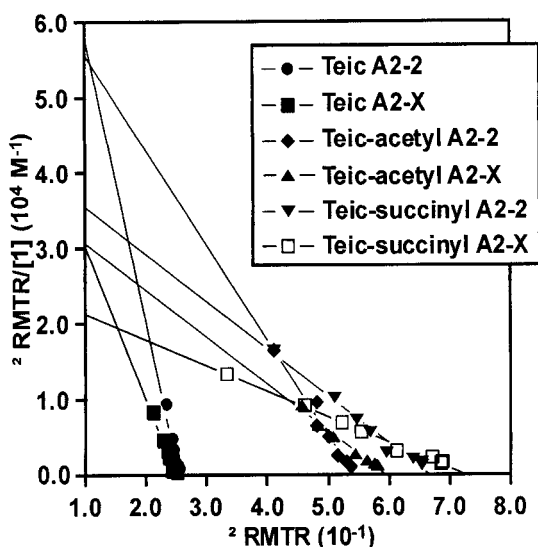
Further proof that derivatization of Teic occurs is that Teic and Teic-acetyl-A2-2 elute at the same place, relative to the internal standards, as do Teic-acetyl-A2-2 and Teic-succinyl-A2-2, respectively. These results also demonstrate that derivatization of Teic has a greater effect on changing the charge than the mass. Teic-A2-X exists in much smaller concentrations than the major form of Teic and upon derivatization yields much smaller peaks for its acetylated and succinylated forms.

Figure 5 is a Scatchard plot of the data for Teic and its derivatives. In this form of analysis  $K_{\text{b}}$  is estimated using a dual-marker form of analysis, which we term the relative migration time ratio (RMTR) (Eq. 1) [25].

$$\text{RMTR} = (t_r - t_s') / (t_s' - t_s) \quad (1)$$

Using this form of analysis, the change in migration time of a receptor is referenced to two noninteracting standards. Here,  $t_r$ ,  $t_s$ , and  $t_s'$  are the measured migration times of the receptor peak, and two noninteracting standard peaks, respectively. In the present experiments,  $t_s$  and  $t_s'$  are the migration times of MO and NAD, respectively. A Scatchard plot can be obtained via Eq. (2).

$$\Delta \text{RMTR}_{\text{R,L}}/[L] = K_{\text{b}} \Delta \text{RMTR}_{\text{R,L}}^{\text{max}} - K_{\text{b}} \Delta \text{RMTR}_{\text{R,L}} \quad (2)$$



**Figure 5.** Scatchard plot of Teic and its derivatives with **1**.

Here,  $\Delta\text{RMTR}_{\text{R,L}}$  is the magnitude of the change in the RMTR as a function of the concentration of peptide. Equation (2) allows for the estimation of  $K_b$  on a relative time scale using two noninteracting standards and compensates for fluctuations in voltage and electroosmotic flow (EOF) in the capillary column that may occur due to changes in viscosity of the buffer.

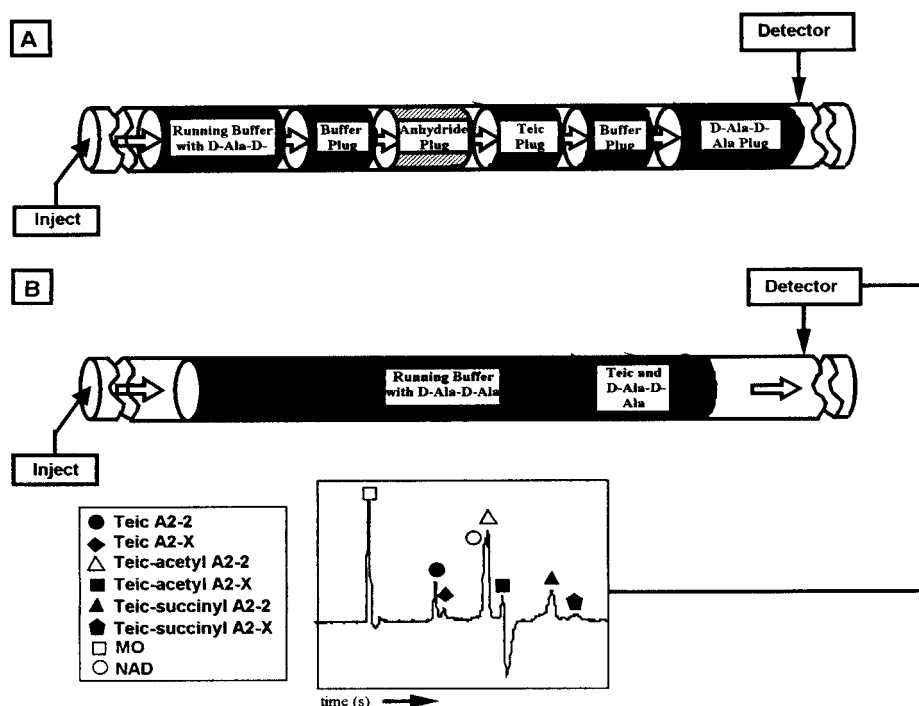
In the second series of experiments, we examined the on-column synthesis of the Teic derivatives coupled to standard ACE techniques to analyze the binding of these receptors to the peptides **2** and **3**. The change in charge upon ligand binding to Teic-succinyl-A2-2 and Teic-succinyl-A2-X precludes the use of PFACE since these peaks migrate slower than the ligand box, thereby, hindering the establishment of a dynamic equilibrium. In these studies the column is first equilibrated with increasing concentrations of the peptide. This is followed by four separate plugs of buffer, Teic sample (also containing the noninteracting standards), anhydride in acetonitrile, and buffer. Electrophoresis proceeds in increasing concentrations of peptide in the running buffer. Overlap of the separate zones of reagent plugs yield the new acetylated and succinylated Teic derivatives. The zone of ligand then migrates into the zone of the Teic derivatives and a dynamic equilibrium is achieved. Figure 6 is a schematic of on-column receptor synthesis coupled to ACE.

Figure 7 shows a representative series of electropherograms of Teic and their derivatives in a capillary filled with increasing concentrations of **2** at 214 nm. With the

addition of increasing concentrations of **2** in the running buffer, Teic shifts to the right (longer migration time) and increases in height because the mass of the newly formed complex is greater than the ligand itself. The inverted peak in Fig. 7 represents the increasing amount of Teic bound to **2** as the concentration of the ligand is increased in the running buffer. It is caused by the dilution of **2** on binding to Teic and its derivatives. At high concentrations of **2** the inverted peak makes analysis of Teic-acetyl A2-X difficult since it elutes at a similar migration time. Hence, some data points were not included in the analysis of Teic-acetyl-A2-2. In the case of **3**, its binding to Teic-succinyl-A2-X was not determined. Figure 8 is a Scatchard plot of data for **2** according to Eq. (2).

Table 1 summarizes the binding data for Teic and their derivatives to ligands **1–3**. A decrease in binding is found upon derivatizing Teic-A2-2 and A2-X. The data obtained by these experiments are consistent with previous work, which show that the binding constant decreases between the glycopeptides and D-Ala-D-Ala target ligands upon derivatization [32]. The extent of this change in binding varies with the glycopeptide as well as the ligand studied.

The data demonstrate that modifying the charge on the N-terminus does change the ability of Teic to complex with D-Ala-D-Ala terminus peptide ligands. Similar results have been observed by other research groups demonstrating that modification of the N-terminus amino group

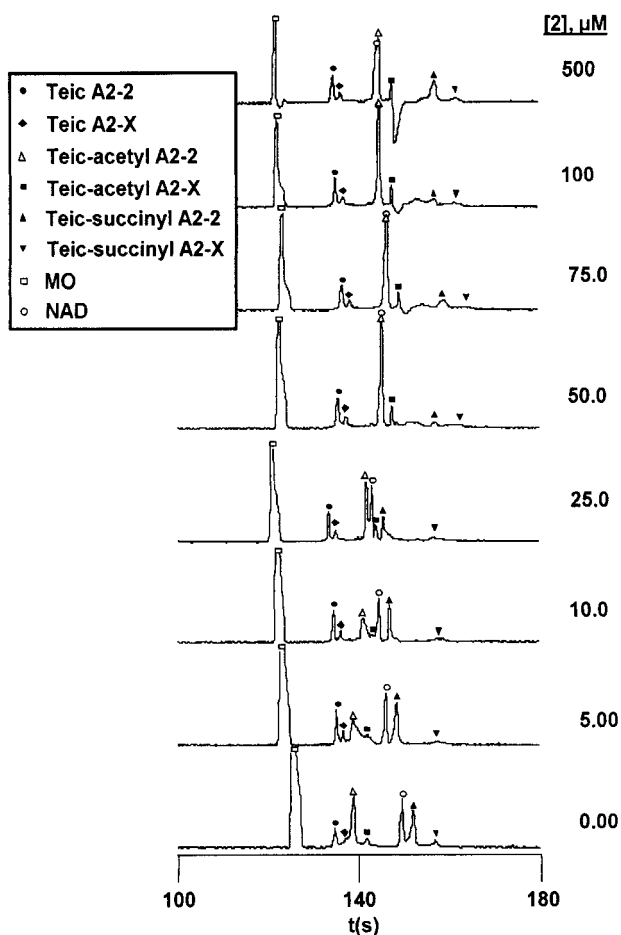
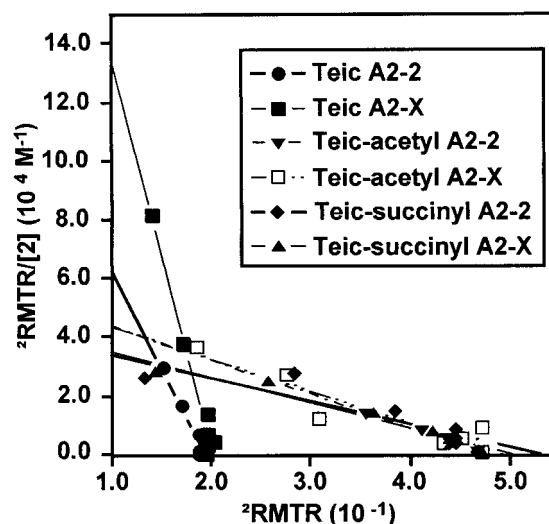


**Figure 6.** Schematic of on-column receptor synthesis coupled with ACE experiment (A) before reaction and (B) after reaction.

**Table 1.** Experimental values of binding constants  $K_b$  ( $10^4 \text{ M}^{-1}$ ) of Teic-A2-2 and A2-X with ligands 1–3 measured by the on-column receptor synthesis PFACE/ACE technique

Antibiotic	$K_b$ ( $10^4 \text{ M}^{-1}$ ) (corr. coeff.)		
	1	2	3
Teic	37 (0.94)	65 (0.95)	140 (0.94)
Teic-A2-X	20 (0.98)	130 (0.98)	140 (0.99)
Teic-acetyl-A2-2	13 (0.98)	7.9 (0.89)	16 (0.98)
Teic-acetyl-A2-X	6.2 (0.99)	8.7 (0.97)	7.6 (0.99)
Teic-succinyl-A2-2	6.2 (0.99)	12 (0.99)	5.0 (0.96)
Teic-succinyl-A2-X	3.4 (0.99)	11 (0.93)	nd

nd, not determined

**Figure 7.** A representative series of electropherograms of Teic and its derivatives in 20 mM phosphate buffer (pH 6.9) containing various concentrations of **2**, using on-column receptor synthesis coupled to ACE. The total analysis time in each experiment was 5.0 min at 20 kV using a 46.5 cm (inlet to detector) 50  $\mu\text{m}$  ID open, uncoated quartz capillary.**Figure 8.** Scatchard plot of Teic and its derivatives with **2**.

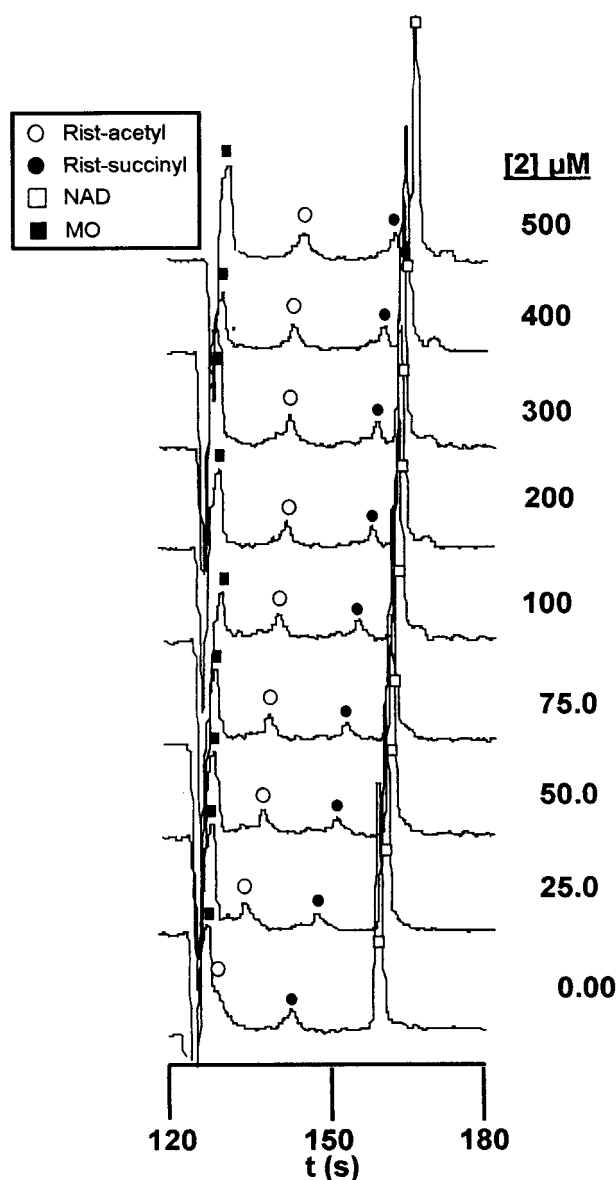
does alter its ability to cause bacterial cell death [15]. However, upon acetylation and succinylation, the extent of binding between Teic and their D-Ala-D-Ala ligands does decrease. It is proposed that the charged N-terminus plays a key role in stabilizing an initial weak coulombic complex between the glycopeptide and D-Ala-D-Ala ligand [33]. This complex only exists for a short time before it undergoes a conformational change to its fully bonded state predominantly involving five hydrogen bonds.

There is variation in the literature with respect to binding data obtained for glycopeptides and their D-Ala-D-Ala target molecules which can be attributed to difference in pH and solvent [28]. The current studies were conducted in phosphate buffer at pH 6.9. Acetonitrile was used as the solvent for the anhydrides to slow hydrolysis in aqueous media. The decrease in binding strength between Rist and Van with increasing mole fractions of acetonitrile is well-known and is caused by the deleterious effect of acetonitrile taking the place of water in a receptor's solvent sphere [28]. This correlates to a predicted hydrophobic effect between the interaction of the alanyl methyl groups of D-Ala-D-Ala ligands with the antibiotics as well as between the sugar residues of the glycopeptides with the ligands.

In the third study, on-column acetylation and succinylation of the antibiotic Rist coupled with PFACE allows for the resolution and determination of binding constants for the new derivatives. While Teic and Van possess a carboxylic acid at its C-terminus, the corresponding group on Rist is esterified. Hence, Rist is positively charged at pH 6.9 and elutes prior to the neutral marker MO. Upon derivatization at the N-terminus, Rist-acetyl is formed

and elutes slightly after MO. Rist-succinyl is negatively charged and differs from Rist-acetyl by a charge of one. In these experiments, we examined the interaction of Rist derivatives to ligands **1** and **2**. Binding constants of these ligands to Rist were determined in separate ACE experiments.

A plug containing increasing concentrations of **2** was injected to partially fill the capillary. A buffer plug was then vacuum-injected into the capillary to separate the



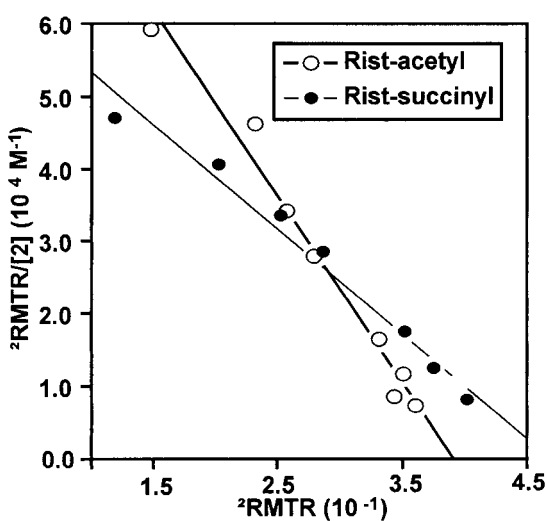
**Figure 9.** A representative series of electropherograms of Rist derivatives in 20 mM phosphate buffer (pH 6.9) containing various concentrations of **2**, using on-column receptor synthesis coupled to PFACE. The total analysis time in each experiment was 5.0 min at 20 kV using a 46.5 cm (inlet to detector) 50  $\mu\text{m}$  ID open, uncoated quartz capillary.

reagents from the ligand plug. The third injection contained a mixture of succinic and acetic anhydride while the fourth plug contained Rist, MO, and NAD. Upon electrophoresis, the overlap of the separate zones of reagent plugs yields the Rist derivatives. Ligand **2** is more negative than the Rist derivatives used in this experiment and, hence, PFACE was used in this study.

Figure 9 shows a representative series of electropherograms of Rist in buffer plugs containing increasing concentrations of **2**. The plug of **2** is not observed in these electropherograms because unlabeled D-Ala-D-Ala ligands do not absorb at the detection wavelength of 254 nm at low concentrations. Upon addition of increasing concentrations of **2** both Rist derivative peaks shift to the right. The complexation between **2** and Rist-acetyl and Rist-succinyl resulted in an increasing negative charge on the derivatives and the complexes are detected later than the uncomplexed forms. At the point of saturation, the Rist derivative peaks no longer shift. Figure 10 is the Scatchard plot for Rist-acetyl and Rist-succinyl and ligand **2**. Table 2 summarizes the binding data for Rist and its derivatives to peptides **1** and **2**. A similar binding trend as that

**Table 2.** Experimental values of binding constants  $K_b$  ( $10^4 \text{ M}^{-1}$ ) of Rist and its derivatives with ligands **1–2** measured by the on-column receptor synthesis PFACE technique

Antibiotic	$K_b$ ( $10^4 \text{ M}^{-1}$ ) (corr. coeff.)	
	<b>1</b>	<b>2</b>
Rist	4.8 (0.99)	10 (0.96)
Rist-acetyl	1.7 (0.99)	3.1 (0.97)
Rist-succinyl	0.9 (0.99)	1.8 (0.98)



**Figure 10.** Scatchard plot of Rist derivatives with **2**.

found for Teic and its derivatives was obtained, whereby, upon derivatization the *N*-terminus binding to D-Ala-D-Ala peptides is weakened.

The decrease in the binding affinity of Teic for **1** and **3** and Rist for **1** and **2** upon acetylation and succinylation demonstrates that electrostatics may play a key role in the affinity of glycopeptides for D-Ala-D-Ala ligands. The extent of this contribution can be measured by the change in free energy of binding upon modification of the antibiotic assuming that the change in affinity is based on charge rather than steric or other effects [33]. The affinity of the acetylated Teic for ligands **1–3** is 18 to 3 times weaker than that of Teic corresponding to a loss of 3–7 kJ/mol in the free energy of binding. The affinity of the acetylated Rist for ligands **1–2** is 3 times weaker than that of Rist corresponding to a loss of 3 kJ/mol in the free energy of binding.

#### 4 Concluding remarks

This report demonstrates the ease of using on-column receptor synthesis coupled to PFACE/ACE and how it can be used to determine binding constants of antibiotics to target molecules. The data presented here can be used to determine how the derivatization of drugs alters their affinity for target molecules. In the present studies we have used on-column synthesis to derivatize Teic and Rist forming the acetylated and succinylated derivatives of each. Subsequent binding constant measurements using either PFACE or ACE have been used to calculate the contribution of electrostatic interaction between the amine terminus of the glycopeptides and the carboxy terminus of the D-Ala-D-Ala ligands. Upon derivatization extent of binding between the derivatives and D-Ala-D-Ala terminus peptides decreases. The current studies demonstrate how on-column techniques can be coupled to ACE to estimate affinity parameters. Further studies demonstrating the versatility of the techniques are in progress.

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