UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
Katedra biochemických věd

Kvantitativní vazba různých analogů vankomycinu na D-Ala-D-Ala za použití povrchové plasmonové resonance

Diplomová práce

ve spolupráci s
UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA
Departamento de química orgánica y farmacéutica

Vedoucí diplomové práce:
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Madrid, Hradec Králové 2009 Ivana Černá

CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
DEPARTMENT OF BIOCHEMISTRY

Quantitative binding of different analogues of vancomycine to D-Ala-D-Ala using surface plasmon resonance

Diploma thesis

in cooperation with
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Madrid, Hradec Králové 2009

Ivana Černá

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

I declare, that I have written this diploma thesis on my own. All references and other sources which were used are named and cited properly in the text.

Acknowledgements

I would like to thank to professor María José Hernáiz Gómez-Dégano and Doc. Ing. Barbora Szotáková, Ph.D for their friendship, patience and all the support they gave me while supervising my diploma thesis.

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ABSTRAKT

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra biochemických věd

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Doc. Ing. Barbora Szotáková, PhD.

Název diplomové práce:

Kvantitativní vazba různých analogů vankomycinu na D-Ala-D-Ala za použití povrchové plasmonové resonance

Tato diplomová práce pojednává o interakcích glykopeptidových antibiotik: teicoplaninu; MDL 63,246; mideplaninu; BI 397 (dalbavancin); A 40926 a vancomycinu s dipeptidem D-Ala-D-Ala. Pro tato antibiotika byly stanoveny a optimalizovány podmínky HPLC analýzy. Pro výzkum vazby uvedených antibiotik na bakteriální stěnu byly připravovány a zkoušeny samotvořící vrstvy (SAM) různých vlastností a různé délky řetězce, prezentující D-Ala-D-Ala. Výsledky dokazují, že nejvhodnější způsob přípravy SAM je inkubace čipu přes noc v etanolovém roztoku alkanthiolu. Nejvhodnější pro studium interakce antibiotikum – D-Ala-D-Ala je SAM tvořena osmiuhlíkovým řetězcem a s několika polyethylenglykolovými skupinami na druhé straně, zakončené karboxylovou skupinou. Na tomto povrchu jsme provedli měření interakcí. Všechny zkoumané glykopeptidy mají k povrchu vyšší afinitu než vankomycin, zvláště BI 397 - dalbavancin.

ABSTRACT

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Biochemical Sciences

Candidate: Ivana Černá
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Title:

Quantitative binding of different analogues of vancomycine to D-Ala-D-Ala using surface plasmon resonance

This diploma thesis discusses the interaction studies of different glycopeptide antibiotics: teicoplanin; MDL 63,246; mideplanin; BI 397 (dalbavancin); A 40926 and vancomycin with D-Ala-D-Ala dipeptide. Firstly, the HPLC analysis conditions for these antibiotics were defined and optimized in order to probe their purity. Then, the interaction studies were carried out, for that various self-assembled monolayers (SAM) were prepared based on different hydrophobicity and length of the chain. These SAMs were functionalized with the dipeptide D-Ala-D-Ala for the study of the binding with the antibiotics. The results suggest that the best way to prepare the SAM is incubation of the chip overnight in the ethanol solution of alcanothiol chain. The most applicable SAM for the study of interaction of antibiotic to D-Ala-D-Ala is formed by alcanothiol chain with a carbon chain of 8 carbons and a tetraethylene glycol chain ending in a carboxylic group. Over this surface we performed the study of interactions. All of the glycopeptidic antibiotics show stronger interactions that vancomycin, especially BI 397 - dalbavancin.

Contents

Abbreviations:

Au: Gold
BIA: Biomolecular interaction analysis
D-Ala-D-Ala: D-alanyl-D-alanine
DNA: Desoxyribonucleic acid
EDC: 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimid-hydrochlorid
HPLC: High performance liquid chromatography
Lac: Lactate
Lys: Lysine
MALDI-TOF: matrix assisted laser desorption ionisation - Time of Flight
MBC: Minimum bactericidal concentration
MIC: Minimum inhibitory concentration
MRSA: Methicillin Resistant Staphylococcus Aureus
NHS: *N*-hydroxysuccinimide
PEG: Poly(ethylene glycol)
RU: Response unit (1 RU = 0,0001°surface plasmon resonance angle shift in the Biacore)
SAM: Self-assembled monolayer
SPR: Surface plasmon resonance

1. INTRODUCTION

The emergence of bacteria that are resistant to vancomycin is a growing problem in clinical practice. Vancomycin acts by binding to the carboxy-terminal D-Ala-D-Ala of the bacterial cell wall mucopeptide precursors and creates a weak point in the cell walls.

Unfortunatelly, resistance to vancomycine is now increasing, and the accompanying increase in the number of deaths from bacterial infections has given new urgency to the search for novel antibiotics of this class.

In the last years new techniques have been developed to study interactions of biomolecules to understand the biological role at molecular level. In this field, some of new techniques that require the immobilization of at least one of the molecules are designed to perform the studies. One of the techniques mentioned above is Surface Plasmon Resonance (SPR). SPR is an optical phenomenon that provides a non-invasive, label-free means of observing binding interactions between an injected analyte and an immobilized biomolecule in real time.

Oligo(ethylene glycol)-terminated self-assembled monolayers resembling the surface of a cellular membrane are being used for the study of adsorption in biosensing.

2. THEORETICAL PART

2.1 Antibiotics

2.1.1 Glycopeptide antibiotics

Glycopeptide antibiotics are complex natural products biosynthesized by several actinomycete genera. They play a significant role in the therapy for Gram-positive bacterial infections.[1] They inhibit bacterial growth by interfering with cell wall biosynthesis.

Glycopeptide antibiotics have a complex structure of polypeptide aglycones, which are glycosylated with mono-, di-, and tetrasaccharides. They are usually further embellished by chlorination, glycosylation, methylation, acetylation and/or sulfation. The clinically used glycopeptides vancomycin and teicoplanin have become last resort antibiotics against multi-resistant Gram positive pathogens. Significant step in research of glycopeptide antibiotics has been done in last 20 years. The resistance among Gram-positive pathogens towards glycopeptide antibiotics has stimulated the research of second-generation glycopeptides with improved properties and expanded antimicrobial spectrum or better pharmacokinetics: MDL 63,246 and A40926. This has created considerable interest in augmenting the structural diversity of glycopeptides by complementing chemical methods, which are limited to few accessible positions, with biological means.[2]

Approximately 100 members of this group have been discovered so far, such as vancomycin, teicoplanin, bleomycin etc. Some of them are very important as the last possible treatment of resistant bacteria infections – methicilin resistant *Staphylococcus aureus*. Recent discoveries demonstrated that glycosidic residues are important in their activity.[3]

Molecular modeling and experimental studies indicate that vancomycin forms a stoichiometric complex with the D-Ala-D-Ala dipeptide via the formation of five hydrogen bonds with peptidic backbone of the glycopeptide (Figure 1).[4] The antibiotic prevents subsequent biochemical processes and leads to the death of the bacteria.

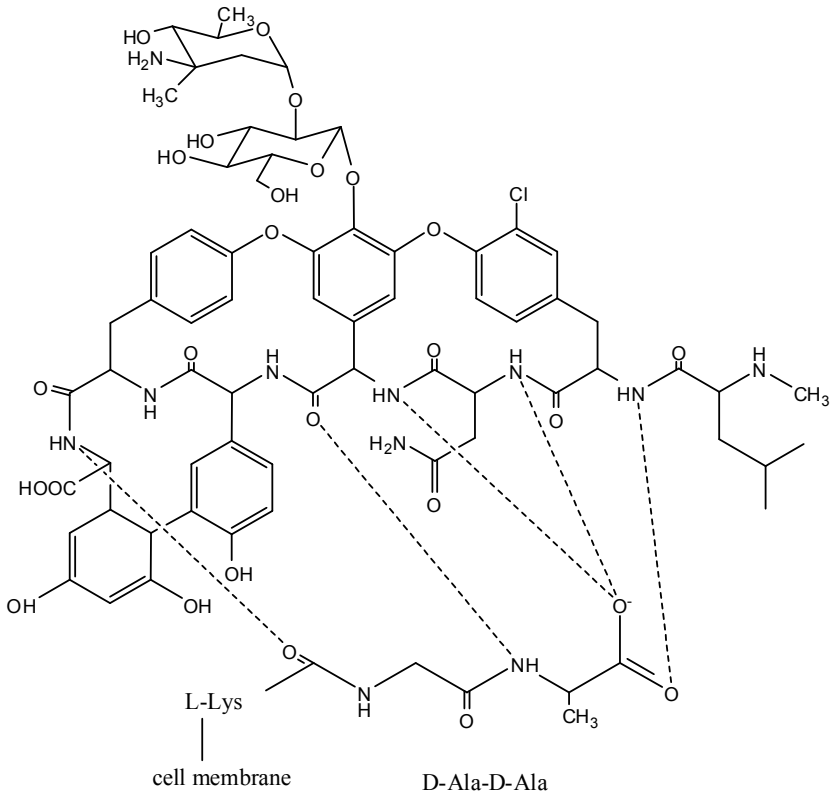

Figure 1: Binding of vancomycin to the D-Ala-D-Ala terminus

The molecular principles of the resistance to antibiotics of this group have been studied quite well. In the glycopeptide-resistant enterococci the C-terminal -Lys-D-Ala-D-Ala peptide fragment is replaced by the depsipeptide fragment -Lys-D-Ala-D-Lac. This makes it impossible to form one of the five hydrogen bonds in the antibiotic-target complex, which leads to a significant decrease in its stability. It was shown on model peptides that the binding constant of vancomycin with Lys-D-Ala-D-Lac is three orders of magnitude lower than that of Lys-D-Ala-D-Ala (Figure 2) [5].

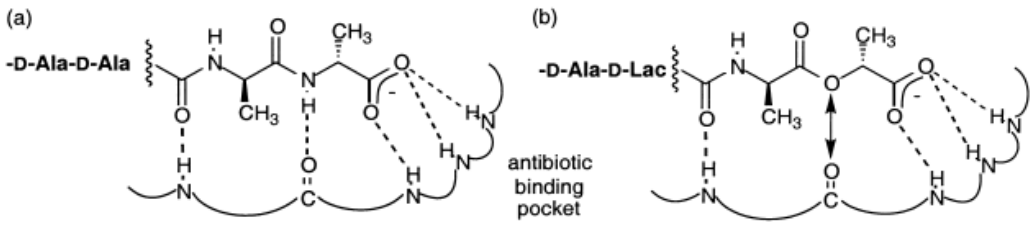

Figure 2: Hydrogen bonding interactions (dashed lines) between the binding pocket of vancomycin and peptides terminating in (a) -D-Ala-D-Ala and (b) -D-Ala-D-Lac. In (b) the repulsive interaction is shown with a double-headed arrow [6]

Teicoplanin

Teicoplanin is a glycopeptide antibiotic derived from *Actinoplanes teicomyceticus* and is chemically related to vancomycin. It has potent *in vitro* activity against Grampositive organisms and has slightly greater activity against *S. aureus* than vancomycin. Teicoplanin has a longer half-life than vancomycin, which is explained by higher capacity to bind serum proteins. It can be administered both intravenously and intramuscularly, and may be less toxic than vancomycin.[7]

It has several potential advantages over vancomycin. Perhaps because of its lipophilic character, is characterized by a higher volume of distribution than vancomycin.[4]

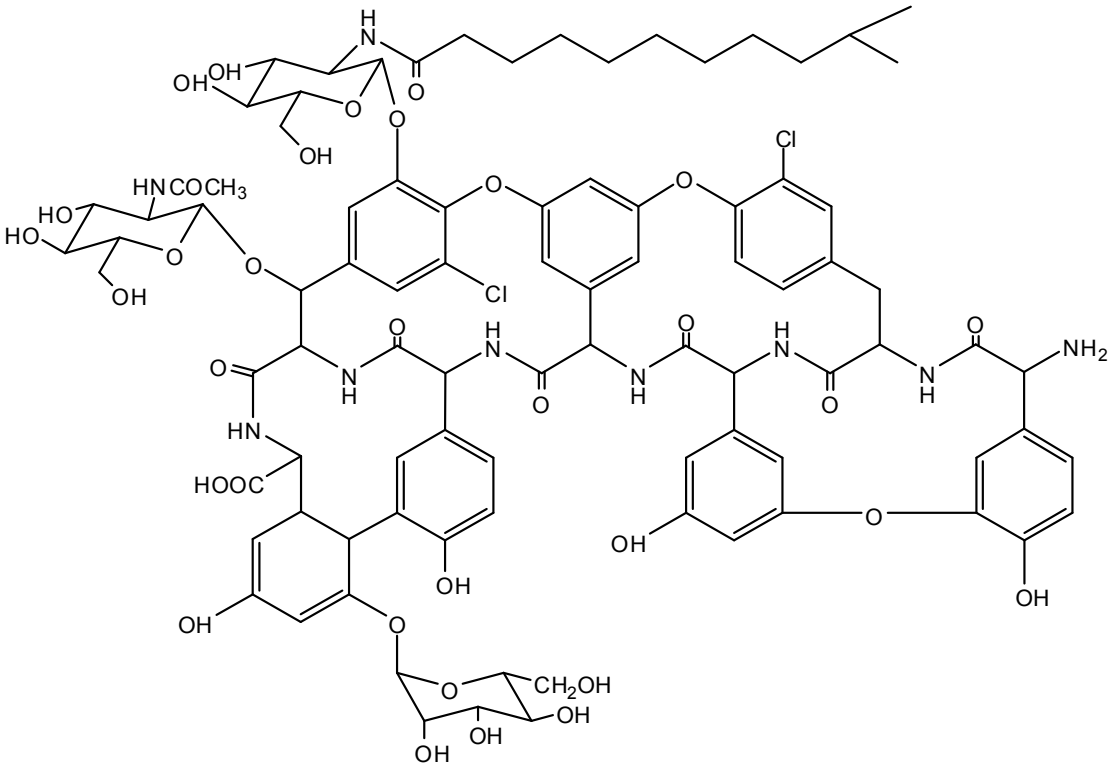

Figure 3: Structure of teicoplanin (Mw=1909,73)

Dalbavancin

Dalbavancin (BI-397) (Figure 4) is a novel second-generation lipoglycopeptide antimicrobial with unique pharmacokinetics and excellent activity against resistant gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus*. While

sharing a similar mechanism of action and spectrum of activity with other glycopeptides, dalbavancin has demonstrated *in vitro* and *in vivo* bactericidal potency superior to that of vancomycin, teicoplanin, and other commonly used antimicrobials.[8]

Dalbavancin is a derivative of the teicoplanin-related glycopeptide A40926 (Figure 5) modified with an amide appendage at the c-terminus and an alteration of the hydrophobic acylglucosamine substituent.

MICs for dalbavancin are markedly lower against many susceptible strains than those of vancomycin and several of the other glycopeptides. However, dalbavancin's MBC/MIC ratios are somewhat higher than for vancomycin and teicoplanin, and dalbavancin's high-level serum protein binding (98%) adversely impacts *in vitro* antibacterial action reducing activity to near-vancomycin levels in some cases. A prolonged half-life also due to the exceptional serum protein binding is observed, but in this case the effect benefits both pharmacodynamic and potential pharmaco-economic properties.[1]

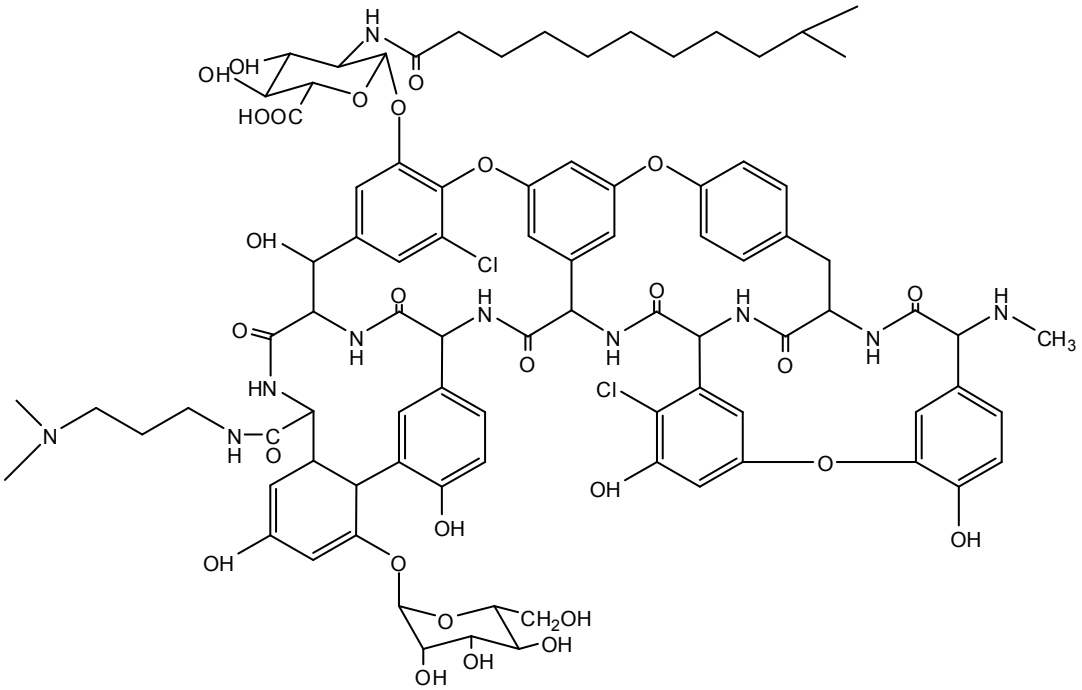

Figure 4: Structure of dalbavancin (BI 397) (Mw=1818,71)

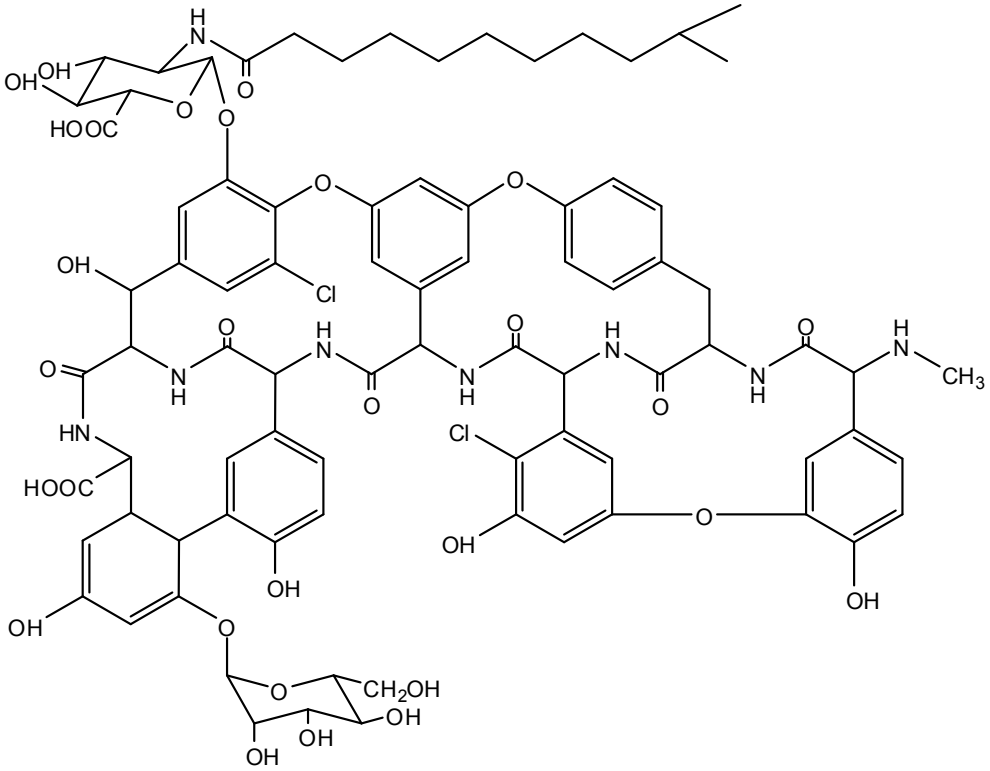

Figure 5: Structure of A 40926 (Mw=1764,61)

The lipoglycopeptide antibiotic A40926 belongs to the teicoplanin family, possessing the heptapeptide structure of the D-alanyl-D-alanine binding glycopeptides.[9]

A40926 and the structurally related teicoplanin have been used as scaffolds for the preparation of semi-synthetic derivatives such as mideplanin (Figure 6), MDL 63,246 (Figure 7) and dalbavancin (Figure 4).[9]

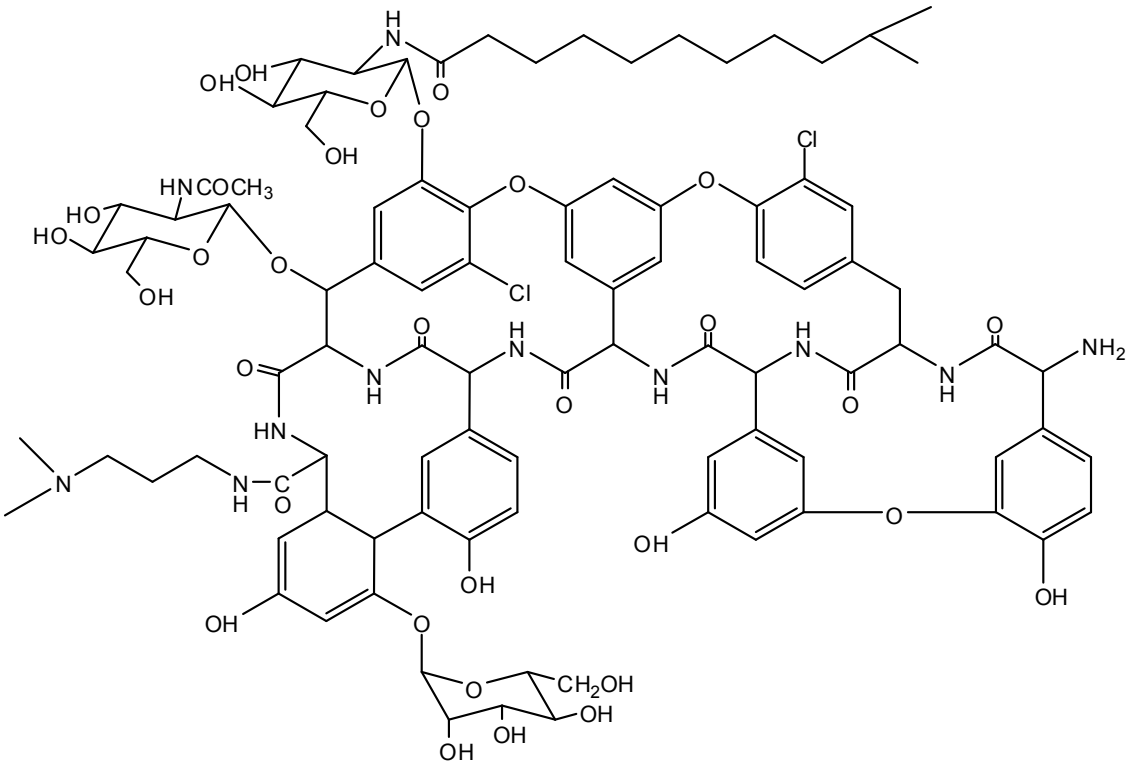

Figure 6: Structure of mideplanin (Mw=1993,89)

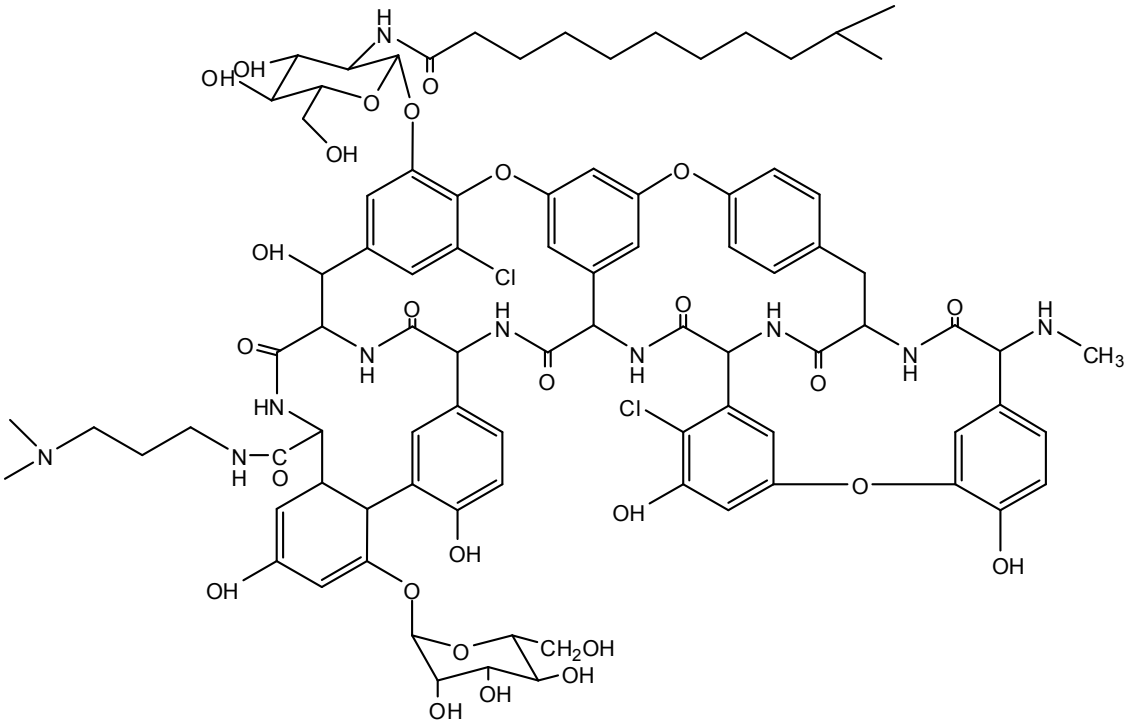

Figure 7: Structure of MDL 63,246 (Mw=1818,75)

Vancomycin

Vancomycin (Figure 8) is a glycopeptide antibiotic that is widely used in the treatment of Grampositive bacterial infections. It was discovered by the pharmaceutical company Eli Lilly in the mid-1950s. *Streptomyces orientalis* were used to produce this antibiotic. It was first used in clinics in 1959. Its importance has increased considerably recently, as it is one of the few antibiotics effective against nosocomial infections, for example, multiple-resistant bacteria – a typical example is Methicillin Resistant Staphylococcus Aureus (MRSA).[3]

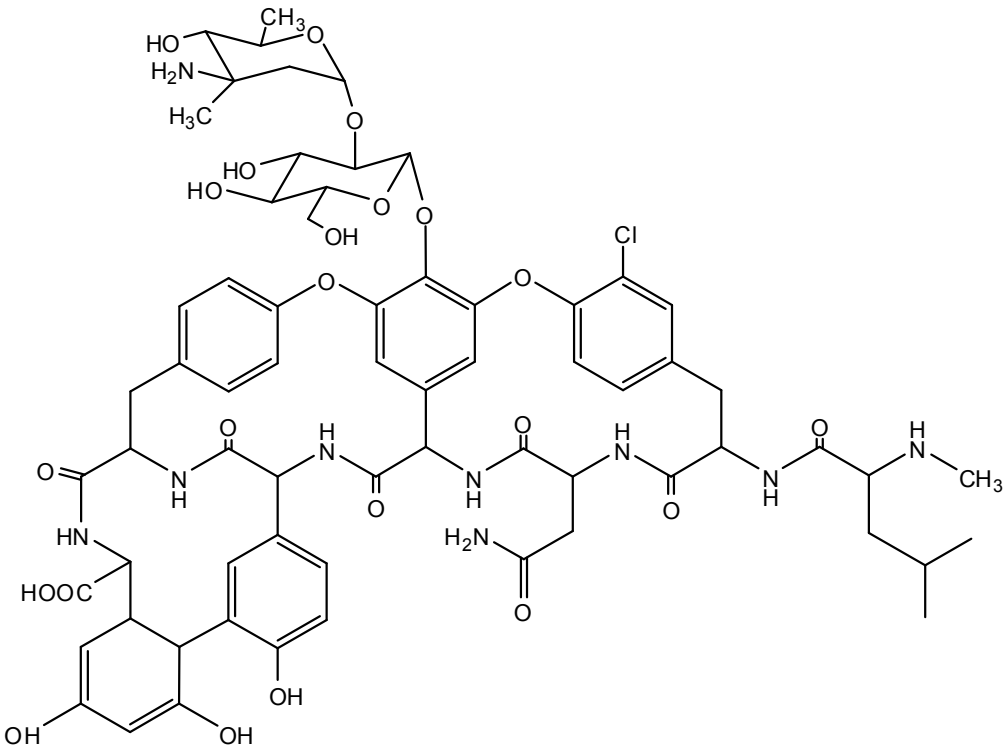

Figure 8: Structure of vancomycin (Mw=1384,83)

2.2 Surface plasmon resonance

2.2.1 Principles of technique

Surface plasmon resonance (SPR) is a method for biomolecular interaction analysis (BIA). It has become a tool for the quantitative and qualitative characterization of reversible interactions between biological macromolecules.

In principle, SPR sensors are thin-film refractometers that measure changes in the refractive index occurring at the surface of a metal film supporting a surface plasmon (Figure 9)[10].

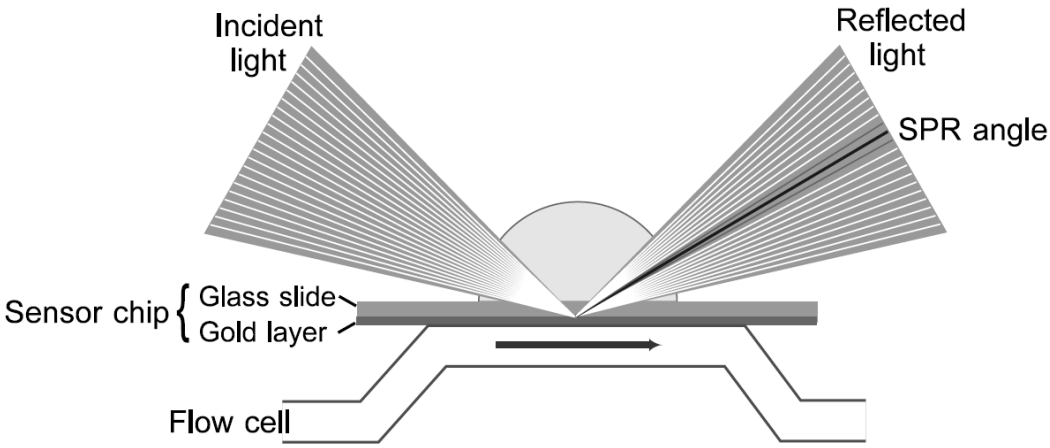

Figure 9: The SPR principle [11]

SPR is a phenomenon that occurs in thin conducting films at an interface between media of different refractive index. In Biacore systems, the media are the glass of the sensor chip and the sample solution. The conducting film is a thin layer of gold on the sensor chip surface.[11]

Biosensor experiments involve immobilizing one reactant on a surface and monitoring its interaction with a second component in solution.[12]

One reactant is covalently attached to the sensor surface. A mobile second reactant at constant concentration is introduced into the buffer flow above the sensor surface, and the progress of complex formation at the sensor surface is monitored. This procedure is followed by the dissociation phase, in which the free mobile reactant is absent from the buffer and the time-course of complex dissociation is recorded. Finally, the sensor surface is regenerated (for example by a short exposure to a buffer at low pH)

to remove the remaining complex. The cycle of association experiment, dissociation experiment, and regeneration is repeated using different concentrations of the mobile reactant. The obtained sequence of binding-progress curves contains information on the chemical rate constants and on the thermodynamic equilibrium constant of the interaction.[13]

SPR biosensors measure the change in refractive index of the solvent near the surface that occurs during complex formation or dissociation. These instruments are capable of characterizing binding reactions in real-time without labeling requirements, no chromophoric group is required. Consequently, SPR biosensors can be used to study the interactions of any biological system from proteins, oligonucleotides, oligosaccharides, and lipids to small molecules, phage, viral particles, and cells.[12]

Qualitative applications range from following a molecule through purification to identifying small molecule leads in a screening mode. Quantitative applications include determining the active concentration of molecules and measuring reaction kinetics and affinity constants. Thermodynamic information can be obtained by measuring reaction rates and equilibrium constants at different temperatures. When experiments are performed carefully, biosensors can be used to determine the stoichiometry and mechanism of the interaction.[12]

Immobilization

For the analysis of binding affinities and kinetics it is crucial that the measured binding reflects exclusively the native interaction of both reactants. Preferably, the macromolecule should be attached with uniform orientation and unrestrained accessibility for the mobile reactant.[13]

A chip which doesn´t need activation is docked, primed and washed in the adequate flow of running buffer. Ligand attachment is normally performed at 25°C unless the ligand is temperature-sensitive. A new sensogram is started in the cell of immobilization. The sample of the ligand is injected as many times as necessary till obtain response units (RU) needed. Finally the surface is washed with buffer or NaCl to clean the surface and avoid further decrease of RU during the experiment.

The normal chemistry used for the immobilization of one of the biomolecules involved in a study is represented in Figure 10.

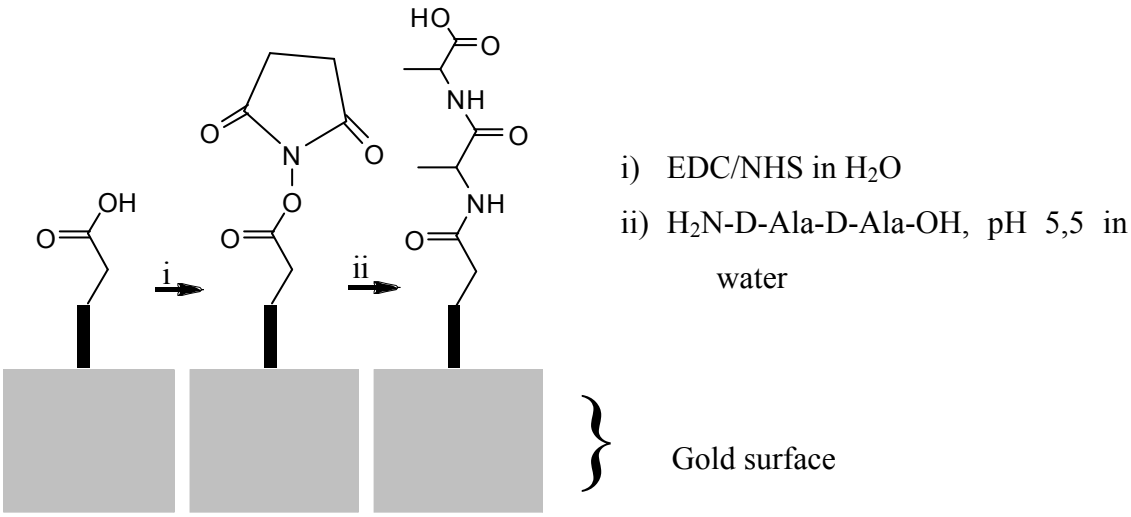

Figure 10: Representation of the surface chemistry for coupling of peptides to a carboxylic acid-presenting self-assembled monolayer

2.2.2 Application of SPR

Currently, several hundred studies on macromolecular interactions using SPR (and related) biosensors have been published in a broad variety of fields. These include cell adhesion molecules, T-cell antigen-receptor and major histocompatibility complex (MHC)-encoded molecules, receptor-ligand interactions, signal transduction, antibody-antigen interactions and antibody engineering, virus research, protein-carbohydrate interactions, protein-DNA and DNA-DNA interactions, interactions involving lipid vesicles or planar bi-layers, and the assembly of a membrane bound quaternary signal transduction complex.[13]

SPR biosensors have been applied in numerous important fields including medical diagnostics, environmental monitoring, and food safety and security.

2.2.2.1 Food quality and safety

The importance of SPR in food quality and safety analysis has increased during the last years. The number of publications continues to increase as well.[10]

Many affinity biosensors have been developed for the food industry.[10,14]

Trends in the area of pathogen detection, but also main techniques, traditional methods and recent developments in the field of pathogen bacteria biosensors have been described.[10,15]

SPR can be used as one of the methods for the detection of pathogens in food and water.[10,16]

An overview of the environmental pollutants and food contaminants was presented.[10,17]

In recent years various pathogens have been targeted by SPR biosensors.[18] In particular they include bacteria, protozoa, fungi and parasites.[10] This review describes the exploitation of exclusively optical SPR biosensors for the direct and indirect detection of pathogenic microorganisms in food chains and the environment. Direct detection is, in most cases, facilitated by the use of defined monoclonal or polyclonal antibodies raised against (a part of) the target pathogenic microorganisms. Indirect detection, on the other hand, is performed by analysis of a humoral immune response of the infected animal or human.[18]

Toxins implicated in food safety include mainly toxins produced by bacteria, fungi and algae.[10] The toxins detected by SPR are Staphylococcal enterotoxin B.[19,20] Medina demonstrated detection of Staphylococcal enterotoxin A using commercial SPR sensor Biacore.[21] Aflatoxin B1 was detected.[10,22]

Another important field in which SPR biosensor technology has been increasingly applied is testing for veterinary drugs residues in food.[10,23] An SPR sensor for the detection of antibiotics in milk was demonstrated.[10,24]

Direct detection of peanut allergens by means of SPR sensor was demonstrated.[10,25]

2.2.2.2 Medical diagnostics

Fast, sensitive, and specific detection of molecular biomarkers indicating normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention presents an important goal for modern bioanalytics. SPR biosensors have been demonstrated to hold promise for the detection of analytes related to medical diagnostics such as cancer markers, allergy markers, heart attack markers, antibodies, drugs, and hormones.[10]

In medical diagnostic is SPR used for detection of cancer markers. The prostate-specific antigen is a marker for prostate cancer, its detection has been reported.[10,26]

The quantitation of a pancreatic cancer marker, carbohydrate antigen (CA19-9) was performed.[10,27]

Protein vascular endothelial growth factor, which plays a role in breast cancer, lung cancer and colorectal cancer was detected using an SPR imaging method.[10,28]

SPR biosensors for the detection of hepatitis virus specific antibodies were reported by several research groups.[10]

SPR is a useful method for detection of drugs. The SPR biosensor-based detection of morphine-3-glucuronide, the main metabolite of heroin and morphine, was demonstrated.[10,29] Detection of the oral anticoagulant warfarin by the SPR method was performed by Fitzpatrick and O´Kennedy.[30]

A marker of pregnancy – human chorionic gonadotropin hormone has been a frequent target of optical biosensor technologies.[10,31]

Measurement of immunoglobulin E antibody levels plays an important role in the diagnostics of allergies. Detection of Ig E antibody using SPR biosensor has been presented.[10,32]

Detection of a marker of heart attack, troponin, in serum was demonstrated.[10,33]

2.2.2.3 Environmental monitoring

Detection of three pesticides – DDT (dichlordifenyltrichloretan), carbaryl and chlorpyrifos by SPR sensor was demonstrated by Lechuga´s group.[10,34]

SPR biosensors for the detection of TNT (trinitrotoluen), heavy metals or dioxins have been extensively researched.[10]

The detection of PCB (polychlorinated biphenyl) 3,3´,4,4´,5-pentachlorobiphenyl was presented.[10,35]

Over the past 5 years, more than 100 SPR biosensors for the detection of a variety of chemical and biological analytes were demonstrated. Most of these biosensors are based on prism coupling and angular or wavelength spectroscopy of surface plasmons. Commercial SPR systems have played an important role in the development of the detection applications due to their increasing spread and the availability of special SPR platforms and kits.[10]

2.2.3 SPR and glycopeptides

A peptide terminating in the sequence Lys-D-Ala-D-Lactate linked by its amino terminus to a acyl chain and anchored in a supported lipid monolayer to mimic the surface of vancomycin-resistant bacteria was presented. SPR analysis was then used to investigate the binding of glycopeptides group antibiotics to the surface. [6,36]

Whitesides and Williams have used SAMs terminating in D-Ala-D-Ala and in D-Ala-D-lactate together with surface Plasmon resonance to show that the covalently linked vancomycin dimer had a much higher binding constant at a surface than in solution. The dissociation of vancomycin dimer from the surface is much slower than that of vancomycin from the same surface.[36,37]

Direct binding of small vancomycin to D-Ala-D-Ala peptide ligands immobilized on a stable and well-packed SAM surface using quartz crystal microbalance was described. The dissociation constant obtained correlated well with values reported in literature. This direct binding method is practical, it provides quantitative binding information, and complicated binding data analysis is avoided.[38]

SPR can be used to directly identify molecules in a tripeptide library that bind tightly to a vancomycin chip. He describes in his paper a procedure, based on direct binding, for identifying tight binding ligands for a receptor immobilized on a sensor chip. As a model he uses vancomycin and a library of 96 tripeptides.[39]

Affinity between a vancomycin polymer and cell wall intermediate mimics of vancomycin resistant bacteria was determined by use of SPR.[40]

Immobilization of glycopeptides on Au sensor chips functionalizes with mixed self-assembled monolayers was described. The biosensors were optimized for the detection of interfering proteins.[41]

3. AIM OF STUDY

The aim of presented diploma thesis was:

1. to determine the conditions of HPLC analysis of glycopeptides antibiotics in order to confirm their purity

2. to prepare suitable SAM for the interaction studies

3. to quantify the interactions between the SAM presenting D-Ala-D-Ala and the glycopeptide antibiotics

4. EXPERIMENTAL PART

4.1. Materials

4.1.1 Antibiotics

Teicoplanin, mideplanin, dalbavancin (BI397), A40926, MDL 63,246 were a generous gift from the investigation group of Francesco Molinari, University of Milano, Italy.

Vancomycine was purchased from Sigma–Aldrich.

4.1.2 HPLC

The mobile phases:

A1 (0,1% trifluoracetic acid)

B1 (acetonitrile)

A2 (0,02 mol/l NaH_2PO_4–CH_3CN (95:5))

B2 (0,02 mol/l NaH_2PO_4 –CH_3CN (25:75)).

The analysis was performed using Agilent HPLC 1100 systems (Agilent Technologies, Palo Alto, CA) analytical column Mediterranea TR-010004 (15 ×0,46 cm, 5 μ) (Tecnocroma) was used. Chemstation Plus Family for LC software was used for data acquisition and analysis.

Acetonitrile was obtained from Scharlau Chemie S.A. All other solvents were from Sigma-Aldrich.

4.1.3 Surface plasmon resonance

All products were used without any further purification step.

D-Ala-D-Ala; O-(2-aminoethyl)-O´-(2-carboxyethyl)polyethylene glycol 3000; O,O´-bis(2-carboxyethyl)dodecaethylene glycol; Vancomycin hydrochloride from

Streptomyces Orientalis; 11-mercapto-undecanoic acid were purchased from Sigma-Aldrich.

α-(11-mercapto.undecanoylamido)-ω-carboxy dodeca(ethylene glycol) and α – thio-ω-carboxy poly(ethylene glycol) from IRIS Biotech.

HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH from PROCHIMIA.

3,3´dithiobis(sulfocuccinimidyl propionate) from Thermo Scientific)

Other solutions used in Biacore:

- mixture of EDC/NHS in the concentration 0,2M EDC and 0,05M NHS.
- 10 mM solutions of acetate buffer (HAc/Ac) of pH from 4.0 to 6.0
- buffer HBS-EP: 10 mM HEPES; pH = 7.4; 150 mM NaCl; 3 mM EDTA (ethylenediaminetetraacetic acid); 0.005 % surfactant P20
- solution of glycine-HCl in water of different pH's: these solutions were prepared from 10 mM glycine and 1M HCl to adjust calculated pH (1-1,5-2-3-5)
- 1 mM NaCl
- water-P20: 0,005 % surfactant P20 in destilled water

SPR measurements were made with a BIACORE instrument (BIACORE 3000). The sensograms and fittings were processed by the software BIAEvaluation (version 4.1, 2003) provided by BIACORE.

Chips, solutions of amino coupling kit, desorb solutions were purchased as well from BIACORE.

4.2 Methods

4.2.1 HPLC

1. Mobile phases: A1 (0,1% trifluoracetic acid), B1 (acetonitrile)

Gradient: minutes (%B1), 0 (0), 40 (100), 50 (100), 50 stop.
Flow rate: 1ml/minute.
Injection volume: 20µl.
Temperature 25°C.

2. Mobile phases: A1 (0,1% trifluoracetic acid), B1 (acetonitrile)

Gradient: minutes (%B1), 0 (12,5), 40 (80), 50 (90), 50 stop.
Flow rate 0,8 ml/minute.
Injection volume: 20µl.
Temperature 25°C.

3. Mobile phases: A1 (0,1% trifluoracetic acid), B1 (acetonitrile)

Gradient: minutes (%B1), 0 (0), 30 (80), 30 stop.
Flow rate 1,0 ml/minute.
Injection volume 20µl.
Temperature 25°C.

4. Mobile phases: A2 (0,02 mol/l NaH_2PO_4–CH_3CN (95:5)), B2 (0,02 mol/l NaH_2PO_4 –CH_3CN (25:75))

Gradient: minutes (%B2), 0 (20), 75 (35), 80 (40), 80 stop.
Flow rate 0,5 ml/minute.
Injection volume: 20µl.
Temperature 25°C.

4.2.2 SPR

4.2.2.1 Preparation of SAM

a) The SAM prepared by incubation

A chip with a gold layer is incubated with 1mM of ethanol-H_2O solution of the molecule we want to immobilize, for 24 hours at 4°C.

Then the chip is rinsed exhaustively with ethanol and water and finally blown dry under stream of nitrogen atmosphere.

b) The SAM prepared in Biacore

The aqueous solution is injected through the cells of the chip. The injections can be repeated till reach the RUs needed.

Flow rate: 2µl/min

Temperature: 25°C

Running buffer: water-P20

The cells are washed after the immobilization with running buffer or NaCl

4.2.2.2 Immobilization

The chip is activated by the injection of the amino coupling kit: EDC/NHS:

Volume: 35µl

Flow rate: 5µl/min

This is followed by injection of the solution of the molecule we want to immobilize. It is repeated until reach the RU needed.

Then follows the injection of ethanolamine

Volume: 35µl

Flow rate: 5µl/min

Temperature: 25°C

Running buffer: HBS-EP

4.2.2.3 Preconcentration

Solutions glycopeptide antibiotics of different pH glycopeptide antibiotics in acetate buffer were injected.
Volume: 10µl
Flow rate: 5µl/min
Temperature: 25°C
Running buffer: HBS-EP
The cells are washed between each injection with running buffer or NaCl

4.2.2.4 *Interaction* assays

Different concentrations of the solutions of glycopeptide antibiotics in HBS-EP buffer were injected.
Volume: 15µl
Flow rate: 5µl/min
Temperature: 25°C
Running buffer: HBS-EP

5. **RESULTS**

5.1 Optimization of HPLC analysis of glycopeptide antibiotics

We followed the conditions determined by Gandolfi et al 2007. Analysis was performed on an Agilent instrument equipped with C18 column, 5µ (15 x 0.46 cm). The solvent system consisted of an aqueous solution of trifluoracetic acid (0.1%) (solvent A) and acetonitrile (solvent B). A linear gradient from solvent A (100%) to solvent B was applied over a period of 40 minutes followed by isocratic elution with solvent B (100%) for an additional time (10 minutes). The flow-rate was 1.0 ml/minute. Injection volume was 20µl and UV detector (254 nm). A method was designed for determination of glycopeptide antibiotics using high-performance liquid chromatography (HPLC). But these conditions were found suitable only for three of the antibiotics: A40926 (Figure 11), BI 397 (Figure 12) and mideplanin (Figure 13).

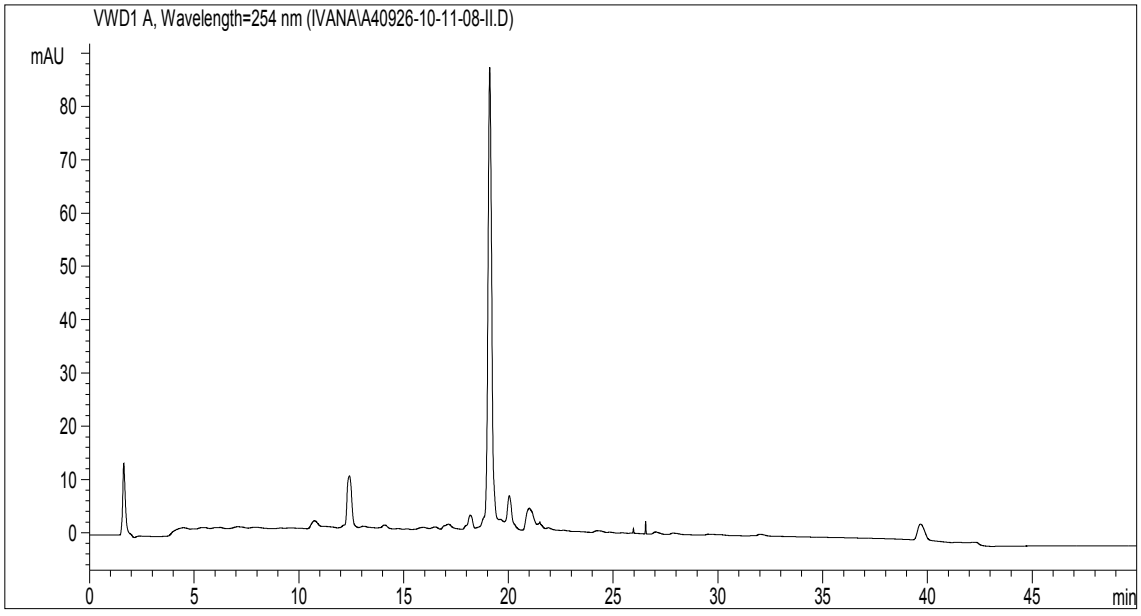

Figure 11: chromatogram of A 40926

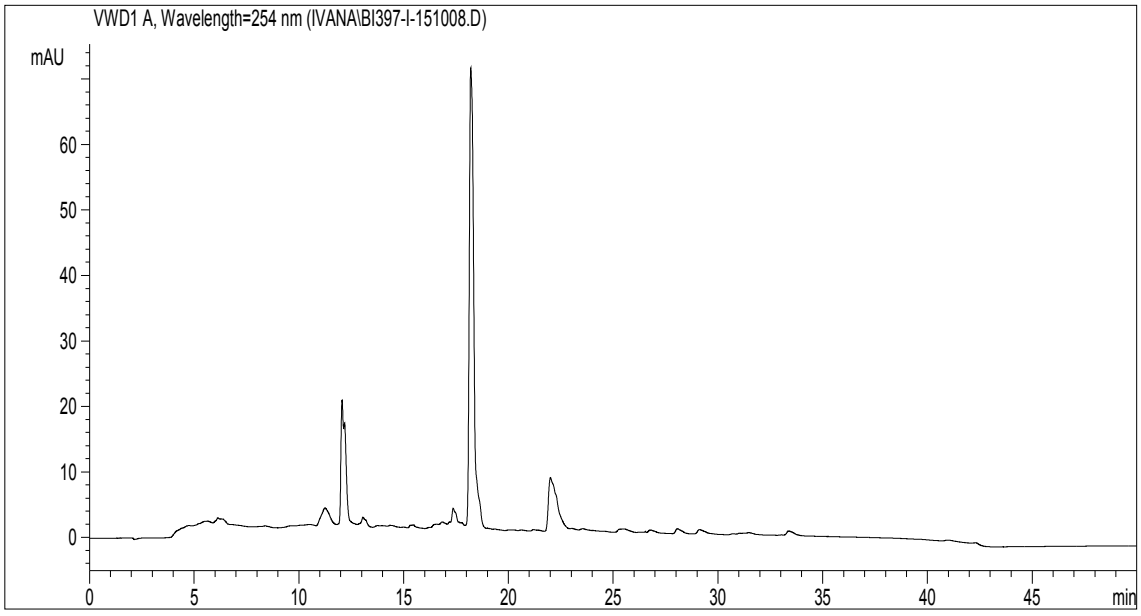

Figure 12: Chromatogram of BI 397

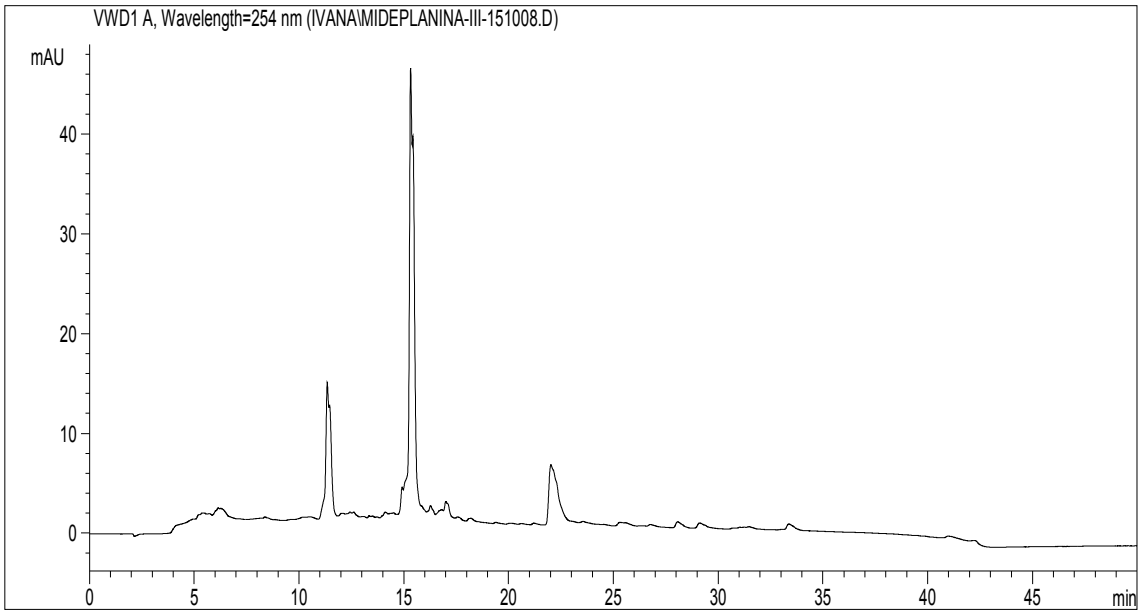

Figure 13: Chromatogram of mideplanin

To determine glycopeptide MDL 63.246 we changed the conditions: the flow-rate was 0.8 ml/minute, a linear gradient from solvent A (87,5 %) to solvent B (80 %) was applied over a period of 40 minutes followed by the gradient from solvent A (20 %) to solvent B (90 %) for an additional time (10 minutes). (Figure 14)

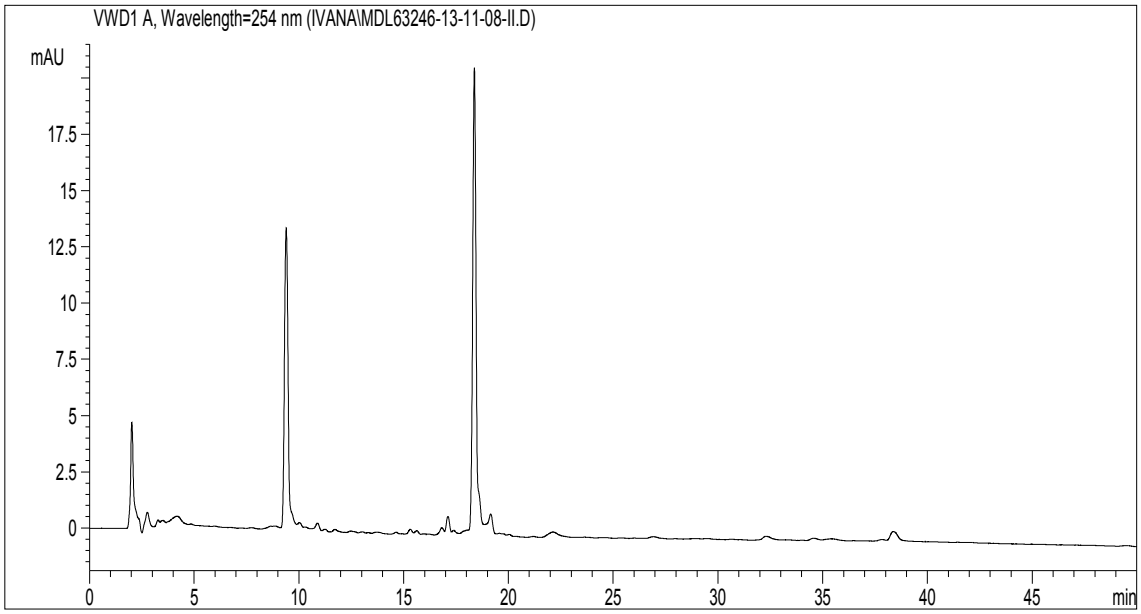

Figure 14: Chromatogram of MDL 63,246

Teicoplanin exists as a complex of several structures. We followed the analytical method of A. Borghi[42]. As mobile phases were used: A 0,02M NaH_2PO_4-CH_3CN (95:5), B 0,02M NaH_2PO_4-CH_3CN (25:75). The conditions of the paper had to be changed and the optimal scheme is: gradient: minutes (%B), 0 (20), 75 (35), 80 (40), 80 stop. Flow rate 0,5 ml/minute. Injection volume: 20µl. Temperature 25°C.

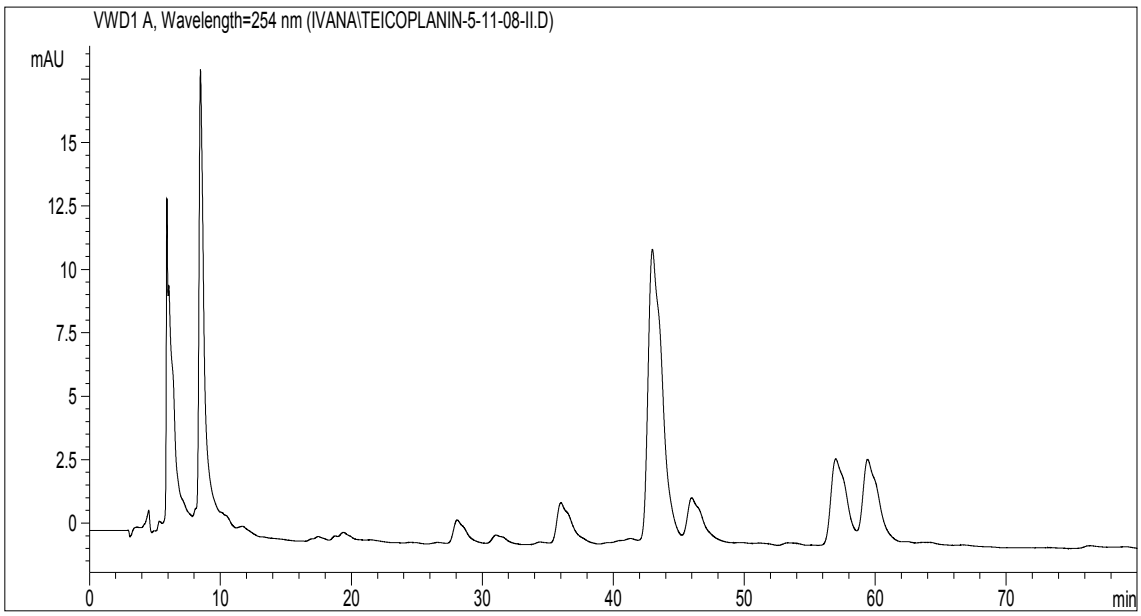

Figure 15: Chromatogram of teicoplanin

To complete the measurements we injected vancomycine – the commercial one. As the best conditions were considered these: mobile phases: trifluoracetic acid (0.1%) (solvent A) and acetonitrile (solvent B). A linear gradient from solvent A (100%) to solvent B (80%) was applied over a period of 30 minutes. The flow-rate was 1.0 ml/minute. Injection volume was 20µl and UV detector (254 nm) (Figure 16)

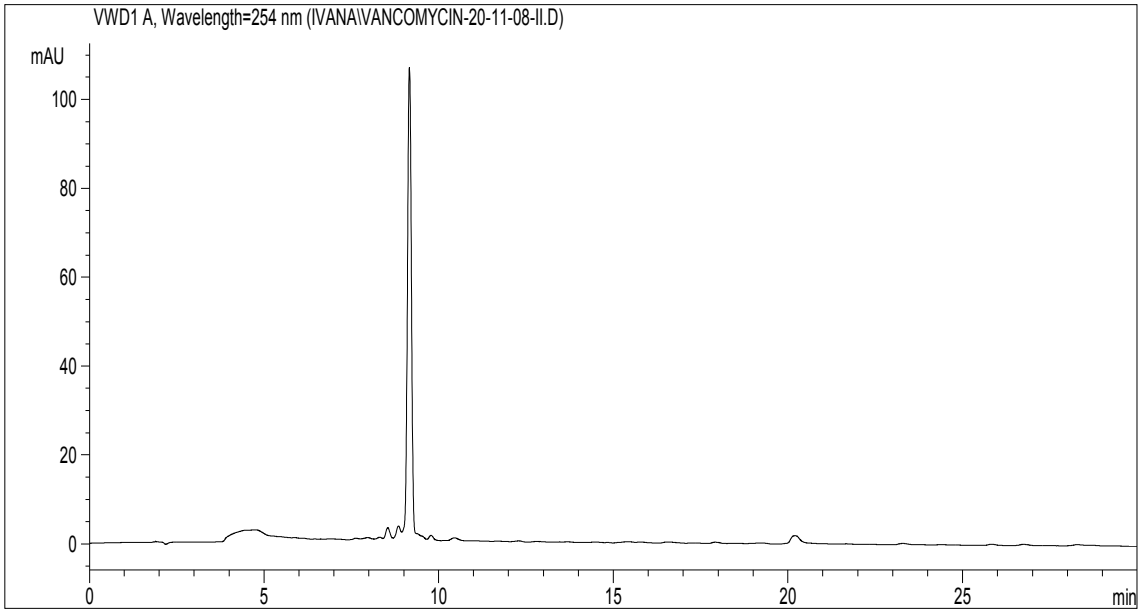

Figure 16: Chromatogram of vancomycine

5.2 Self-assembled monolayers

5.2.1 SAM of 11- mercapto-undecanoic acid

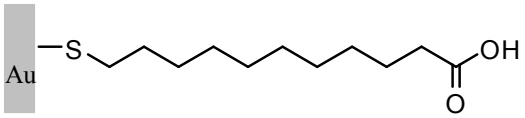

Figure 17: Schematic representation of the SAM formed by mercapto-undecanoic acid

A chip with 11- mercapto-undecanoic acid was prepared by incubating the Au sensor chip with a solution of 11- mercapto-undecanoic acid (1mM in ethanol) for 24 hours at 4°C. After the incubation the chip was rinsed exhaustively with ethanol, ethanol-water and water and finally blown dry under stream of nitrogen atmosphere.

5.2.2 SAM of α – thio-ω-carboxy poly(ethylene glycol)

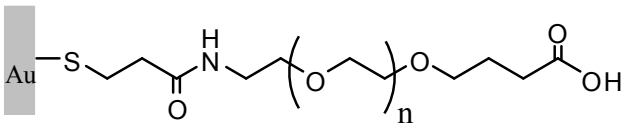

Figure 18: Schematic representation of the SAM formed by α–thio-ω-carboxy poly(ethylene glycol)

This SAM was prepared by two injections of 40µl α–thio-ω-carboxy poly(ethylene glycol) (100µg/ml) at flow rate 2µl/min.
The final number of RUs reached is 300.

5.2.3 SAM of α-(11-mercapto-undecanoylamido)-ω-carboxy dodeca(ethylene glycol)

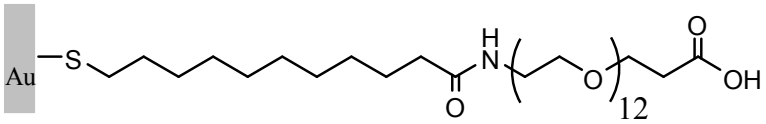

Figure 19: Schematic representation of the SAM formed by α-(11-mercapto-undecanoylamido)-ω-carboxy dodeca(ethylene glycol)

This SAM was prepared by two injections of 60µl α-(11-mercapto-undecanoylamido)-ω-carboxy dodeca(ethylene glycol) (100µg/ml) at flow rate 2µl/min. The final number of RUs reached is 200.

5.2.4 SAM of HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH

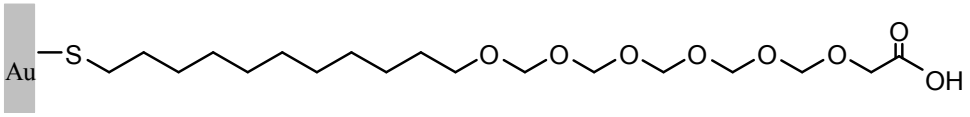

Figure 20: Schematic representation of the SAM formed by HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH

A chip with SAM formed by HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH was prepared by incubating the Au sensor chip with a solution of HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH (1mM in ethanol) for 24 hours at 4°C. After the incubation the chip was rinsed exhaustively with ethanol, ethanol-water and water and finally blown dry under stream of nitrogen atmosphere.

5.3 Effect of the change of the flow rate

The flow rate also has an influence on the measured dissociation, since at 5µl/min the dissociation rate is slower than at 25 µl/min, further indicating the importance of rebinding.[43] The binding was measured at four different flow rates 5, 10, 15, 20 µl/min over the surface of 11-mercapto-undecanoic acid in running buffer HBS-EP. Concentration of the antibiotics was 50µg/ml.

In this case the effect of rebinding isn´t so strong. There is noticeable that the dissociation is faster with the flow 20 µl/min. But the importance is insignificant. The other measurements were performed with the flow 5 µl/min to save the samples.

The results of this measurement are demonstrated in the following graphic charts:

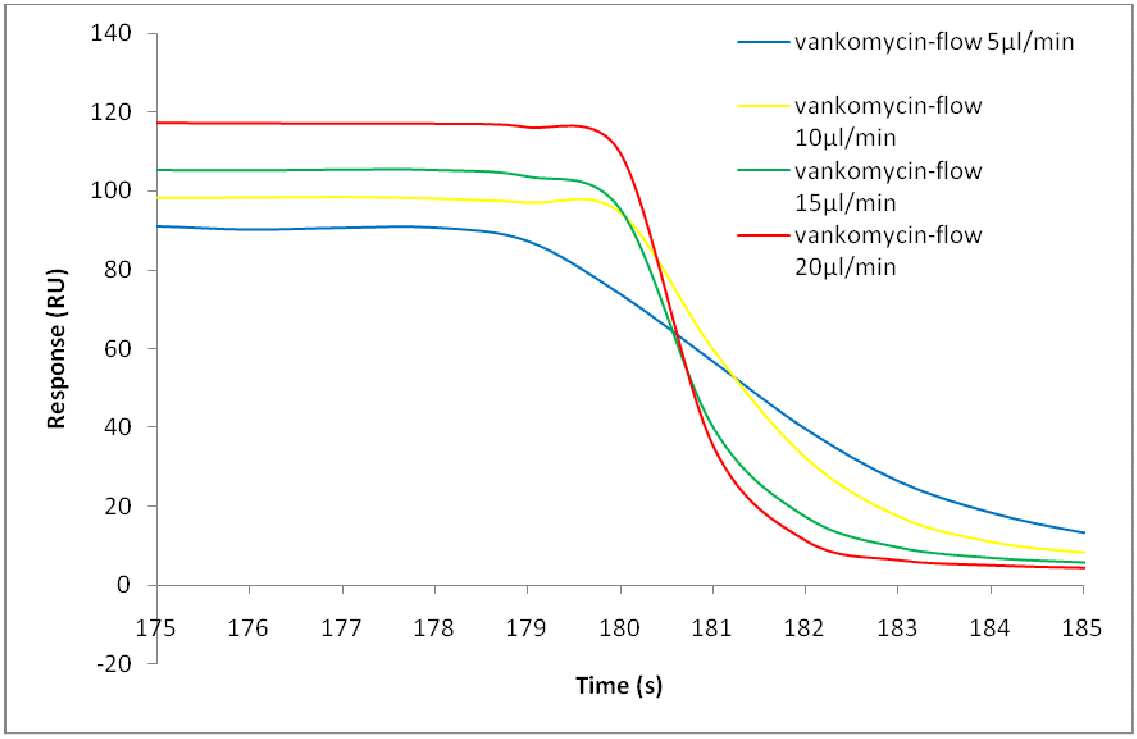

Figure 21: Vancomycin flown over the surface of 11-mercapto-undecanoic acid at different flows

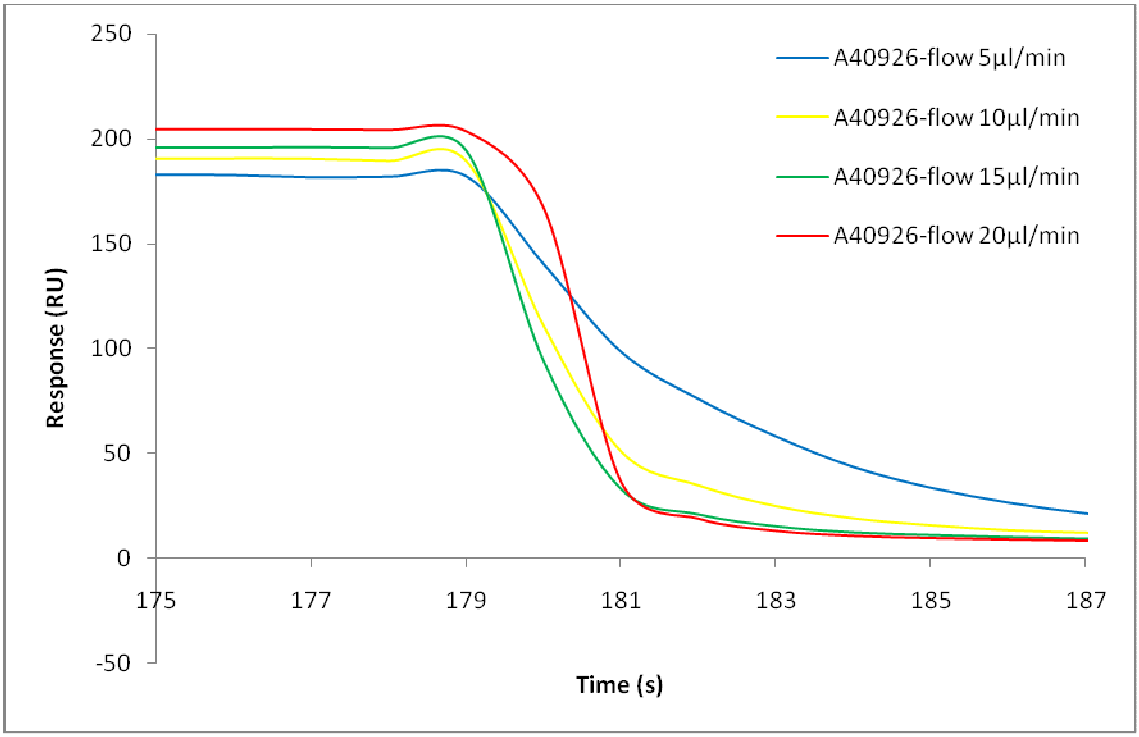

Figure 22: A40926 flown over the surface of 11-mercapto-undecanoic acid at different flows

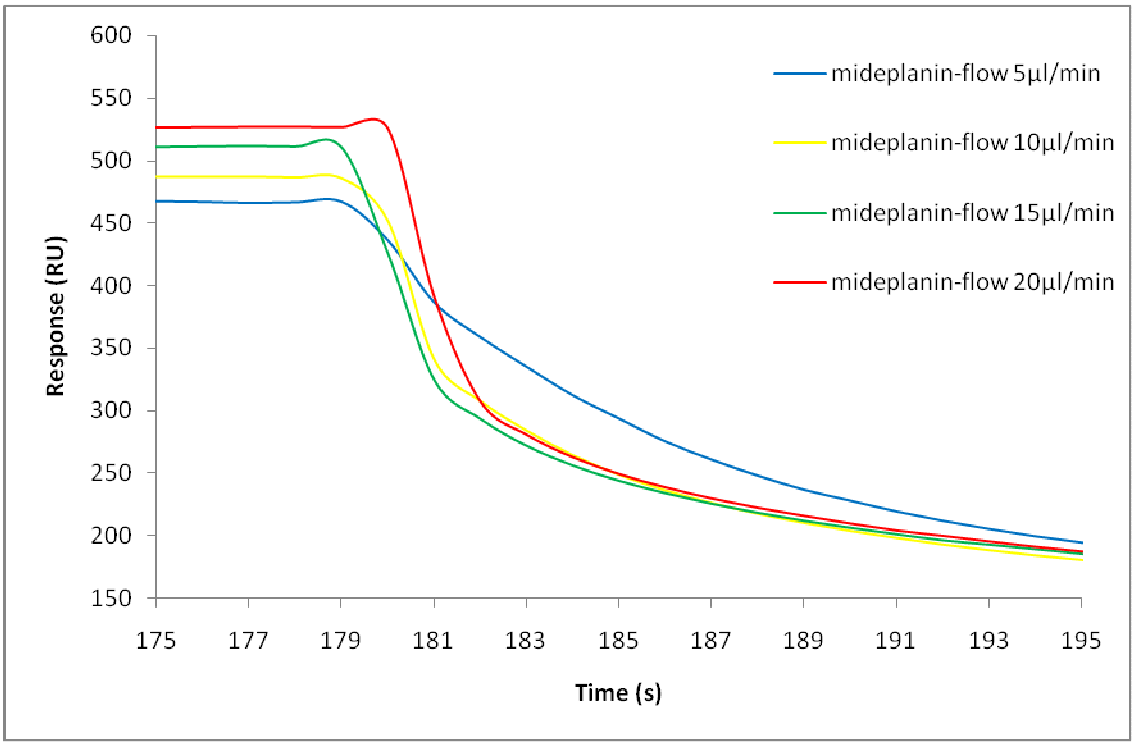

Figure 23: Mideplanin flown over the surface of 11-mercapto-undecanoic acid at different flows

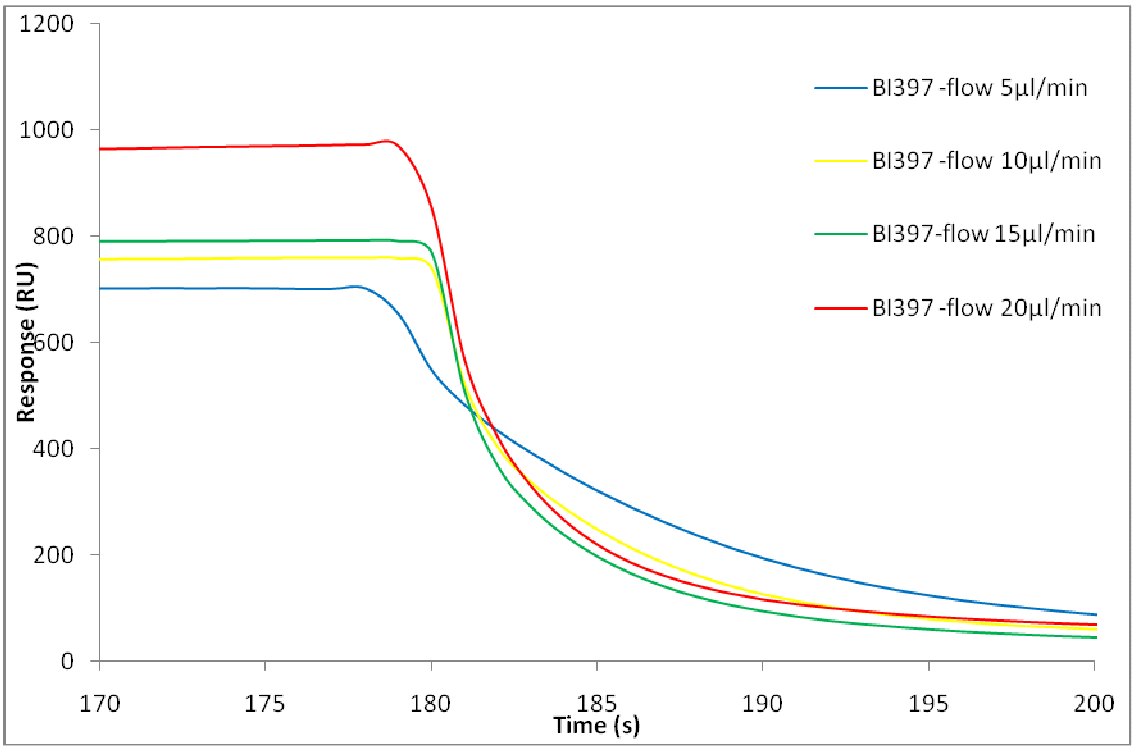

Figure 24: BI 397 flown over the surface of 11-mercapto-undecanoic acid at different flows

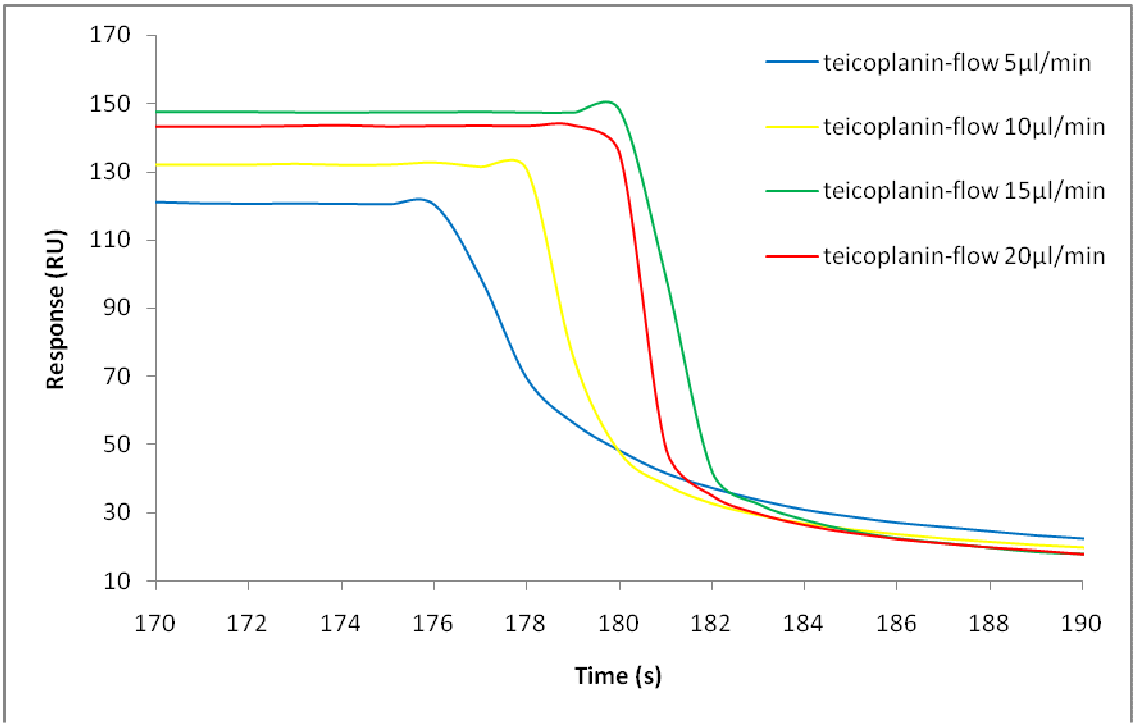

Figure 25: Teicoplanin flown over the surface of 11-mercapto-undecanoic acid at different flows

5.4 Interaction assays

Data were prepared for analysis by adjusting the response prior to injection to zero and adjusting the time of each injection to zero. Affinities were calculated from analysis of equilibrium binding levels at varying antibiotic concentration.

The steady state binding level is related to concentration according to the equation:

$$R_{eq} = \frac{K_A C R_{\max}}{1 + K_A C n}$$

where n is a steric interference factor specifying how many binding sites are on average blocked by binding one analyte molecule. R_{max} is the theoretical binding capacity, which will differ from an experimentally measured value if n is not 1. R_{eq} is obtained from the sensograms. C is the concentration of antibiotic. K_A is the affinity constant (M^{-1}).

Vancomycin

The surface treated with the reagents for conjugation served as negative control to avoid considering glycopeptide-independent binding. Sensorgrams were recorded in this channel routinely to assess extent of signal. In parallel experiments, solutions with increasing vancomycin concentration were passed over the chip surfaces with the derivatives.

The interaction curves obtained for vancomycin are shown in Figure 26 and we can see a fast association rate that reaches the steady state very fast, and also a rapid dissociation rate. Due to the fact that both association and dissociation phases occur so rapidly, we carried out a steady-state affinity study, and the resulting value of apparent K_A obtained for that fitting was $K_A = 1.78 \times 10^4\ M^{-1}$

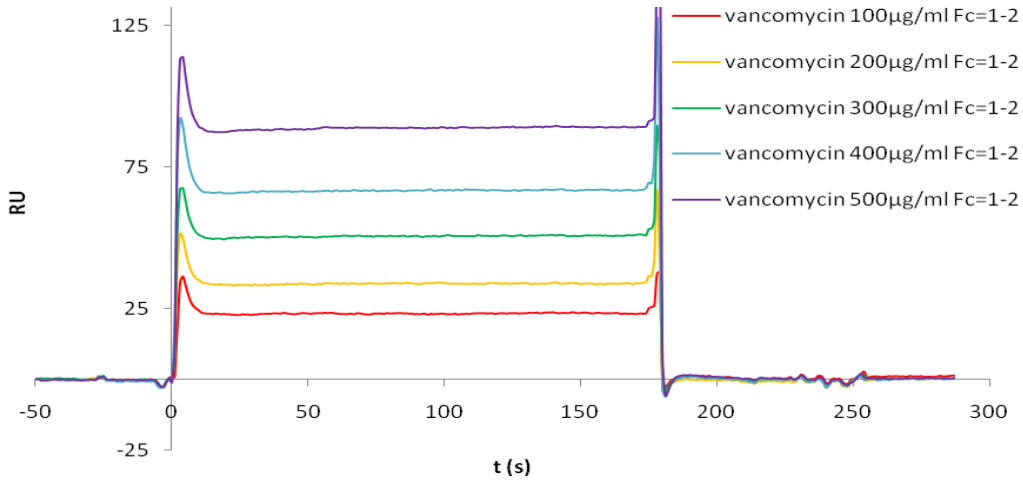

Figure 26: The SPR response (RU) of binding of vancomycin to HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH SAM presenting D-Ala-D-Ala.

Teicoplanin

As shown in Figure 27, the association phase for teicoplanin was also very fast reaching the steady state rapidly, and the dissociation phase was also very fast. The apparent K_A value calculated for this interaction was 3.94 x 10^4 M^{-1}. These values are both in the same magnitude range and in good agreement to the mentioned affinity data for Vancomycin.

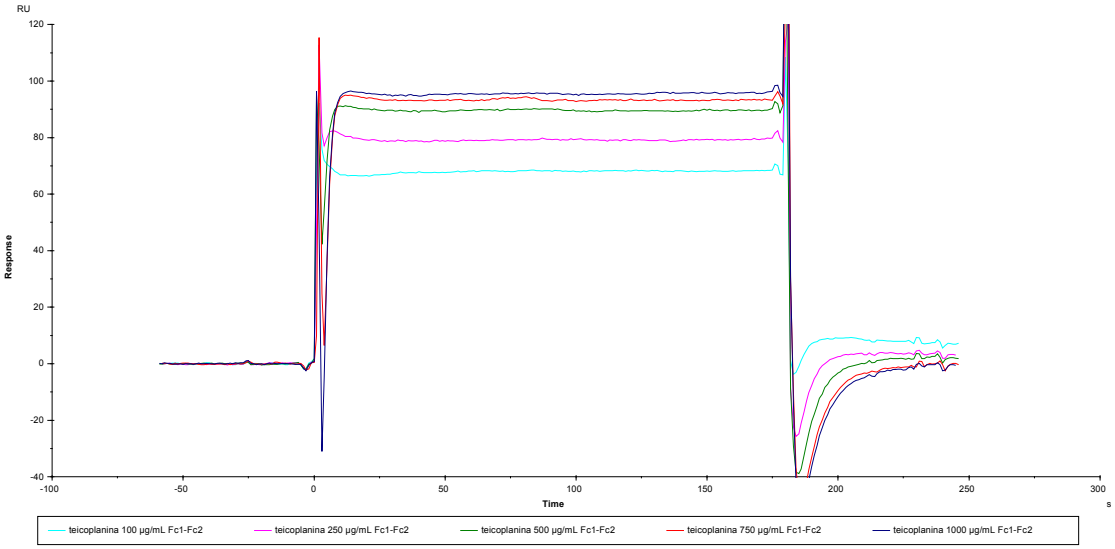

Figure 27: The SPR response (RU) of binding of teicoplanin to HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH SAM presenting D-Ala-D-Ala.

The K_A value was obtained from nonlinear fit of the data in plot of R_{eq} (response at equilibrium) versus the concentration of the antibiotic. (Figure 28)

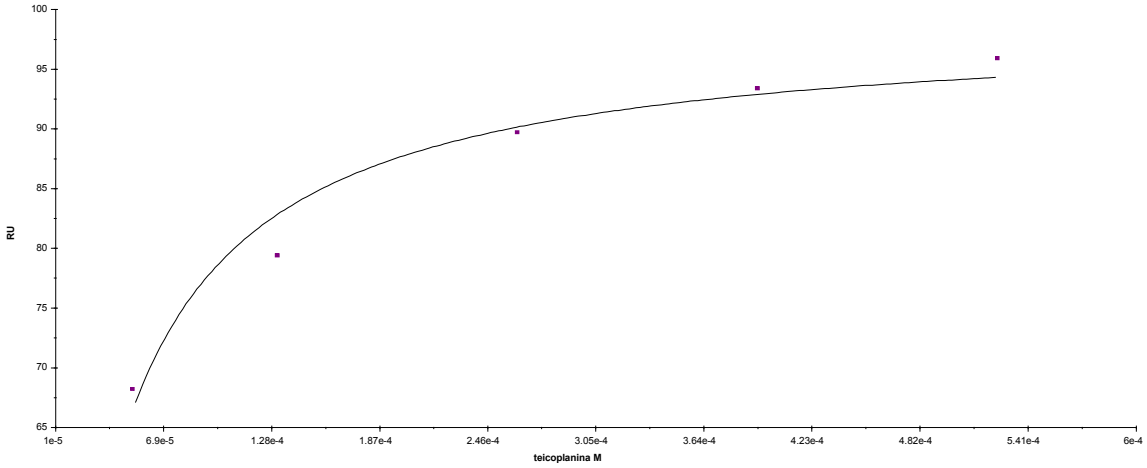

Figure 28: Steady state affinity of the interaction between teicoplanin and D-Ala-D-Ala immobilized on the HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH surface.

Dalbavancin (BI 397)

In the case of dalbavancin (BI 397) a relatively fast association phase (k_{on}=38.7 M^{-1} s^{-1}) and a gradual dissociation phase (k_{off} = 1 x 10^{-3} s^{-1}) are shown in the curves for the binding of the glycopeptides and immobilized D-Ala-D-Ala (Figure 29). The binding abilities are characterized by the respective dissociation constants K_D, where K_D=k_{off}/k_{on} and K_A=1/K_D. A K_D of 2.8 x 10^{-5} M and K_D of 3.57 μM were obtained, which indicate stronger interactions that the one observed with the others glycopeptides.

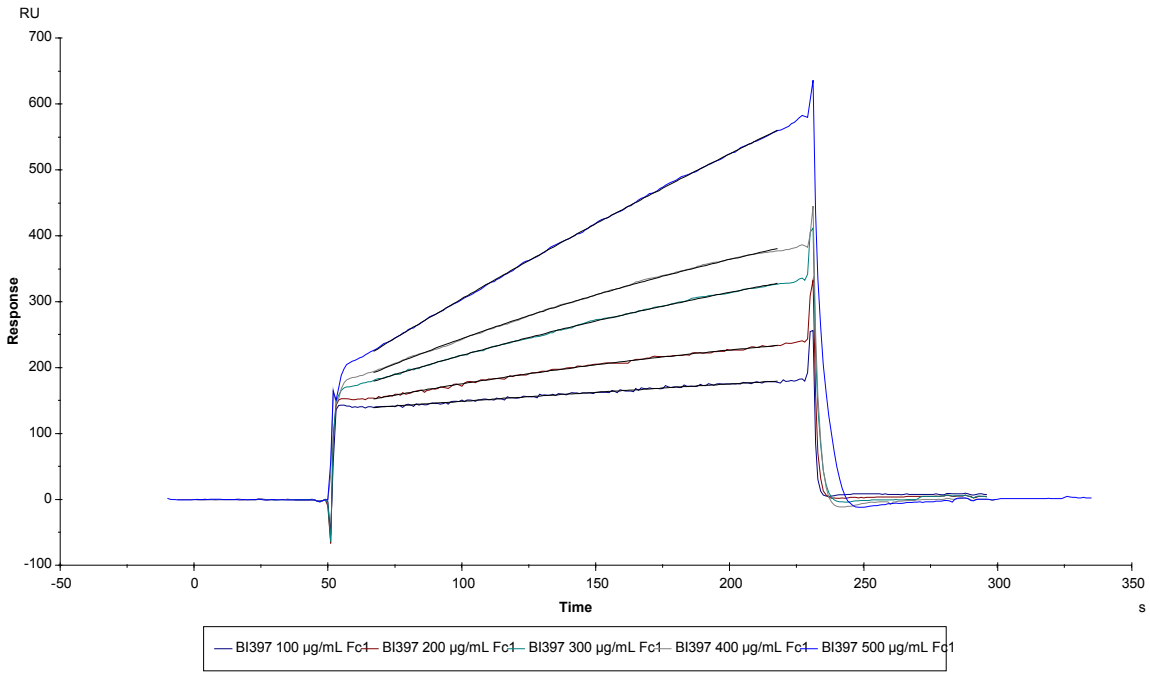

Figure 29: The SPR response (RU) of binding of BI 397 to HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH SAM presenting D-Ala-D-Ala.

A 40926

As shown in Figure 30, the association phase for A40926 was also very fast reaching the steady state rapidly, and the dissociation phase was also very fast. The apparent K_A value calculated for this interaction was 2.34 x 10^3 M^{-1}. These values are both in the same magnitude range and in good agreement to the mentioned affinity date for Vancomycin.

This K_A value was obtained from nonlinear fit of the data in plot of R_{eq} (response at equilibrium) versus the concentration of the antibiotic (Figure 31).

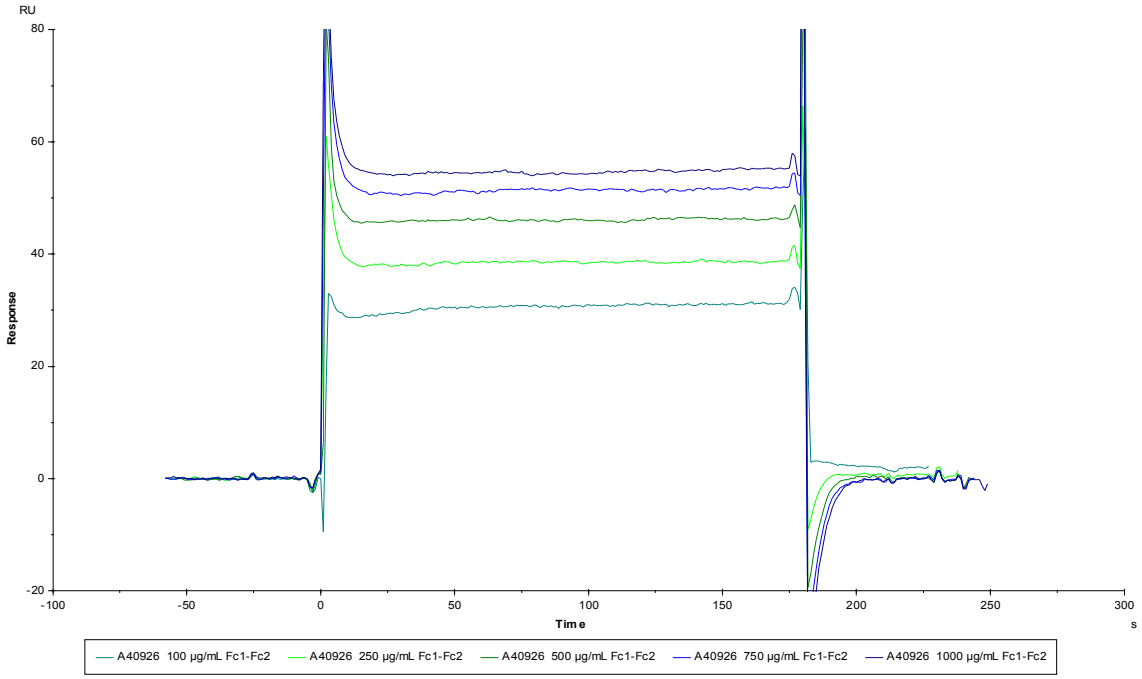

Figure 30: The SPR response (RU) of binding of A 40926 to HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH SAM presenting D-Ala-D-Ala.

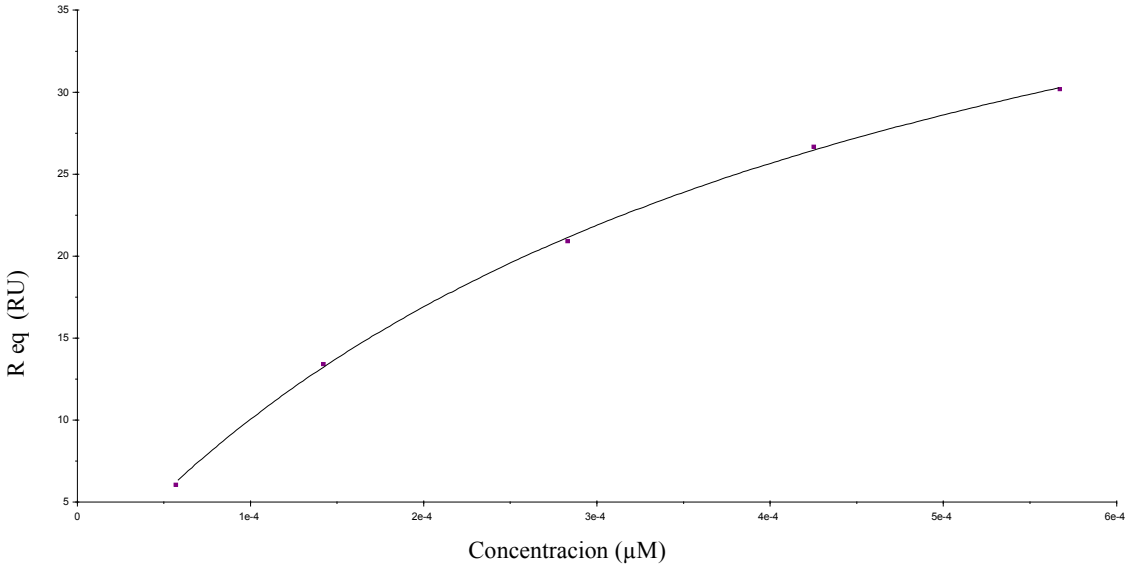

Figure 31: Steady state affinity of the interaction between A 40926 and D-Ala-D-Ala immobilized on the HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH surface.

6. DISCUSSION

In this study we demonstrated, by Surface Plasmon Resonance, that all the novel antibiotics from the glycopeptide group are more active than vancomycin.

The initial HPLC method to probe the purity of the glycopeptide antibiotics was taken from Gandolfi et al.[9] This method had to be adapted to our conditions. For the antibiotics: mideplanin, BI 397 and A 40926 these are: linear gradient from solvent A (100%) to solvent B was applied over a period of 40 minutes followed by isocratic elution with solvent B (100%) for an additional time (10 minutes). The flow-rate was 1.0 ml/minute. Where the solvent A is trifluoracetic acid (0.1%) and the solvent B is acetonitrile.

Because the sample of MDL 63,246 contained 12,5% of acetonitrile it was found necessary to change the percentage proportion of solvents: linear gradient from solvent A (87,5 %) to solvent B (80 %) was applied over a period of 40 minutes followed by the gradient from solvent A (20 %) to solvent B (90 %) for an additional time (10 minutes).

Chromatograms of teicoplanin in conditions with these solvents were not reproducible, so we had to search a new method. According to the paper of A. Borghi[42] as mobile phases we used: A 0,02M NaH_2PO_4-CH_3CN (95:5), B 0,02M NaH_2PO_4-CH_3CN (25:75). The conditions of the paper had to be changed and the optimal scheme is: gradient: minutes (%B), 0 (20), 75 (35), 80 (40), 80 stop. Flow rate 0,5 ml/minute.

Our aim was to prepare several SAMs of different properties as hydrofobicity and length of the chain.

Muñoz et al (2005) demonstrated that PEG-based biosensor chip offer an alternative to dextran-based chips, which are now the most used. Alcanothiol SAMs on gold are well organized, densely packed and very stable against chemical and mechanical stress. Several research groups are engaged in investigation of SAMs, using different tail groups chain lengths and spacers. Svedhem et al. (2001) presents the synthetic strategy based on coupling of monofunctionalized oligo (ethylene glycol) amines to ω-mercaptocarboxylic acids to generate corresponding amides. [44]

We followed this method and tried to optimize it to prepare the SAMs by reaction *in situ* in Biacore. But due to the problems with air bubbles we couldn´t obtain

the confirmation of RUs. The greatest disadvantage was, that we didn´t have any confirmation of the final compound.

One of the ways we tried in the beginning was to synthesize a new compound for the immobilization.[45] But the molecular weight obtained from MALDI-TOF Mass Spectrometry didn´t correspondent with the molecular weight expected. We suppose that the problem occurred during the lyophilization of the product.

At last we started to use the commercially available compounds of alcanothiol chain ending in a carboxylic group to immobilize D-Ala-D-Ala.

The SAMs were prepared by incubating the Au sensor chip with the solution of alcanothiol overnight or by several injections of the solution of alcanothiol *in situ*.

When transport influences dissociation, rebinding occurs. A ligand that dissociates from a receptor may rebind to another receptor rather than escape into the bulk fluid. In our case the effect of rebinding was demonstrated as insignificant by the changes of the flow-rate. There is noticeable that the dissociation is faster with the flow 20 µl/min, but in order to save the samples we continued the measurements at flow 5 µl/min.

The interactions were performed over the surface of HS-$(CH_2)_{11}$-EG_6-OCH_2-COOH SAM presenting D-Ala-D-Ala. Other SAMs have been prepared, but after several problems with the results of interactions, we decided to continue the measuremnts only over the mentioned SAM.

Other problems appeared when cleaning the surface after injectons. pH lower than 2 destroys the surface, as well as the contain of acetonitrile in the sample of MDL 397.

Tseng et al (2005) presents using surface plasmon resonance to quantify the bindig of several peptides to the immobilized vancomycin. The equilibrium affinity constant of D-Ala-D-Ala and vancomycin is 1,53 x 10^4 M^{-1}, which corresponds with our affinity constant 1,78 x 10^4 M^{-1}.

Tseng et al (2007) describes in his paper direct binding of a small vancomycin to D-Ala-D-Ala peptide ligands immobilized on a stable and well packed SAM surface.

Direct binding measurements showed that vancomycin associates with the D-Ala-D-Ala – containing peptide with an affinity of 37 x 10^4 M^{-1} confirmed by SPR.

The affinity constants of the novel glycopeptide antibiotics have not been publicated yet. Our measurements show stronger interaction of the antibiotics BI 397 – dalbavancin and teicoplanin than vancomycin. The affinity constant of BI 397 – dalbavancin is $K_A = 3{,}57 \times 10^4$ M^{-1}. And the affinity constant of teicoplanin is $K_A = 3{,}94 \times 10^4$ M^{-1}.

In the case of vancomycin, teicoplanin and A40926 the interaction curves demonstrate that the association and dissociation was very fast, so we could calculate only the affinity constant in the steady state.

Only the interaction of dalbavancin enabled to calculate the association and dissociation constants.

The interaction results depend on the surface peptide coverage. Covalent attachment of peptides to the SAM surface does not allow the correct adjustment of the surface position of the peptides necessary for cooperatiave binding of an antibiotic. As well as the number of cycles of injections and cleans affect the quality of the surface respons.

7. CONCLUSION

A methodology for the HPLC determination of glycopeptide antibiotics was set up. Two main strategies were carried out in optimizing the method.

Different surfaces presenting D-Ala-D-Ala have been prepared for interactions of the glycopeptide antibiotics. Only the SAM of the polyethylene glycol with the chain of 12 carbons showed good properties, it means that the specific binding was higher than non-specific interaction.

We have developed an efficient and versatile strategy to immobilize D-Ala-D-Ala on a biochip surface and then used as presenting surface for SPR studies or high-throughput screening for novel glycopeptide antibiotics.

Interaction studies of D-Ala-D-Ala and glycopeptides:

The results presented so far reveal the bioreactivity of the derivatives presented D-Ala-D-Ala on a surface for the different glycopeptides used in this Thesis. Toward this aim we herein present a facile strategy for immobilized D-Ala-D-Ala as model system to study glycopeptide antibiotics binding to their target in the bacteria. Bioactivity of the surface was tested with different members of the family of glycopeptide antibiotics: teicoplanin, A 40926, dalbavancin (BI 397) and vancomycin in a SPR assay. All of them show stronger interactions that vancomycin, especially BI 397 – dalbavancin.

8. SUMMARY IN CZECH

Glykopeptidová antibiotika

Glykopeptidová antibiotika jsou přírodní komplexy získávané biosynteticky. Působí baktericidně na G+ bakterie včetně anaerobů: stafylokoky, enterokoky a streptokoky.

Glykopeptidová antibiotika blokují syntézu peptidoglykanu buněčné stěny bakterií vazbou na postranní peptid kyseliny muramové. Účinek glykopeptidů je omezen na gram-pozitivní bakterie, protože jejich velká molekula neproniká zevní membránou gram-negativních bakterií.

Vankomycin je antibiotikem užívaným k léčbě infekcí vyvolaných rezistentními gram-pozitivními bakteriemi nejčastěji, má ale některé významné nevýhody, především značnou toxicitu, potřebu monitorování hladin, nutnost intravenózního podávání a hrozbu narůstající rezistence. Teikoplanin se od vankomycinu liší zejména dlouhým eliminačním poločasem (90 hodin) a vazbou na plazmatické proteiny přesahující 90 %.

V nedávné době začala vznikat rezistence i k těmto antibiotikům. To mělo za následek rozvoj výzkumu nových struktur. V této práci jsme se zabývali několika z nich: mideplaninem, dalbavancinem (BI 397), A 40926 a MDL 63,246. Tato druhá generace glykopeptidových antibiotik se vyznačuje především rozšířeným spektrem účinku a lepší farmakokinetikou.

Rezonance povrchových plasmonů

V posledních letech se stále více obrací pozornost k využití optických biosenzorů jako prostředků pro komplexní charakterizaci interakcí biologických makromolekul obecně. Jedním z široce používaných biosenzorů je přístroj švédské firmy Biacore založený na tzv. rezonanci povrchových plasmonů (surface plasmon resonance, SPR). Jedná se o optický jev umožňující charakterizaci proteinových interakcí v reálném čase za použití nativních neznačených molekul. K SPR dochází na rozhraní tenkých kovových filmů s dielektrikem při totálním odrazu světla.

Měří se úhel odrazu, který vypovídá o změnách optických vlastností povrchové vrstvy čipu s imobilizovaným ligandem, které jsou způsobeny interakcí mezi tímto ligandem a proteinem rozpuštěným v tekuté mobilní fázi. Měření probíhá kontinuálně

v reálném čase, čímž jsou získána data vedoucí k popisu kinetiky proteinové interakce. Časový průběh SPR signálu je označován jako sensorgram. Používá tzv. rezonanční jednotky (resonance units, RU).

Metoda má několik výhod: umožňuje pracovat s nativními neznačenými molekulami, vyžaduje relativně malé množství proteinu (1 až 10 mg), je vysoce citlivá, detekuje i velmi slabé interakce, není časově náročná, doba jedné analýzy se pohybuje okolo 10 minut, je široce použitelná, umožňuje analyzovat peptidy, proteiny a jejich směsi, nukleové kyseliny, polysacharidy, lipidy, buněčné membrány, viry či dokonce celé nativní buňky.

Cílem této práce bylo optimalizovat podmínky HPLC analýzy za účelem stanovení čistoty vzorků antibiotik, připravit vhodné samotvořící vrstvy a kvantifikovat interakce mezi samotvořící vrstvou prezentující D-Ala-D-Ala a glykopeptidovými antibiotiky.

Pro zavedení metody HPLC analýzy jsme postupovali podle článku Gandolfi et al.[9] V případě antibiotik A 40926, dalbavancinu (BI 397), mideplaninu a MDL 63,246 gradientová eluce začínala na 100% fáze A (0,1% trifluoroctová kyselina) po dobu 40 minut se zvyšoval podíl fáze B (acetonitril) až na 100% a následovalo 10 minut 100% fáze B. V případě teikoplaninu jsme použili upravené podmínky podle článku Borghiho se spolupracovníky.[42] Mobilní fáze A obsahovala 95% 0,02M NaH_2PO_4 a 5% acetonitrilu. Fáze B obsahovala 25% 0,02M NaH_2PO_4 a 75% acetonitrilu. Gradientová eluce probíhala po dobu 75 minut z 20% na 40% fáze B.

Připravili jsme několik samotvořících vrstev pro prezentaci D-Ala-D-Ala o různých vlastnostech a délkách řetězce. Nejvýhodnější se ukázalo použití vrstvy tvořené několika uhlíkatým řetězcem a na druhé straně s několika polyetylen glykolovými skupinami.

Při disociaci ligandu z povrchu senzoru se může někdy vyskytovat tzv. „rebinding“. Jde o to, že ligand po uvolnění do volného roztoku je zachycen dalším vazebným místem. Z měření, které jsme provedli s průtokovými rychlostmi od 5 do 20 µl/min, vyplývá, že v našem případě je tento efekt zanedbatelný.

K měření interakcí jsme používali vrstvu tvořenou $HS\text{-}(CH_2)_{11}\text{-}EG_6\text{-}OCH_2\text{-}COOH$ s imobilizovaným dipeptidem D-Ala-D-Ala. Při měření se zjišťovala interakce s tímto povrchem a zároveň s povrchem kontrolním, jehož hodnota udává míru

nespecifické interakce. Výsledné senzogramy byly upraveny k výpočtu afinitních konstant v programu BIAevaluation. Z testovaných vzorků antibiotik vyplývá, že všechna mají vyšší afinitu k povrchu než vankomycin, zvláště dalbavancin (BI 397).

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