Globomycin: An Interesting Antimicrobial Depsipeptide

Discovering New Active Analogues

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Chemistry, School of Chemistry and Physics of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The research was financially supported by NRF.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

As the candidate's supervisor, I have approved this thesis for submission.

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PLAGIARISM DECLARATION

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ABSTRACT

In South Africa and other African countries infectious diseases are reported to be the main health problem and results into death. This is the reason why the pharmaceutical industry has tried to develop new antibiotics to fight these diseases. Medicinal chemists are now being challenged to fight these diseases as few drugs are available and micro-organisms have become resistant towards the available antibiotics. Gram-negative bacteria are one of those bacteria and it is difficult to inhibit it. Globomycin a "head-to-tail" cyclodepsipetide has grown interest in the pharmaceutical industry since it can specifically inhibit Gram-negative by its mode of action. Other Globomycin analogues synthesised have shown activity also against Gram-positive bacteria. In this regard new active Globomycin analogues are to be synthesised in this project to increase the activity of the antibiotic against Gram-negative and Gram-positive bacteria. Since the lipidic chain of Globomycin is aliphatic and is important for its activity, in this project it is studied what will happen on the activity of the peptide if some changes are made on the aliphatic chain such as introducing a carbonyl or sulfonyl group on the lipidic chain. The analogues were synthesised using Fmoc SPPS strategy on 2-CTC-resin as the solid support, cyclised then purified using the SEMI-PREP HPLC. This was the first synthesis of Globomycin in solid phase where cyclization was achieved by lactamization reaction. In total four analogues were synthesised namely **Fmoc-Globomycin**, **di-Hexyl-Globomycin**, **Hexanoic-Globomycin** and **Hexanesulfonyl-Globomycin**. Antimicrobial activity studies then followed where both linear and cyclic Globomycin analogues were tested against both Gram-negative and Gram-positive bacteria strains. The results illustrated that only the cyclic di-Hexyl-Globomycin analogue was active against both bacteria strains. This demonstrated that the cyclic state of the compound is very important and also the aliphatic nature. The results also agree with the work done by the Kogen group that the L-allo-Thr and L-allo-Ile units are not very important.

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When I joined this group last year I never thought by this time I will be writing my thesis. There are a lot of people that helped me.

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LIST OF ABBREVIATIONS

ACN: Acetonitrile

DCM: Dichloromethane

DIEA: N, N-Diisopropylethylamine

HPLC: High-Performance Liquid Chromatography

TFA: Trifluoroacetic acid

DMSO: Dimethylsulfoxide

TIS: Triisopropylsilane

AcOH: Acetic acid

DMAP: 4-Dimethylaminopyridine

THF: Tetrahydrofuran

MIC: Minimum Inhibition Concentration

NMR: Nuclear Magnetic Resonance

SPPS: Solid Phase Peptide Synthesis

EDC.HCl: N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

NI: No Inhibition

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CHAPTER 1

Introduction

1.1 Cyclic depsipeptides

A family of compounds called cyclic depsipeptides have showed a broad spectrum of biological activities such as antibacterial, antitumor, antifungal, anti-inflammatory effects, etc that have spotlighted them as head compounds to find new drugs to face all kinds of diseases[1, 2].

Cyclic depsipeptides can be defined as a type of peptides in which at least one amide bond is substituted by an ester bond. The naturally occurring cyclic depsipeptides have been mainly isolated from marine sources and microbial organisms. One of the recently discovered is the promising antibiotic Teixobactin, produced by a soil bacteria. In this kind of habitat with limited resources, the microbes use the antibiotic to kill off their food and water competitors.

Several years ago, a cyclic depsipeptide was isolated, Globomycin, which created high expectative because of its peculiar mode of action. Nowadays, after a period it has been neglected, the need of new antibiotics because of bacterial resistance growth, have made Globomycin a compound of high interest.

1.2 Background history on Globomycin

In 1978 a new antibiotic was isolated from four different strains of *Streptomyces* which exhibited selectivity against gram-negative bacteria by formation of spheroplasts. This characteristic merited it the name of Globomycin.[3] The first physico-chemical properties illustrated that it was a molecule with a melting point of 115°C, a molecular formula of $C_{32}H_{57}N_5O_{9,}$ soluble in a number of solvents such as methanol, ethyl acetate, chloroform, etc, however, slightly soluble in water.[4] Spectroscopical analysis such as IR revealed the presence of an ester bond, NMR revealed the presence of N-methyl group, and the hydrolysate of Globomycin indicated the presence of serine, threonine, glycine and one more unidentified amino acid. At that time, the final structural elucidation [5] concluded that the peptide antibiotic was composed of L-serine, L-allo-threonine, glycine, L-allo-isoleucine, N-methyl leucine and

3-hydroxy-2-methylnonaic acid (the absolute conformation of the two last residues was ambiguous) forming a cyclic structure as depicted in **Figure 1**. Interestingly, the determination of this structure showed as first time the existence of L-allo-Thr and L-allo-Ile as natural occurring amino acids.

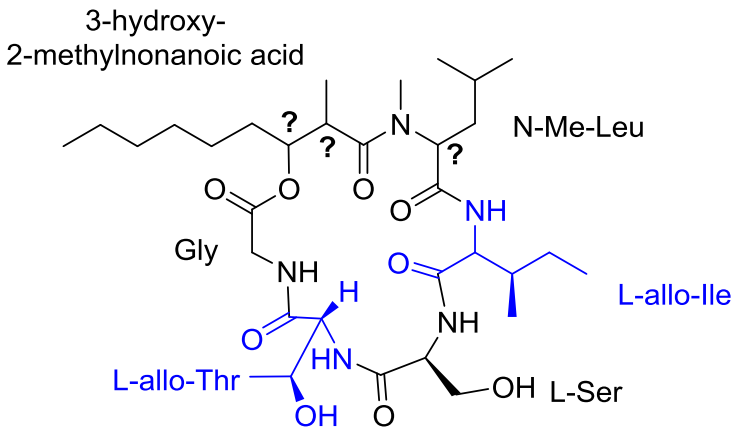


Figure 1: Initial established chemical structure of Globomycin. In blue are highlighted the two *allo* residues found for first time together in a natural compound. The question marks are placed on the ambiguous chiral centers

In the same year (1978), other Globomycin derivatives were discovered such as SF-1902 A_5, SF-1902 A_{4a}, SF-1902 A_2, SF-1902 A_3 and SF-1902 A_{4b} which had related physico-chemical and biological properties with Globomycin. These derivatives were isolated from *Streptomyces hygroscopicus* SF-1902 which resembles one of the producers of Globomycin. These derivatives differed from Globomycin as they had different length of the lipidic chain and some had valine instead of L-allo-isoleucine.

Although Globomycin aroused great interest from the point of view of its biological activity, it was not until two decades later when the conformation of all its residues was completely confirmed by x-ray crystallography (**Figure 2**) and the first chemical synthesis was reported.[6]

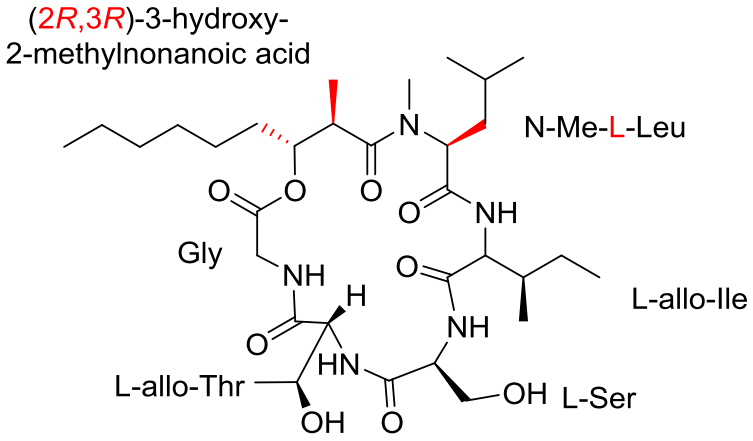


Figure 2: Final chemical structure of Globomycin determined by XRD. In red are highlighted the last stereocenters assigned by this technique.

1.3 Mechanism of Action

The first observation about Globomycin mode of action was its activity against certain, but not all, Gram-negative bacteria and total inactivity against Gram-positive bacteria. In *E. coli*, it was observed the formation of spheroplasts in the presence of Globomycin, hence it was established their inhibition of the bacterial cell wall synthesis.[3]

Gram- positive and gram-negative bacteria differ in their cell envelope. While gram-positive bacteria have a thick cell wall constituted by peptidoglycan, gram-negative bacteria have a thinner peptidoglycan layer surrounded by an outer membrane (**Figure 3**).[7]

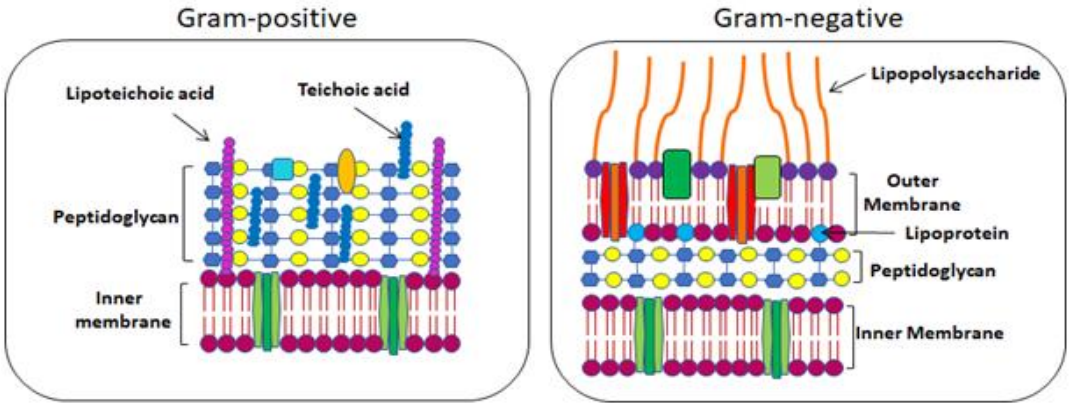


Figure 3: Differences in the structure of cell wall of gram-positive and gram-negative bacteria.

The outer membrane is composed of phospholipids, lipoproteins, proteins, and lipopolysaccharides (LPS). The LPS are located in the external face of the outer membrane contributing to the maintenance of the structural integrity of the bacteria and act as a barrier for many chemical attacks. The lipoproteins are located mainly in the inner layer of the outer membrane, as the connectors of the outer membrane to the peptidoglycan layer.

Globomycin mode of action was demonstrated to be related with the biosynthesis of lipoproteins. When Inukai and co-workers studied it in deep, they determined that Globomycin mechanism of action involved an inhibition of the enzyme that converts prolipoprotein to lipoprotein, since they found an accumulation of prolipoprotein in the envelope.[8] For better understanding of this mode of action in **Figure 4** is represented as a simplified scheme of general lipoprotein processing and sorting pathway in Gram-negative bacteria.

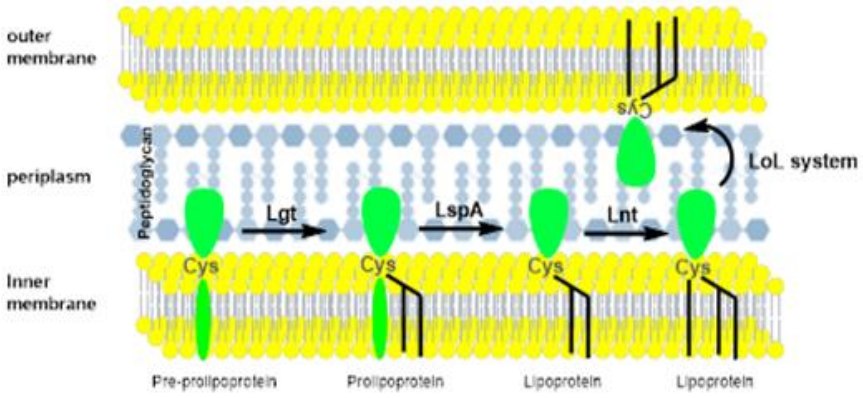
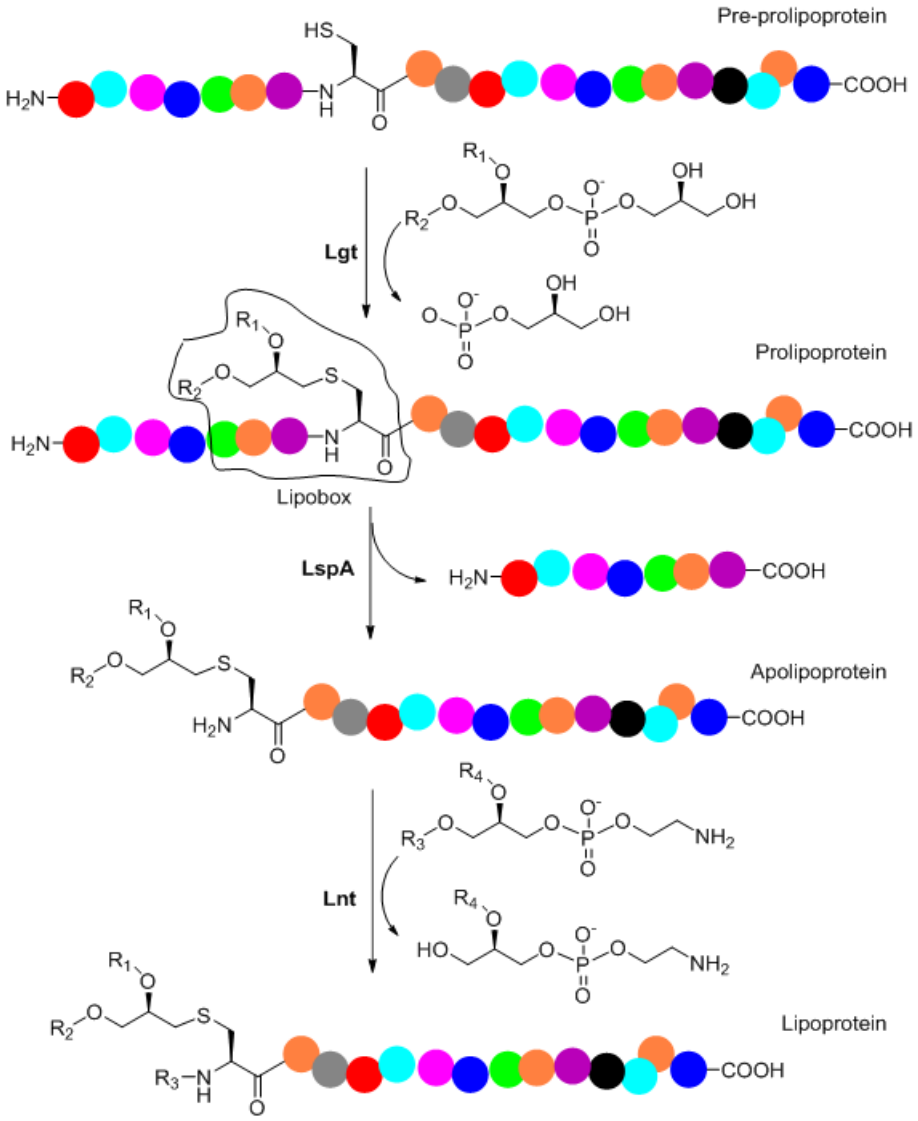


Figure 4: Schematic representation of Lipoprotein processing

Briefly, after the cytoplasmatic synthesis of the pre-prolipoprotein and its anchorement into the inner membrane, the first step in the pathway modification consists of the addition of a phosphatidylglycerol to the sulfhydryl group of the cysteine (Cys) which is catalized by the enzyme phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt). After the acylation, the signal peptide is cleaved by the enzyme lipoprotein signal peptidase II (LspA). Then, the process continues with a second acylation, this time carried out on the N-terminal of the Cys residue and finally the lipoprotein is translocated to the outer membrane by the localization of lipoprotein enzymes that conform the LoL system (**Scheme 1**).[9, 10]



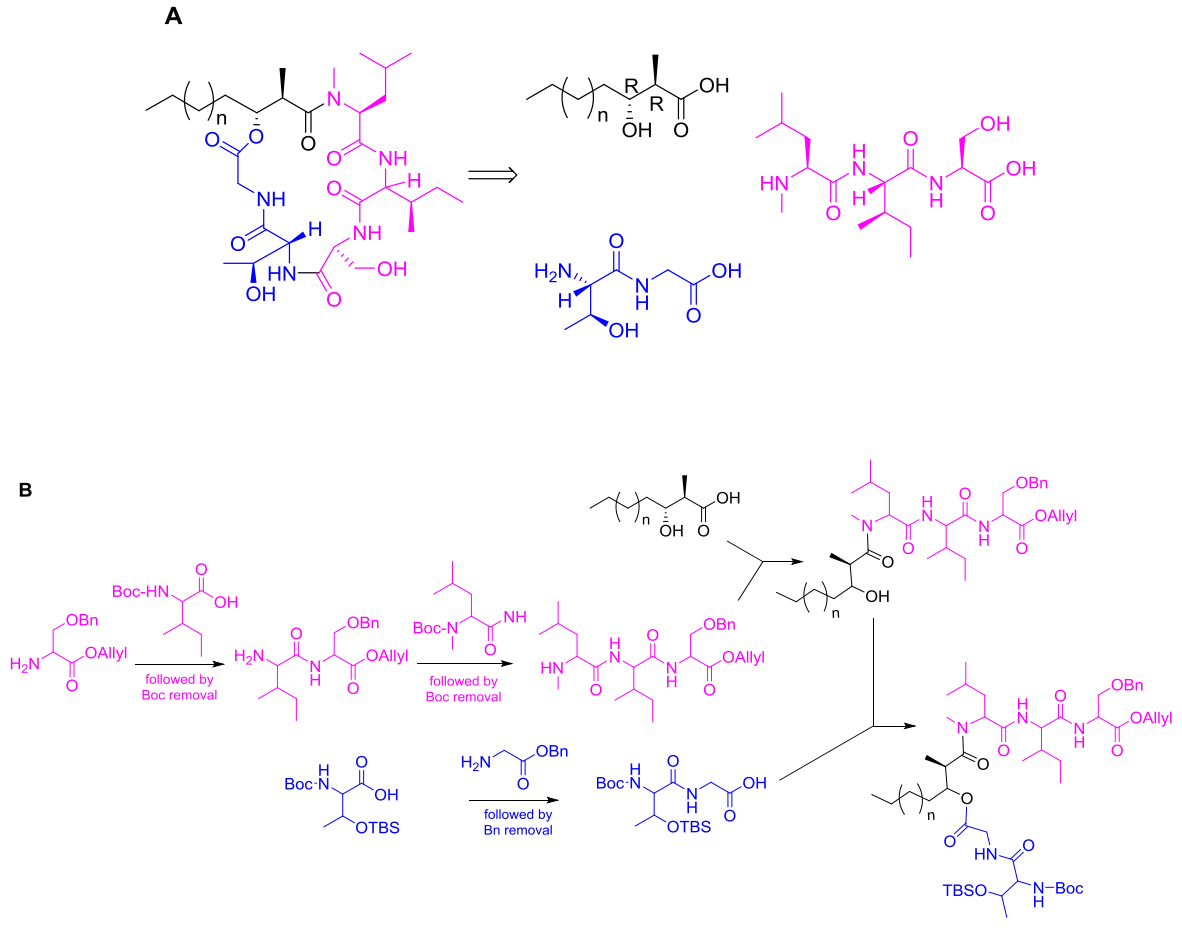
Scheme 1: Chemical transformation carried out in each step of the lipoprotein processing.

Globomycin specifically inhibits LspA,[11] a small protein of only 169 amino acids residues. The enzyme recognizes a consensus sequence present in the signal peptide of the prolipoprotein that always ends in a Cys residue. This sequence of four amino acids is called lipobox and the most repetitive is LAGC. Globomycin imitates a signal peptide substrate binding with high affinity the active site and avoiding the access to the active site for real lipoprotein substrate.[12]

Finally, it worth to remark that the characteristic that more attracted the attention towards the mode of action of Globomycin was its ability to cross the strong barriers as the LPS, outermembrane and periplasmic space, to reach its target located in the inner membrane.

1.4 Chemical synthesis

As it was mentioned previously, the first total synthesis of Globomycin was delayed more than two decades after its finding. It was in 2000 when the group of Kogan and co-workers designed the first synthetic route based in a convergent approach in solution.[6] The idea behind was to be able to determine the final conformational structure at the same time that open a way to synthesise Globomycin and other analogues to start structure-activity (SAR) study.[13-15] In their design, they fragmented Globomycin in three parts: one fragment was the β-hydroxy carboxylic acid, second fragment was L-*allo*-threonine and glycine moiety, and third fragment was N-Methyl-leucine, L-*allo*-isoluecine and L-serine moiety (**Scheme 2A**). Each fragment was synthesised independently with the corresponding protection in the required functional groups and after the assembly of the three units (**Scheme 2B**).

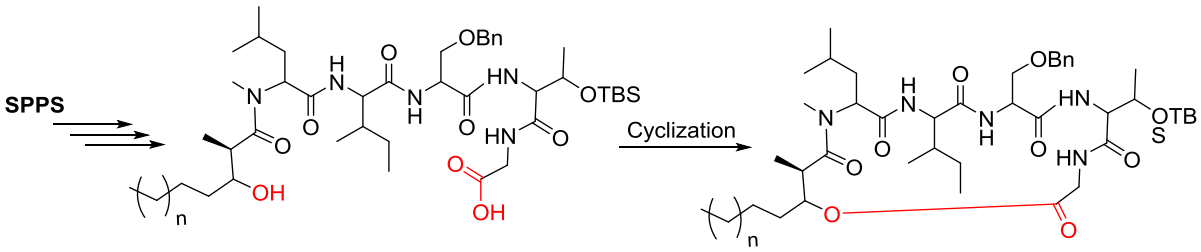


Scheme 2: Convergent **s**ynthesis of Globomycin in solution developed by Kogen group.

Finally, after the removal of Boc protecting group present at the Thr α-amino group, and the allyl protecting group of the α-carboxylic group of the Ser residue, the macrolactamization was afforded. The cyclic compound was fully deprotected to obtain Gobomycin.

This research team prepared a large number of analogues following this methodology, their antimicrobial activities will be discussed along the next section.

Any other synthetic scheme was proposed until several years later, when the first and only Solid-phase synthesis report was published. This work by Sarabia *et al.*[16] represents a very different approach, not only because of the use of solid-phase peptide synthesis (SPPS) methodology if not because instead to carry out the cyclization through a macrolactamization they propose a macrolactonization (**Scheme 3**).

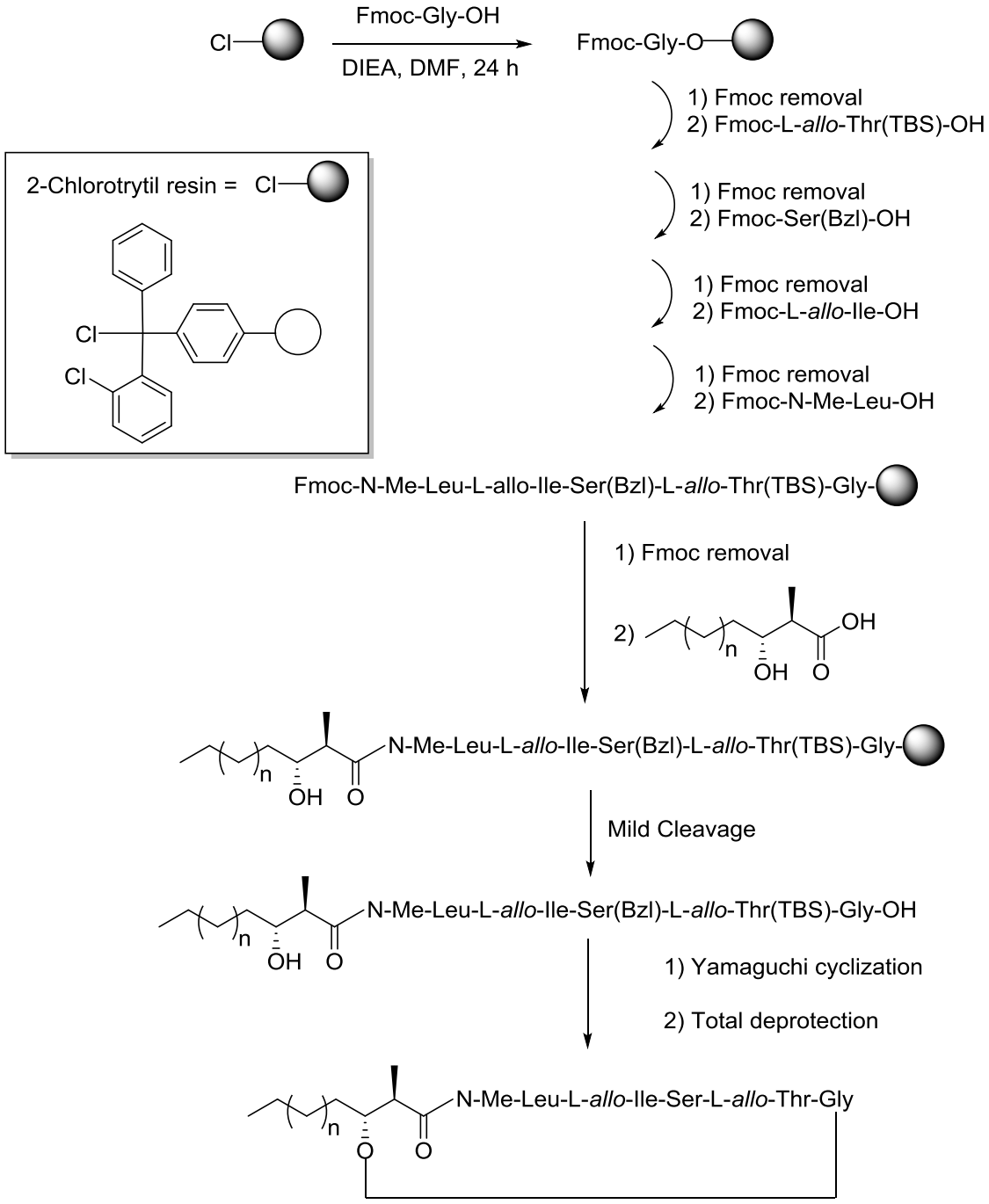


Scheme 3: Globomycin cyclization through a ester bond formation (lactonization).

To build up the linear peptide they used 2-Chlorotrityl (2-CTC) resin that allows the release of the carboxylic acid peptide while the side chains remain protected.[17] After the loading of the first amino acid (Fmoc-Gly-OH) by esterification in presence of *N*,*N*-Diisopropylethylamine (DIEA), the peptide elongation until the N-MeLeu residue was carried out using Fmoc-amino acid derivatives by means of N,N'-diisopropylcarbodiimide (DIC) and hydroxybenzotriazol (HOBt) as coupling cocktail. The Fmoc group was removed using by standard treatment with 20% piperidine in *N*,*N*-dimethylformamide (DMF).

Regarding the β-hydroxy acid also they presented an innovative synthetic methodology based in their previous works[18, 19] on asymmetric epoxidation for the stereoselective synthesis. To incorporate this moiety into the peptide chain they used diethyl cyanophosphate (DEPC) as coupling reagent and a double coupling was performed to ensure total reaction of the amine group.

Once the peptide chain was completed, it was cleaved from the resin under mild conditions keeping in place the side chain protecting groups. Then, the ester bond between the C-terminus of the peptide and the alcohol group of the β-hydroxy acid was done in solution following the Yamaguchi method.[20] Finally, all the permanent protecting groups (Bzl and TBS) were removed. The full synthetic scheme is shown in **Scheme 4**. The HPLC and NMR analysis was then performed to confirm the peptide [16].



Scheme 4: Synthesis of Globomycin in Solid Phase Peptide Synthesis

1.5 Structural-Activity Relationship studies

Although Globomycin was the major component isolated from *Streptomyces hygroscopicus* SF-1902, other related compounds were found in the mixture. The main differences lied in the alkyl lipidic chain length and some analogues have a Val residue in place of L-allo-Ile[21]. From the natural occurring analogues, it could be extracted the first conclusions about SAR:

(i) As much longer is the alkyl lipidic chain, higher activity and (ii) L-allo-Ile is not a crucial residue.

The initial Kogen's group SAR studies,[14] corroborated the first conclusion before. They prepared an analogue with longer alkyl lipidic chain than the isolated natural analogues and they found an improvement in the antimicrobial activity even expanding its activity toward gram-positive bacteria. Moreover, later they proved that the stereochemistry of the β-carbon is also crucial in the lipidic chain.[15] In the first study, they also did the following replacements:

1- L-allo-Ile unit by Ile: the compound maintains almost the same activity; thus, it is not a crucial residue.

2- L-allo-Thr unit by Thr: total loss of activity.

3- L-allo-Thr unit by Thr-OMe: the activity was kept.

4- L-allo-Thr unit by EtGly: the activity was kept.

The conclusion from these three (2-4) replacements is that, when the hydroxyl function is there, the conformation is important, otherwise this functional group is not needed then no conformational matters thus affect the activity.

5- N-Me-Leu unit by Leu: total loss of activity; this is a crucial residue

In the extension of this work,[15] more substitutions were carried out:

6- N-Me-Leu unit by N-Et-Leu: very poor activity

7- N-Me-Leu unit by Pro: total loss of activity

8- N-Me-Leu unit by Homo-Pro: total loss of activity

Substitions 6 to 8 confirmed than N-Me-Leu is a crucial residue.

9- Ser unit by Ser-OMe: total loss of activity; OH is crucial for activity.

10- Gly unit by N-MeGly: total loss of activity: H on this position plays an important role.

For better understanding, **Figure 5** summarizes the main conclusion from the SAR studies carried out by Kogen's group.

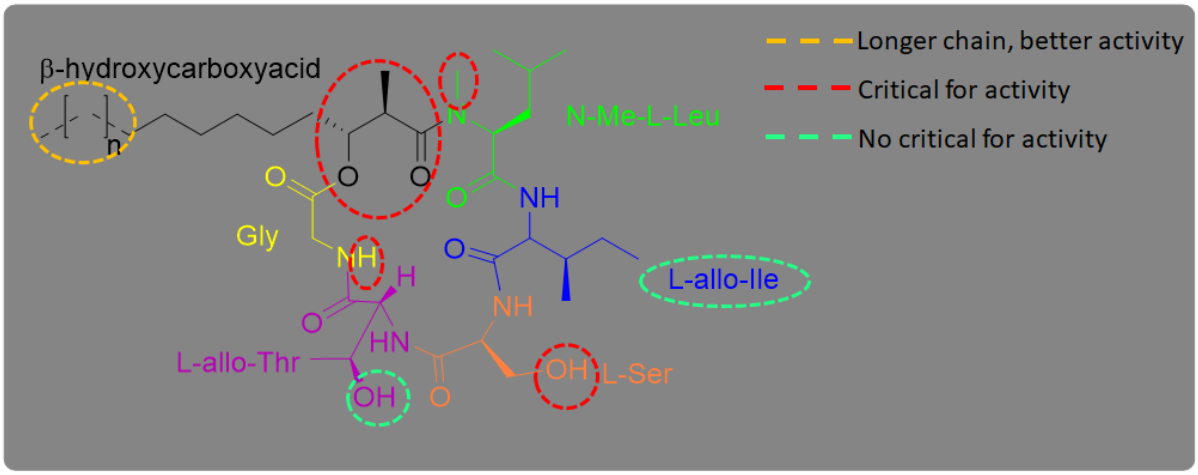


Figure 5: SAR studies from the Kogen group

In this context and considering that our group is interested in developing new strategies to fight microbial diseases, we called our attention to this intriguing peptide. In order to develop a synthetic strategy and at the same time to start a structure-activity relationship study, we propose the synthesis of several analogues where L-Val was used in the position of L-allo-Ile, because Val is already in some natural analogues; where L-allo-Thr is substituted by the L-Thr, which is much more economical affordable and keeping the configuration at the α-carbon that is part of the cycle; and where the β-hydroxy linker is substituted by the amino acid D-Ser, which is also a β-hydroxy acid and has the same configuration than the β-hydroxy linker at the α-carbon (**Figure 6**). Finally, taking advantage of the presence of the amino group in the D-Ser, we propose to incorporate hydrophobic groups through that function (**Figure 7**).

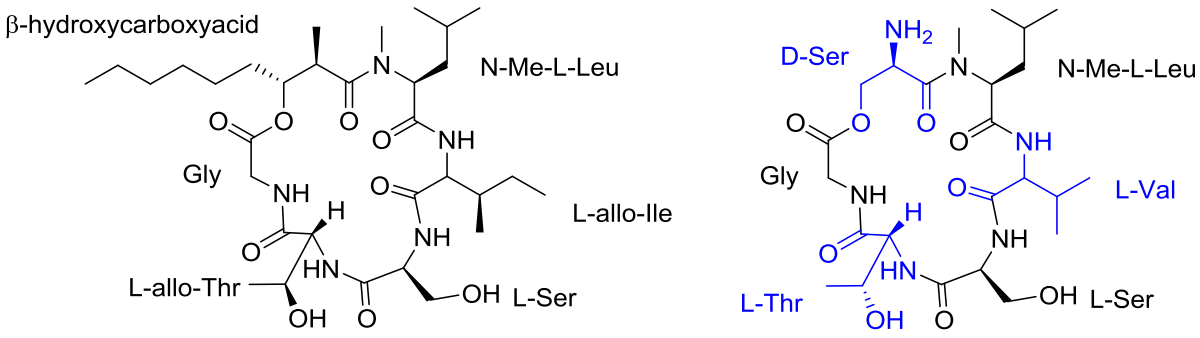


Figure 6: Structure comparison between Globomycin and the scaffold used in our study. In blue are highlighted the differences

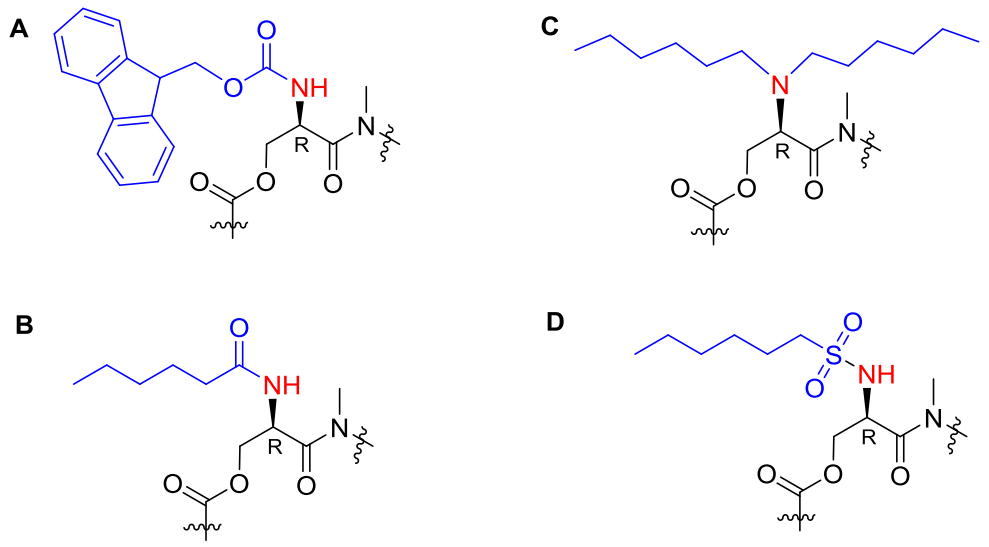


Figure 7: Globomycin analogues to be synthesised in this project. A) Fmoc-Globomycin, B) Hexanoic-Globomycin, C) di-Hexyl-Globomycin and D) Hexanesulfonyl-Globomycin

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CHAPTER 2

Results and Discussion

2.1 Chemical Synthesis

Globomycin analogues were synthesised on SPPS using 2-chlorotrityl chloride (2-CTC) resin. The first amino acid was loaded on the resin using DIEA in DCM. The rest of protected amino acids (Fmoc/tBu) were incorporated using DIC and OxymaPure as coupling reagent. After the incorporation of the protected amino acid, the Fmoc group was removed with 20% piperidine in DMF. After the elongation of the linear chain, the peptide was cleaved from the resin with 95% TFA in the presence of scavengers. The cyclization was done in solution at 10^{-4} M using DCM as a solvent and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), OxymaPure, and DIEA as activation method at a basic pH. In total four analogues were synthesised. The first analogue has Fmoc on the D-Ser, the second one was synthesised by substituting Fmoc from the first analogue with hexanoic acid. The third analogue had di-substituted hexyl on the NH_2 group of D-Ser. The fourth analogue had hexanesulfonyl instead of Fmoc. The peptides were then purified using semi preparative HPLC.

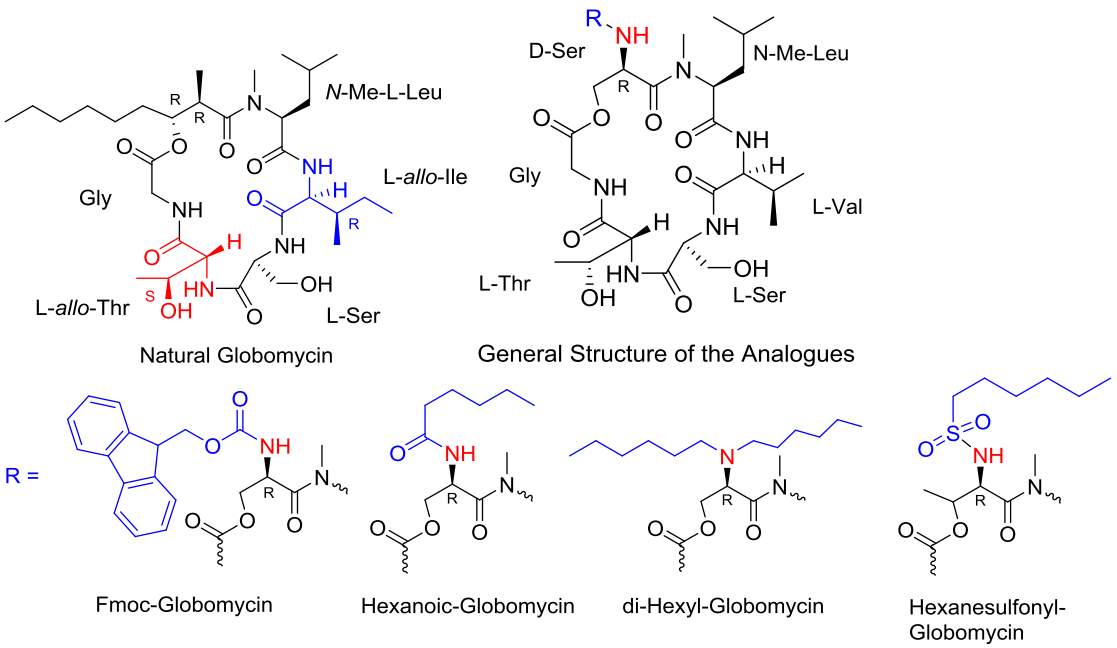
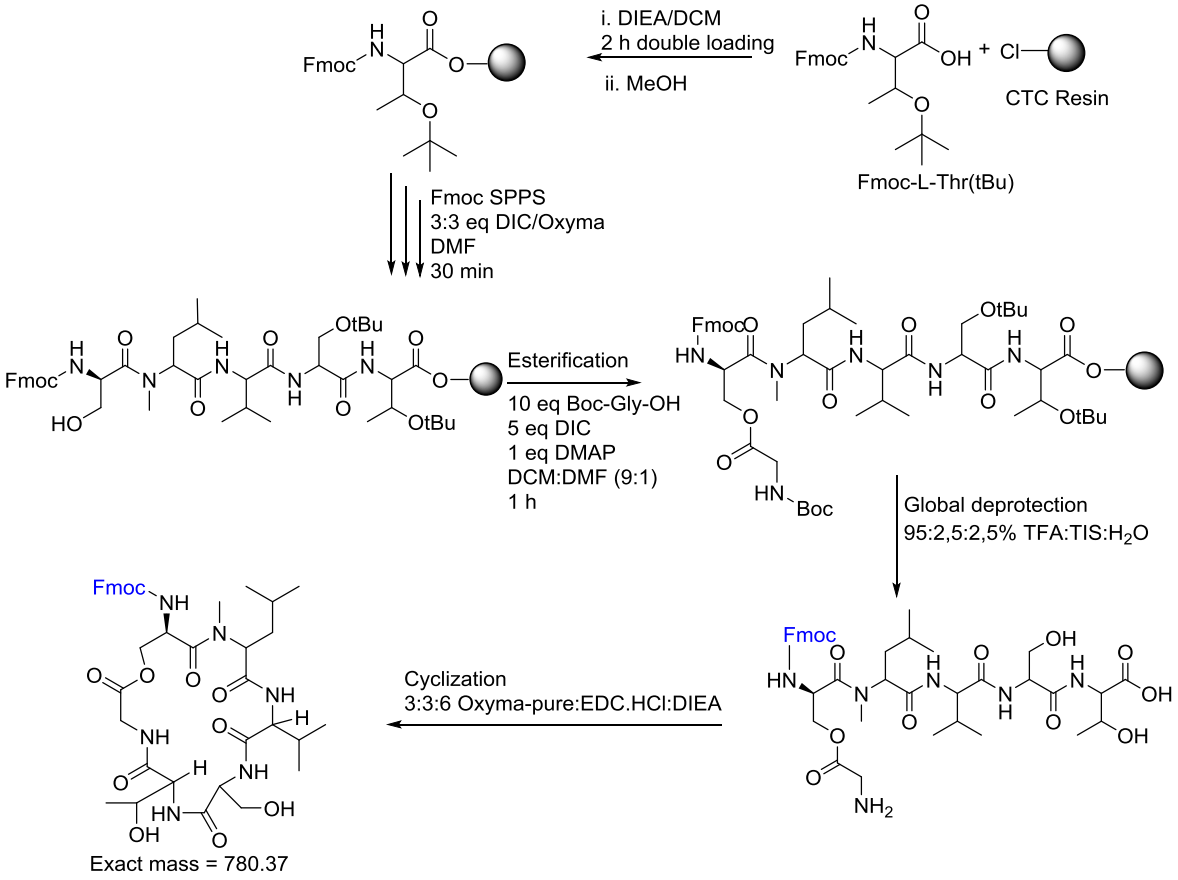


Figure 1: Chemical structure of natural Globomycin and general structure of the analogues synthesised with the R-group defined.

The first analogue was named Fmoc-Globomycin as the Fmoc group was not removed from D-Ser (**Figure 1**). The synthetic scheme of the analogue is shown in **Scheme 1**. The synthesis of the peptide was challenging as the choice of the protecting groups to be used was very vital. Fmoc protected amino acids were used for the chain elongation except the esterification step where Boc-Gly-OH was used instead. This was done to keep the Fmoc group on the D-Ser so it will be available during cyclization and prevent any side reactions. The esterification step was done between the hydroxyl group of the side chain of D-Ser and the carboxylic group of Gly. The esterification step was a crucial step during peptide chain elongation as it takes place with low yield presumably because of inter or intra chain interactions. During the synthesis double coupling of Gly was performed to ensure 100% yield.



Scheme 1: Synthetic scheme of the first analogue (Fmoc-Globomycin)

Once the linear peptide was synthesised, it was purified before cyclization. This was due to large amount of impurities obtained when cyclised without purifying. The peptide was cyclized in solution at low concentration of 0.0001 M. This was due the fact that if the peptide cyclization solution is concentrated the peptide will only "see" itself and dimerize. The cyclization point is also important. For this synthesis the cyclization point was between

the carboxylic group of L-Thr and the amine group Gly. This was a good choice since Gly does not have the side chain and all the other amino acids used have side chains excepts for N-MeLeu which is more challenging to use as the cyclization point since it is difficult to introduce another amino acid after the N-methylated amino acid even in solid phase synthesis. The other alternative cyclization point is between Gly and D-Ser but this was not favoured as lactonization reaction was going to occur between the hydroxyl group of the side chain of D-Ser and the carboxylic group of Gly forming an ester bond. This reaction is more difficult as compared to lactamization due to the poorest nucleophilicty of the hydroxyl group *vs* the amine ones. Thus a priori, the chosen cyclization point was a good choice.

2.2 Analysis

After the synthesis, the sample was analysed by HPLC and LC-MS. When comparing the HPLC chromatograms of the linear Fmoc-Globomycin and the cyclic Fmoc-Globomycin (**Figure 2**), it is observed that the peak shifted from lower retention time (7.638 min) to higher retention time (9.764 min) indicating non-polarity of the compound after cyclization. LC-MS analysis (**Figure 3**) indicated that after cyclization 18 m/z is lost from the compound which indicated a loss of H_2O molecule thus the compound became more non-polar since the hydroxyl group is lost from Thr and an hydrogen is lost from Gly thus an amide bond is formed. The same pattern is observed for all the analogues and the HPLC and LC-MS chromatograms are shown from **Figure 4** to **Figure 9**.

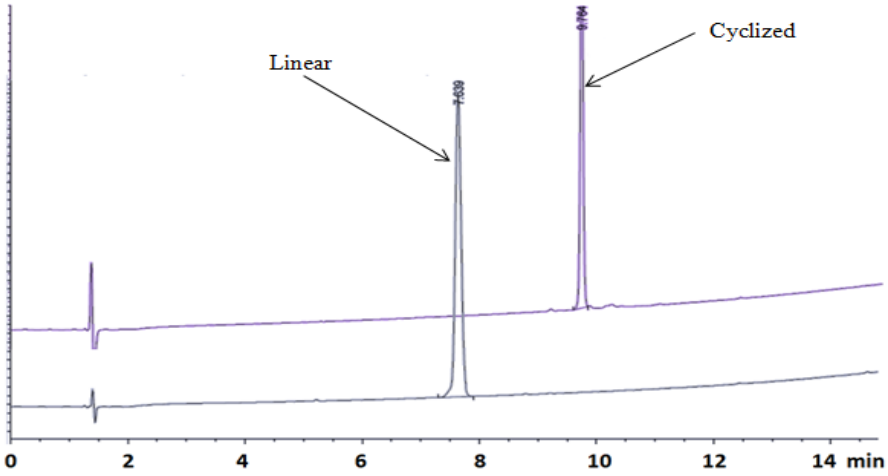


Figure 2: HPLC Chromatogram of linear and cyclic Fmoc-Globomycin in 5-95% of 0.1% TFA in ACN

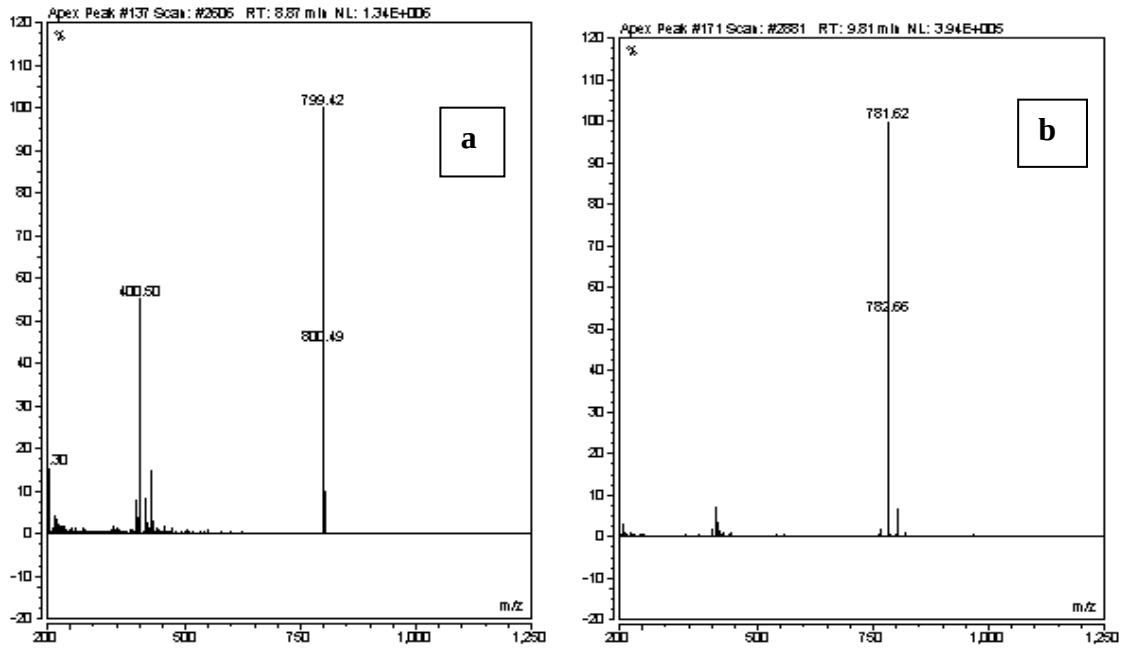
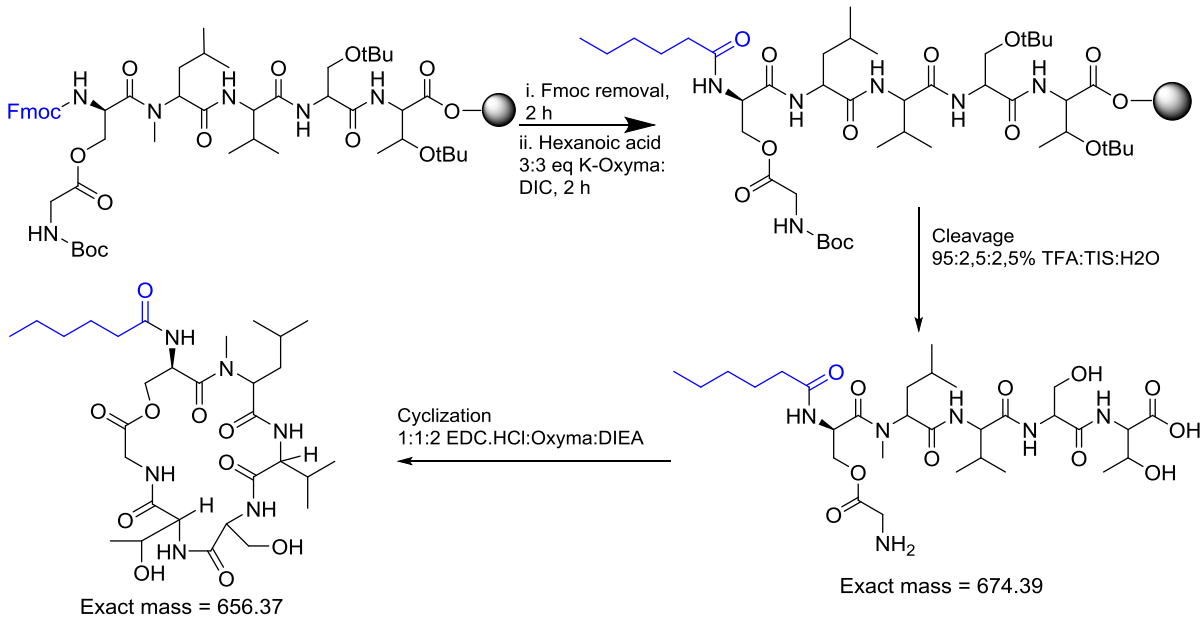


Figure 3: MS spectra of linear (a) and cyclic (b) Fmoc-Globomycin (from LC-MS)



Scheme 2: Synthetic scheme of the second analogue (Hexanoic-Globomycin)

The second analogue was synthesised the same way as the first analogue (**Scheme 2**) and after the esterification, the Fmoc group was removed from D-Ser and hexanoic acid was incorporated on the resin. The peptide was cleaved from the resin and cyclization was done using 1:1:2 equivalence of EDC.HCl-Oxymapure-DIEA. After cyclization the linear peptide was still observed but both linear and cyclic were purified using SEMI Preparative HPLC and

purity of the compound was checked on analytical HPLC. Greater than one (>1) mg of each compound was obtained and submitted for biological activity studies.

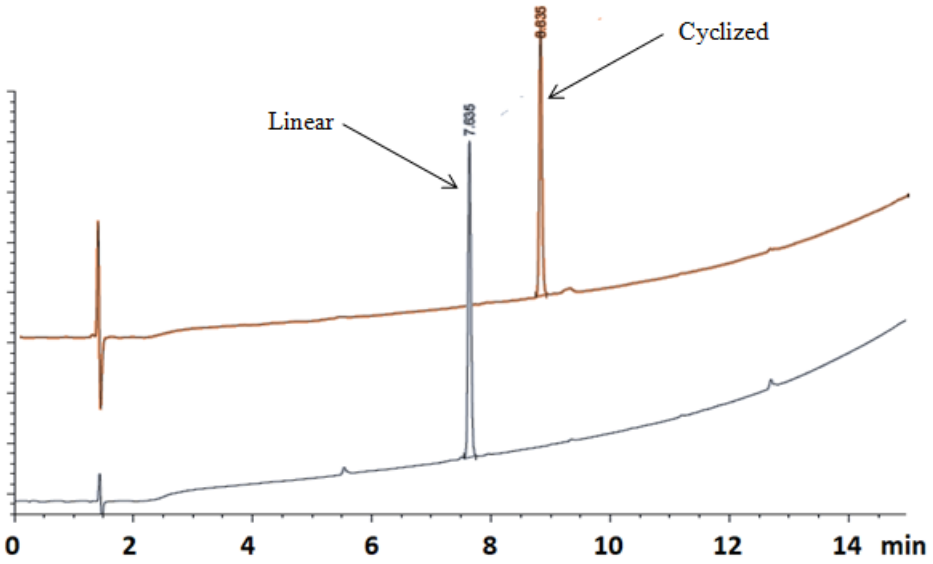


Figure 4: HPLC chromatogram of linear and cyclic Hexanoic-Globomycin in 5-95% of 0.1% TFA in ACN

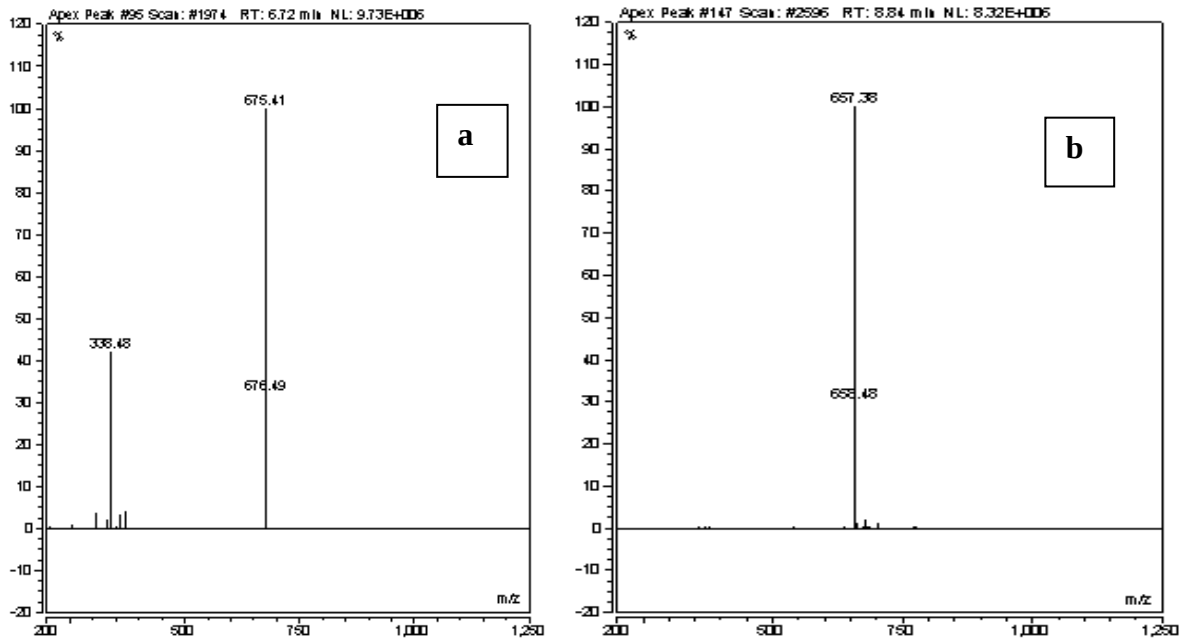
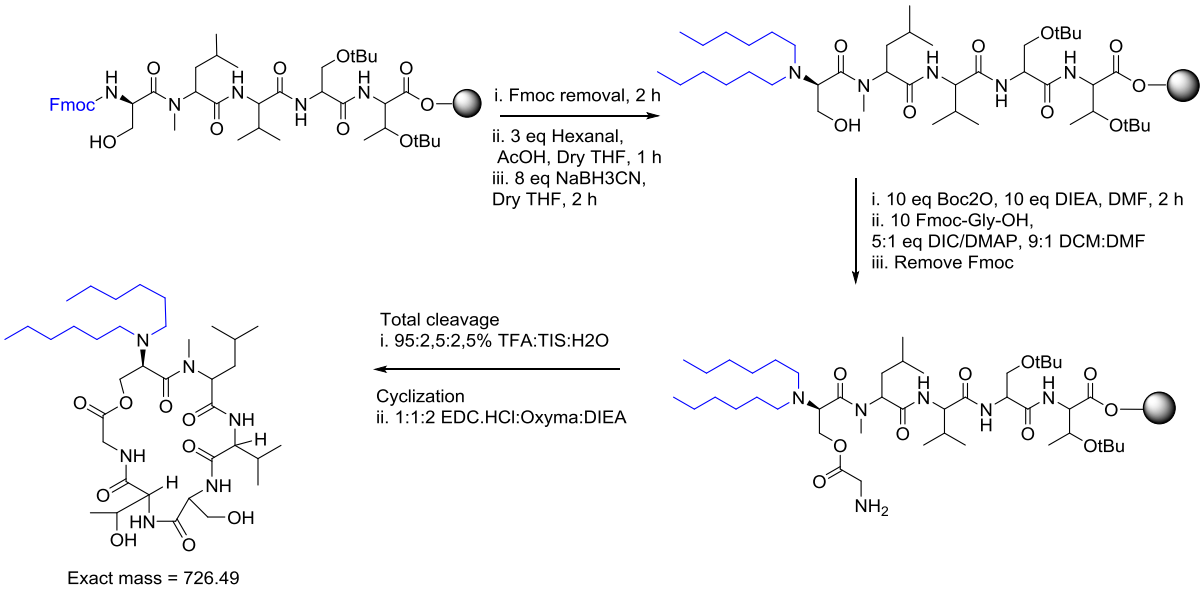
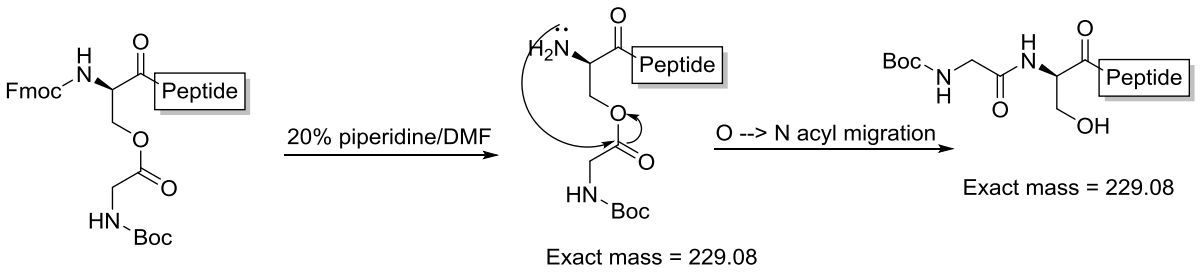


Figure 5: MS spectra of linear (**a**) and cyclic (**b**) Hexanoic-Globomycin (from LC-MS)



Scheme 3: Synthetic scheme of the third analogue (di-Hexyl-Globomycin)

For the third analogue in **Scheme 3**, Fmoc group was removed before the esterification due to the side reaction that was occurring when Fmoc was removed after carrying out the esterification. This was because Fmoc is removed under basic conditions, and in these conditions the amine liberated can attack the carbonyl of the ester. Then there is rearrangement of protons that take place as shown in **Scheme 4**. This reaction is called O → N acyl migration [22]. The problem with this rearrangement is that it cannot even be detected by LC-MS as it has the same mass for both the product and the by-product. As it is obvious, after the occurrence of this reaction, there is not any free amine able for carrying out a reductive amination. Therefore the alkylation was done before the esterification.



Scheme 4: Side reaction occurring when Fmoc is removed after esterification (O → N acyl migration) [1].

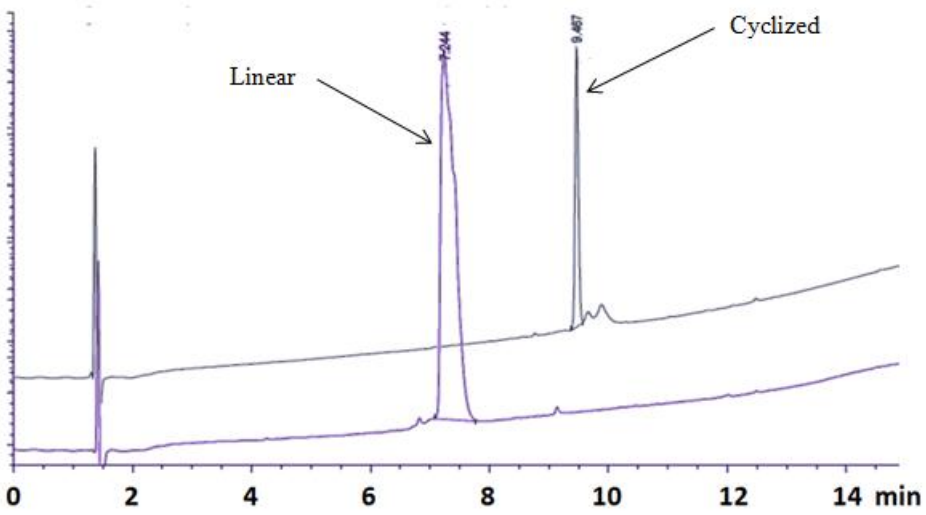


Figure 6: HPLC chromatogram of linear and cyclic di-Hexyl-Globomycin in 5-95% of 0.1% TFA in ACN

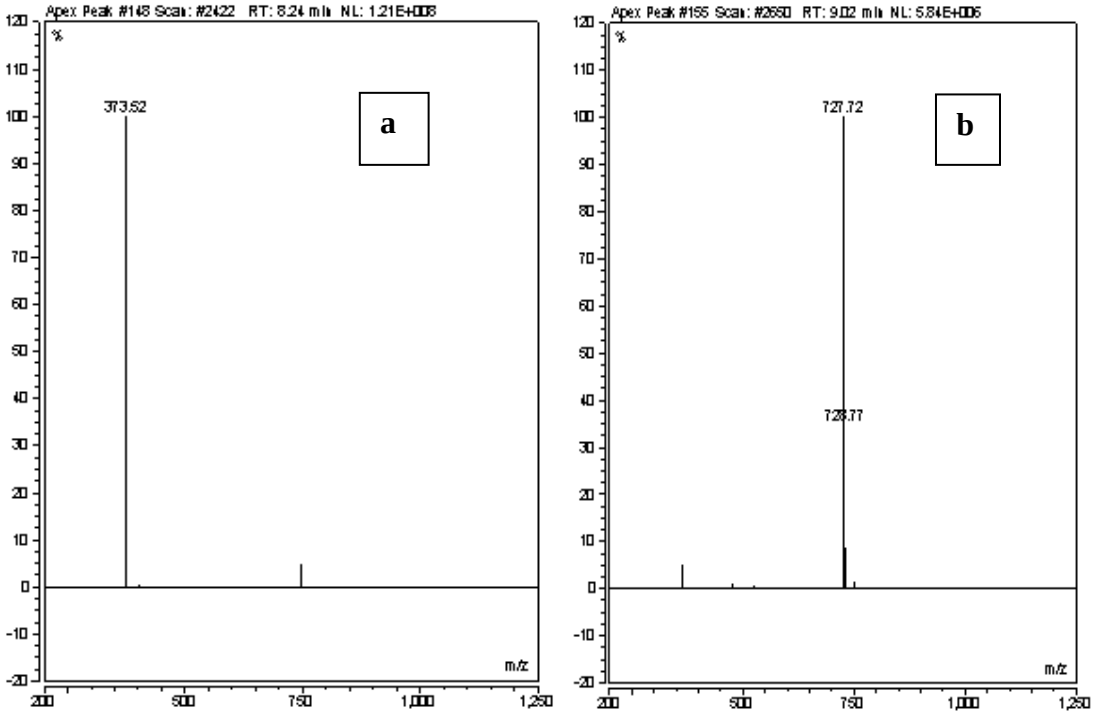
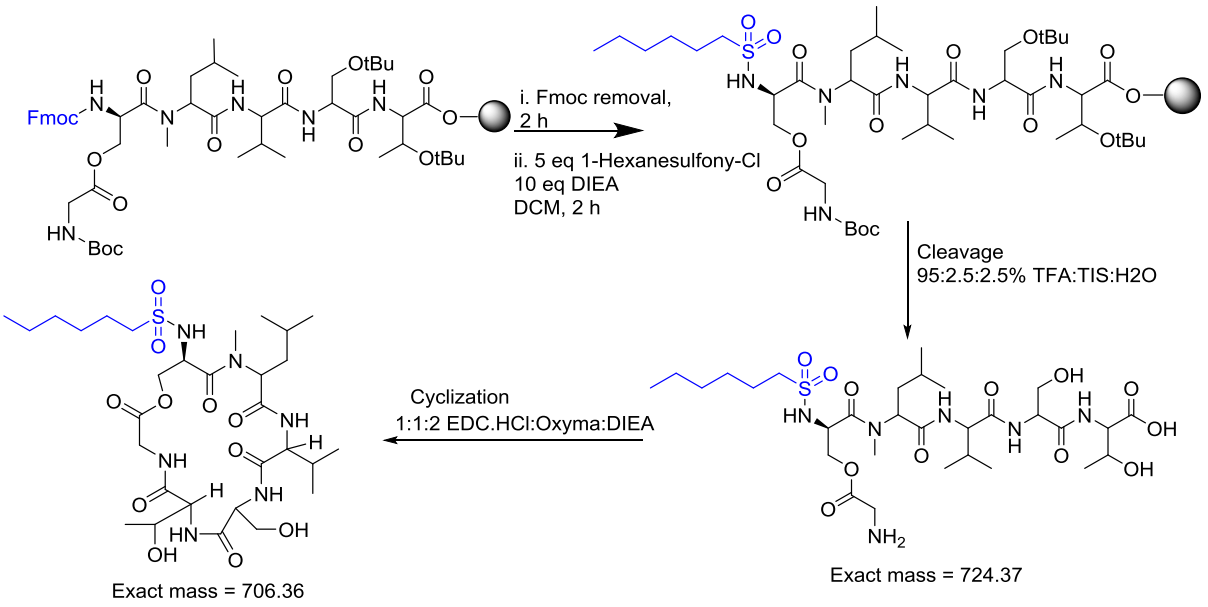


Figure 7: MS spectra of linear (**a**) and cyclic (**b**) di-Hexyl-Globomycin (from LC-MS)

The mass spectra of the linear analogue 3 showed a (M+2)/2 (ion +2) peak as the dominant peak instead of M+1 as compared to the mass spectra of the other analogues.



Scheme 5: Synthetic scheme of analogue four (Hexanesulfonyl-Globomycin)

Synthetic **Scheme 5** shows the synthesis of the fourth analogue where Fmoc was substituted by hexanesulfonyl. After purification of the cyclic compound not enough sample was obtained for biological testing thus only the linear was tested and only the linear LC-MS chromatogram is shown in **Figure 9**.

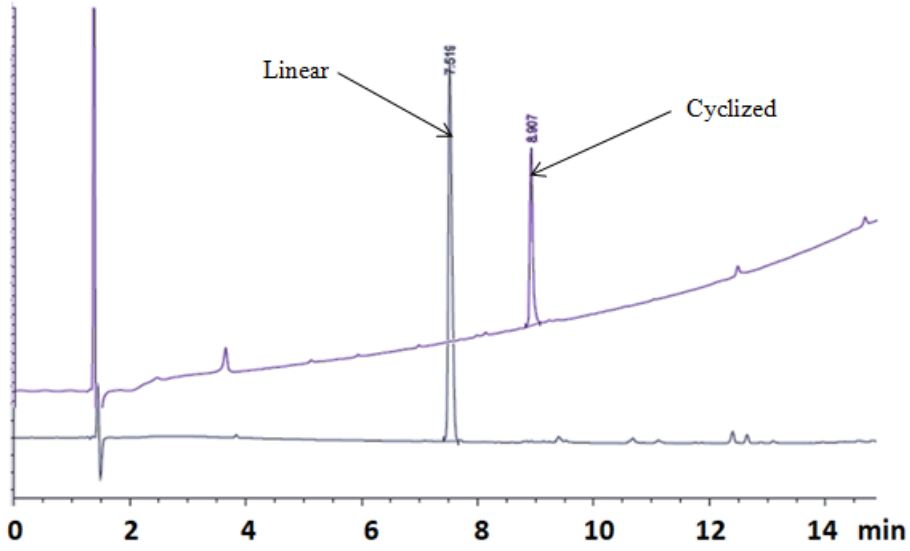


Figure 8: HPLC chromatogram of linear and cyclic Hexanesulfonyl-Globomycin in 5-95% of 0.1% TFA in ACN

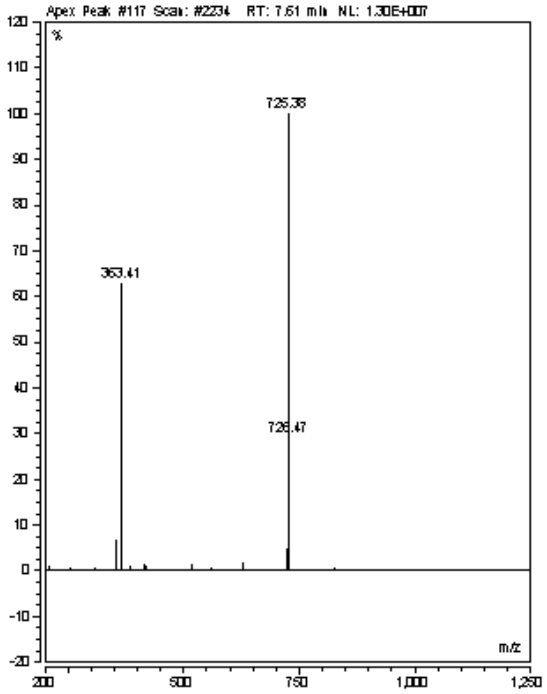


Figure 9: MS spectra of linear Hexanesulfonyl-Globomycin (from LC-MS)

2.3 Antimicrobial activity studies

After the purification and analysis of the Globomycin analogues using HPLC and LC-MS, their antimicrobial activity tests were then done against two Gram-positive bacteria strains namely *Staphylococcus aureus* and *Bacillus subtilis* and one Gram-negative bacteria strain *Escherichia coli* (**Table 1**). The compounds were dissolved at 1 mg/mL in 90% DMSO solvent system due to their poor solubility in water. To check if 90% DMSO had an effect on the bacteria strains it was also tested against them. It was found that it had no inhibition (NI). The MIC was reported at a concentration of 100 µg/mL.

The results showed that only **cyclic di-Hexyl-Globomycin** was active at this concentration against the all the bacteria strains. This could be due to the two aliphatic chains attached in the lipidic chain position since the hexyl group was di-substituted on the linker. This also agreed with the work reported by the Kogen group [2] that the longer the alkyl chain, more active is the Globomycin analogue. Thus two hexyl groups were attached on the amine group meaning longer or more aliphatic is the alkyl chain. The other cyclic analogues were not active at this concentration this could be due to the carbonyl group present on the lipidic chain. All the linear analogues were inactive. This could be due to the fact that the natural

Globomycin is cyclic thus the cyclic nature had an effect on the activity since even the **linear di-Hexyl-Globomycin** was also inactive.

The antimicrobial activity results obtained from this project also agreed with the SAR studies performed by the Kogen group [15]. They stated that the L-allo-Thr and L-allo-Ile units are not very important for biological activity. This is also seen on these analogues when L-allo-Thr was substituted by L-Thr and L-allo-Ile was substituted by L-Val. However, the cycle-di-Hexyl-Globomycin analogue was active.

Table 1: MIC (µg/mL) for Globomycin analogues

	Gram-positive		**Gram-negative**
Globomycin derivatives	***S. aureus* ATCC 29213**	***B. subtilis* ATCC 6051**	***E. coli* ATCC 25822**
Cycle Fmoc	>100	>100	>100
Cycle di-Hexanal	**100**	**50**	**100**
Cycle Hexanoic	>100	>100	>100
Linear Fmoc	>100	>100	>100
Linear Hexanal	>100	>100	>100
Linear Hexanoic	>100	>100	>100
Linear Hexanesulfonyl	>100	>100	>100
90% DMSO	NI	NI	NI

NI: No inhibition

Experimental Procedure

2.4 Materials

All the reagents and solvents used were obtained from different commercial suppliers and were used without further purification unless stated otherwise. For analytical HPLC; Agilent 1100 system was used and to process data; Chemstation software was used. Solvent A: 0.1% TFA in H_2O; Solvent B: 0.1% TFA in ACN. A flow rate of 1 mL/min and UV detection wavelength of 220 nm were used. LC-MS analysis was done using Thermo Scientific Ultimate 3000 using a Phenomex Luna C_{18} column (36 µm, 4.6 × 150 mm) and data was processed using Chromeleon 7 software. Solvent A: 0.1% formic acid in H_2O; Solvent B: 0.1% formic acid in ACN. Crude peptide was purified on SEMI-Preparative HPLC, Young Lin Instrument Autochro-3000. Same solvent system and wavelength detection as analytical HPLC is used. The column used was a Phenomenex Luna C_{18} (2) column (10 µm, 10 × 250 mm), with a flow rate of 7 mL/min.

2.5 Methods

2.5.1 Synthesis of linear peptide

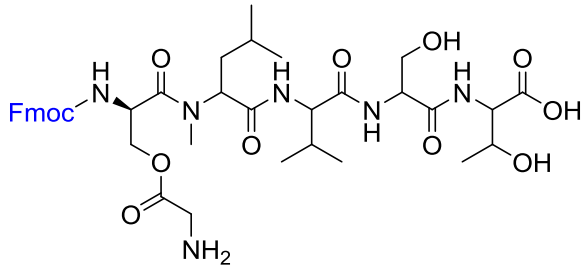
The peptide was synthesised using Solid Phase Peptide Synthesis (SSPS) on a 10 mL polypropylene syringe fitted with a polyethylene filter disc using 128 mg of 2-CTC (0.78 $mmolg^{-1}$ loading) resin as the solid support. The resin was first activated with 50% thionyl chloride in DCM for 4 hours. Then filtered and washed with DCM several times. The first amino acid, Fmoc-Thr(tBu)-OH (0.397 mg, 0.1 mmol, 1 equiv) was loaded to the resin using DIEA (174 µL, 1 mmol, 10 equiv) in 0.5 mL of DCM and left on the shaker for 1 hour. This was repeated twice for complete loading. The resin was then capped with 100 µL of methanol for 30 min, then washed with DCM (3 × 10 mL, 1 min). The Fmoc group was then removed using 20% piperidine in DMF (2 × 10 mL, 10 min) then washed with DMF (3 × 10 mL, 1 min) only. The coupling of the next amino acids was achieved using Fmoc-AA-OH-DIC-OxymaPure (3:3:3 equiv. respect to the resin). After each coupling step, the Fmoc group was removed using 20% piperidine in DMF (2 × 10 mL, 10 min) and resin washed with DMF (3 × 10 mL, 1 min). When the tetrapeptide was achieved (H-N-Me-Leu-Val-Ser-Thr-OH), some beads of resin were treated with TFA-Triisopropylsilane (TIS)-H_2O (95:2.5:2.5) for 1 hour. The solution was then precipitated with diethyl ether, centrifuged, discards diethyl ether

and dried under vacuum. The compound was then dissolved in 10%AcOH in H_2O, filtered and injected on HPLC and LCMS to monitor the reaction (this process is called "mini cleavage"). The HPLC and LCMS results showed that the coupling of the amino acids was complete. Double coupling of Fmoc-D-Ser-OH (98 mg, 0.3 mmol) was carried out since it was coupled on the N-methylated peptide. The Fmoc group was not removed at this stage to protect the amine group during esterification point of the hydroxyl group of the D-Ser. Again after this stage a mini cleavage was done to monitor the reaction which showed complete coupling.

The esterification was carried with Boc-Gly-OH (175 mg, 1 mmol, 10 equiv), followed by addition of 4-dimethylaminopyridine (DMAP, 12.2 mg, 0.1 mmol, 1 equiv) and DIC (78.3 µL, 0.5 mmol, 5 equiv) in DCM-DMF (9:1) for 1 hour. Double esterification was done to ensure complete incorporation. After this stage a mini cleavage was done again to monitor the reaction and complete esterification was obtained. Once the linear peptide was successfully synthesised, it was purified using SEMI-preparative HPLC in 20-80% of 0.1% TFA in ACN method for 30 min. The peak of the product was collected on a test tube and purity was checked on analytical HPLC. Then the samples were freeze dried to remove H_2O and ACN.

2.5.2 Synthesis of linear analogues

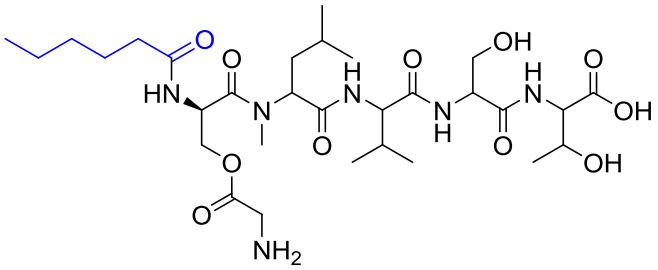
2.5.2.1 Synthesis of linear Fmoc-Globomycin analogue



Exact mass = 798.38

The Fmoc group from D-Ser was not removed. Cleavage from the resin was carried out TFA-TIS-H_2O (95:2.5:2.) for 1 hour. The solution was precipitated with cold diethyl ether, centrifuged and the ether was discarded, then the peptide was dried under bench vacuum to remove all the ether. After the purification of the linear peptide, a white powder (73.4 mg) was obtained.

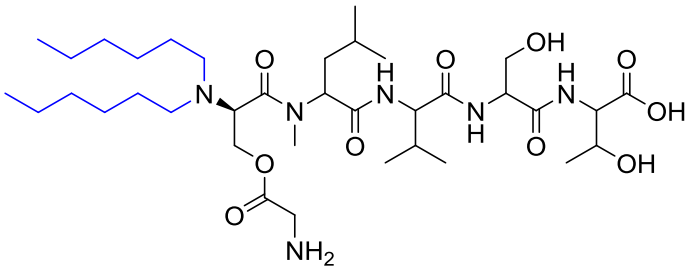
2.5.2.2 Synthesis of linear Hexanoic-Globomycin analogue



Exact mass = 674.39

The Fmoc group was removed from D-Ser using 20% piperidine/DMF for 1 hour. The resin was filtered and washed with DMF (3 × 10 mL, 1 min). Mini cleavage was done to monitor the reaction. The hexanoic acid (12.5µL, 0.1 mmol, 1 equiv) was then incorporated on the resin using DIC (47 µL, 0.3 mmol, 3 equiv) and K-Oxyma (54.06 mg, 0.3 mmol, 3 equiv) in 0.5 mL of DMF for 2 hours with shaken. Mini cleavage was done to check complete incorporation of hexanoic acid. The peptide was cleaved from the resin using TFA-TIS-H_2O (95:2.5:2.5) for 1 hour. This treatment also removed the side protecting group (tert-butyl). The solution was precipitated with diethyl ether, centrifuged, the ether discarded and dried under bench vacuum. The linear peptide was then purified rendering a white powder of 38 mg.

2.5.2.3 Synthesis of linear di-Hexyl-Globomycin analogue

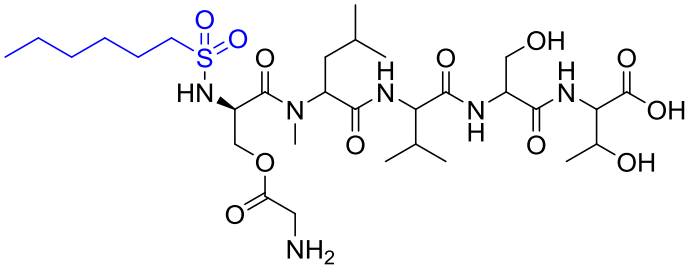


Exact mass = 744.50

After the removal of the Fmoc group from D-Ser before the esterification point, hexanal (36 µL, 0.3 mmol, 3 equiv) was added on the resin using AcOH (60 µL) in dry tetrahydrofuran (THF) for 1 hour, followed by sodium cyano borohydride (50 mg, 0.8 mmol, 8 equiv) in dry THF was added on the resin for 2 hours. This treatment renders mostly the incorporation of two hexyl moieties on the amine group. The resin was then washed with DMF (3 × 10 mL, 1 min) only. Mini cleavage was done to monitor the reaction. The esterification step then

carried using Fmoc-Gly-OH (297.31 mg, 1 mmol, 10 equiv) with DIC-DMAP (5:1) in DCM-DMF (9:1) solution for 1 hour. The treatment was repeated. The mini cleavage was done again to monitor the reaction. Fmoc group was removed using 20% piperidine in DMF for 10 min (×2). After the mini cleavage it was observed that the linear peptide was successfully synthesised. The peptide was cleaved from the resin as previously, purified, rendering a white powder (24 mg).

2.5.2.4 Synthesis of linear Hexanesulfonyl-Globomycin analogue



Exact mass = 724.37

After Fmoc group was removed from the D-Ser, 1-hexanesulfonyl chloride (63.3 µL, 0.5 mmol, 5 equiv) was added onto the resin using DIEA (174 µL, 1 mmol, 10 equiv) in DCM (0.5 mL) for 2 hours. Mini cleavage was done after this point to monitor the reaction. It was found that the reaction was not complete, thus the treatment was repeated to force the reaction into completion. Mini cleavage was done again and it was seen that the linear peptide was successfully synthesised. The peptide was cleaved from the resin as previously, purified, and a white powder was obtained (20 mg).

2.5.3 Cyclization step

For all the four analogues, the cyclization step was the same. This was done in 100 µmol/L dilution, using EDC.HCl-OxymaPure-DIEA (3:3:6) for overnight in DCM. The linear peptide was place in a round bottom flask and DCM was added, followed by EDC.HCl, OxymaPure, and then DIEA. The reaction was then stirred at room temperature while monitoring with HPLC every 2 hours. After the reaction was the complete (consumption of the linear peptide was achieved), the DCM was removed and the crude peptide was purified using SEMI-preparative HPLC in 30-80% of 0.1% TFA in ACN for 30 min. The purity of the compounds was checked on analytical HPLC and LC-MS was used to confirm the mass of the compounds. The compounds were then lyophilised and white powder was obtained. For all

the analogues > 1 mg of cyclic peptide was obtained except for cyclic Hexanesulfonyl-Globomycin analogue.

2.5.4 Biological activity studies

The antimicrobial activity tests were done by testing all the Globomycin analogues (Linear and cyclic) against three different strains namely *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6051 and *Escherichia coli* ATCC 25822. The first two strains were Gram-positive bacteria and the last one was Gram-negative bacteria respectively. All these strains were obtained from American Type Culture Collection (ATCC). The Minimum Inhibition Concentration (MIC) was determined using the broth micro dilution method following the Clinical and Laboratory Standards Institute (CLSI), 2017 guidelines. Firstly, the bacterial strains were sub-cultured onto Mueller Hinton agar and incubated at 37°C for 24 hours prior to use. Then stock solutions of the analogues at 1 mg/mL in 90% DMSO were prepared. Followed by two-fold dilutions of each compound were made in cation-adjusted Mueller Hinton broth (CAMHB) in a 96-well microplate. Distilled H_2O was used to prepare the bacterial inoculum, matched to a 0.5 McFarland standard, and added to make a final volume of 100 µL in each microlitre well. The plates were incubated for 24 hours at 37ºC under aerobic conditions. The MIC was then recorded as the lowest concentration at which no visible growth occurred. Standard drug, media and bacterial growth control wells were included in each plate. The positive control was Meropenem and a blank solution of 90% DMSO in H_2O was also tested to make sure that the solvent does not affect the bacterial strains[3]. The assay was performed in duplicates to confirm the results.

2.6 References

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2. Kiho, T.; Nakayama, M.; Yasuda, K.; Miyakoshi, S.; Inukai, M.; Kogen, H., Structure-activity relationships of globomycin analogues as antibiotics. *Bioorg. Med. Chem.* **2004,** *12* (2), 337-361.

3. Monaim, S. A. H. A.; Noki, S.; Ramchuran, E. J.; El-Faham, A.; Albericio, F.; Torre, B. G. d. l., Investigation of the N-Terminus Amino Function of Arg10-Teixobactin. **2017,** *22* (10), 1632.

CHAPTER 3

CONCLUSION

Globomycin analogues were successfully synthesized on SPPS. Cyclization was done on solution phase by lactamization reaction. After the synthesis and purification of the compounds, antimicrobial activity studies then followed where both linear and cyclic analogues were tested against both Gram-positive and Gram-negative bacteria. Results indicated that only cycle di-Hexyl-Globomycin was active at 100 µg/mL. From these results few conclusions can be drawn, that is, the cyclic nature of the peptide is important; this was illustrated by the di-Hexyl analogue where the cyclic was active while the linear was inactive. All the linear compounds were inactive. The aliphatic state of the lipidic chain is vital. This is observed when only the cycle di-Hexyl-Globomycin was active while the cycle-Fmoc and cycle-Hexanoic-Globomycin analogues were inactive and this could be due to the carbonyl group present in the lipidic chain. The L-allo-Thr and L-allo-Ile moieties are not important; again this was seen when cycle-di-Hexyl was active while it has L-Thr and L-Val instead of the amino acids mentioned above.