UNIVERSITAT DE BARCELONA

Facultat de Farmàcia
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Final Year Degree's Project

Pharmacy Degree

SYNTHESIS OF VISIBLE LIGHT PHOTOSWITCHABLE ANTIBIOTICS AS A TOOL TO COMBAT RESISTANCE

PAULA DEL CUERPO I LÓPEZ

Organic Chemistry

Pharmaceutical Chemistry, Physical Chemistry and Instrumental Techniques

Department of Pharmacology, Toxicology and Therapeutic Chemistry

Facultat de Farmàcia i Ciències de l'Alimentació

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Acronyms

Alloc: Allyloxycarbonyl

AMR: Antimicrobial resistance

Asn: Asparagine

Boc: *tert*-butoxycarbonyl

CEBA: Tetra-*ortho*-chloro-diethylelene-azobenzene amino acid

DCM: Dichloromethane

DIC: *N*,*N*'-Diisopropylcarbodiimide

DMF: Dimethylformamide

Fmoc: 9-Fluorenylmethyloxycarbonyl

Gln: Glutamine

HPLC: High Performance Liquid Chromatography

IBEC: Institut de Bioenginyeria de Catalunya

Leu: Leucine

MEBA: Tetra-*ortho*-methoxy-diethylelene-azobenzene amino acid

MS: Mass Spectrometry

Orn: Ornithine

Phe: Phenylalanine

Pro: Proline

SPPS: Solid Phase Peptide Synthesis

TES: Triethylsilane

TFA: Trifluoroacetic acid

Trt: Triphenylmethyl (trityl)

Tyr: Tyrosine

UV: Ultraviolet

Val: Valine

Abstract

The appearance and rise of antibiotic resistance is a public health concern that will lead to increasing pressure on the world's healthcare systems. Previously treatable infections are set to become one of the major causes of mortality worldwide due to the development of resistance. New drugs as well as new strategies are needed to combat this situation; in this context, modulation of antibiotic activity is a useful tool to prevent the rise of further resistance.

Photopharmacology allows spatiotemporal control of antibiotic activity through light and is an attractive area of research in the development of new antibiotic strategies. Photocontrolled antibiotics could be released into the environment inactivated after acting on the body, removing evolutionary pressure on microorganisms and decreasing the rise of resistance.

One such photoswitchable class of molecules are azobenzenes. Most azobenzenes isomerize under UV light, hostile to life, and while reports of harmless, red-shifted tetra-*ortho*-methoxy-azobenzenes exist since 2011, their use was limited due to low yields and harsh reaction conditions used to prepare them.

More efficient and versatile synthetic pathways to prepare tetra-*ortho*-methoxy-azobenzenes were developed recently. This opened up new synthetic and application possibilities for this family of compounds, such as their use in solid phase peptide synthesis as amino-acid analogues.

A library of photoswitchable analogues of the antibiotic peptide Tyrocidine A that incorporate a tetra-*ortho*-methoxy-azobenzene amino-acid was prepared and purified, verifying its compatibility with conventional SPPS methods. In the future, it could be used to test and study isomerization under red light and *in vitro* biological activity.

Key concepts: photopharmacology, azobenzenes, solid phase peptide synthesis

Resum

L'aparició i augment de la resistència als antibiòtics és un problema de salut pública que augmentarà la pressió sobre els sistemes sanitaris del món. Les infeccions que prèviament s'havien pogut tractar es convertiran en una de les principals causes de mortalitat a tot el món a causa del desenvolupament de resistències. Es necessiten nous fàrmacs i noves estratègies per combatre aquesta situació i la modulació de l'activitat antibiòtica és una eina útil per a prevenir noves resistències.

La fotofarmacologia permet el control espai-temps de l'activitat antibiòtica a través de la llum i és una àrea atractiva de recerca en el desenvolupament de noves estratègies. Els antibiòtics "fotomodulats" s'alliberarien inactivats després d'actuar, eliminant la pressió evolutiva sobre els microorganismes i disminuint l'aparició de noves resistències. Una classe de fotocommutadors són els azobenzens. La majoria dels azobenzens s'isomeritzen sota la llum UV, perjudicial pels organismes vius, i tot i que al 2011 es van descriure tetra-*orto*-metoxi-azobenzens compatibles amb la vida, el seu ús ha estat limitat a causa dels baixos rendiments i les fortes condicions de reacció emprades.

Recentment, s'han desenvolupat rutes sintètiques eficients i versàtils per a preparar tetra-*orto*-metoxi-azobenzens. Això ha obert noves possibilitats sintètiques i aplicacions per a aquests compostos, com en la síntesi de pèptids en fase sòlida com a anàlegs d'aminoàcids.

S'ha preparat i purificat una biblioteca d'anàlegs fotocommutables del pèptid antibiòtic tirocidina A substituïts amb un derivat aminoàcid de tetra-*orto*-metoxi-azobenzè, verificant la seva compatibilitat amb la metodologia convencional de SFS. En el futur, es podrien emprar per a estudiar la isomerització sota llum vermella i l'activitat biològica *in vitro*.

Paraules clau: fotofarmacologia, azobenzens, síntesi en fase sòlida

Integration of fields

The major framework within which this project is developed is Organic Chemistry, as the objectives were centred around organic synthesis of novel photoswitchable peptides from their constituent amino acids. All experimental work has been undertaken in and under the surveillance of the Organic Chemistry Unit of the Department of Pharmacology, Toxicology and Therapeutic Chemistry of the UB Faculty of Pharmacy and Food Sciences.

Of great importance is also the aim with which these peptides were prepared, that is to study their antibiotic properties, and particularly the innate capability for their activity to be modulated through light, both conditioned by the synthetic addition of our tetra-*ortho*-methoxy-diethylelene-azobenzene amino acid (MEBA) photoswitch. This justifies Pharmaceutical Chemistry as another secondary academic field of relevance.

Finally, the photochemical and physicochemical properties of photoswitchable compounds play a key role that determines which procedures are feasible and which are not, as well as help identify and purify the final product with techniques such as High-Performance Liquid Chromatography (HPLC), Mass Spectroscopy (MS) and Reverse Phase Column Chromatography. Thus, the academic field of Physical Chemistry and Instrumental Techniques is also involved as a secondary field.

Sustainable development goals

SDG 3, Good Health and Well-Being is the SDG with which this work is most involved, since it aims to further research a healthcare issue that will become increasingly concerning in the following years and thus adds to the body of work that can be used to advance the health and well-being of our society. Of particular relevance are point 3.b, which aims to support research and development in “vaccines and medicines against transmittable and non-transmittable diseases that primarily affect developing countries”, which this project tries to help with by developing new therapies against the emergence of antibiotic resistance that would inevitably disproportionally affect those countries with less resources; and point 3.d, which proposes to “strengthen the capacity of all countries, in particular developing countries, for early warning, risk reduction and management of national and global health risks”, which by developing new strategies to combat resistances that try to reduce future risk of antibiotic resistance development and help management of existing and new resistances, this project directly contributes to.

SDG 6, Clean Water and Sanitation is a secondary SDG with which this project also helps, albeit indirectly. By decreasing the evolutionary pressure that environmental pathogens are exposed to, the load of potentially life-threatening pathogens without effective treatment is also decreased, making water and food consumption safer in developing countries. In this context, it helps with target 6.1 “By 2030, achieve universal and equitable access to safe and affordable drinking water for all”, target 6.2 “By 2030, achieve access to adequate and equitable sanitation and hygiene for all […]”, target 6.3 “By 2030, improve water quality by […] minimizing release of hazardous chemicals and materials […]” and lastly, target 6.6 “By 2030, protect and restore water-related ecosystems, including mountains, forests, wetlands, rivers, aquifers and lakes”.

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1. INTRODUCTION

1.1. ANTIBIOTIC RESISTANCE

Bacterial antibiotic resistance was first detected soon after the discovery of the first antibiotic, penicillin-G, in 1928. The first ever documented cases of penicillin-specific resistance were registered soon after its production and beginning of widespread use in 1940; an *E. coli* strain capable of producing the first ever beta-lactamase. In 1942 some *S. aureus* strains were already resistant to penicillin, and about 18 years later most strains were. In 1967, reports started surfacing documenting cases of penicillin resistant *S. pneumoniae* strains.[1,2] These events constituted the first pieces of evidence of rapid bacterial adaptation to combat antibiotics and promoted further development of other antibiotics and pharmacological strategies to bypass or slow the development of resistance.[3]

In recent years, due to misuse and over-prescription of antibiotics, among other factors, previously easily treatable infections have become difficult to treat due to the development of bacterial strains with multi-resistance to antibiotics; this has grave repercussions on public health as well as an economic impact on our health-care systems.[4] The acquisition of antimicrobial resistance (AMR) by bacteria is partly due to the accumulation of antibiotics in the environment, which increases evolutionary pressure on these microorganisms and accelerates the appearance of AMR.[5]

In the face of this public health paradigm, it has become increasingly necessary to design new cautionary antibiotic use protocols to slow the appearance of new resistances, as well as developing new antibiotics that not only aim to bypass established AMR via a new mechanism of action, but also strive to prevent further resistance appearance by finding clever ways in which these new substances can avoid getting targeted by bacterial resistance mechanisms. [6]

One such way of doing this is by developing **photoswitchable antibiotics**, antimicrobial molecules that are able to switch between two different isomers with different geometries that result in a change in biological activity. Light grants spatiotemporal control and provides a means to deactivate the drug after its therapeutic use.[7] The latter feature is of particular interest when designing new antibiotics that diminish the chances and rate at which new resistances appear. By promoting the excretion of an inactivated

antibiotic from the body we prevent it from exerting evolutionary pressure on environmental bacteria when it finds itself in their presence. This is relevant to preventing AMR, as it is a well-documented factor conducive to the appearance and increased rate of new resistances.[5,7]

1.2. PHOTOPHARMACOLOGY AND PHOTOSWITCHES

Photopharmacology has emerged in recent years as a novel therapeutic approach to overcoming long-standing challenges in medicine adjacent fields such as medicinal chemistry. It involves the use of light to switch from a pharmacologically inactive form of a drug to an active one or, when useful, the opposite, so as to control its activity. This allows for control over where and when the drug is made active or inactive, which has already proven to be beneficial in diminishing adverse effects caused by off-target interactions and in modifying the drug's overall pharmacokinetical profile.[7,8,9]

The design of these therapies can be approached through multiple strategies: a common one that is put into practice in this thesis is the use of photoswitchable moieties, molecules or chemical motifs that undergo isomerisation upon being irradiated with a certain frequency of light. Such transformations can lead to the breakage and formation of bonds to form completely chemically new species (Figure 1), otherwise known as constitutional isomers, or may only lead to different stereoisomers of the photoswitch. In both cases, these reactions may be completely irreversible, even when irradiation is stopped, partially reversible, or completely reversible once the light stimulus is removed.[8]

These chemical changes can have important implications on the physical and chemical properties of both the isolated photoswitch or the molecule to which it is attached. Because the pharmacological properties of drugs are intrinsically linked to their physical and chemical properties, the modulation of these properties through a photoswitch alters its pharmacological activity, either activating it or deactivating it.[9]

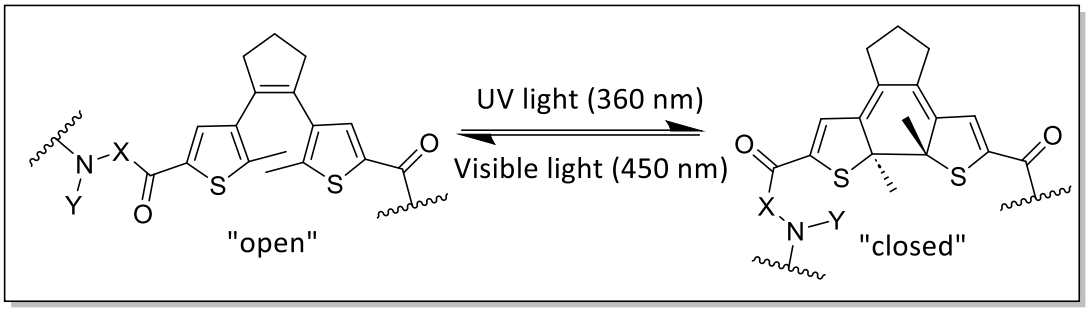

Figure 1: Diarylethene-based photoswitch, an example of a photoswitch that alternates between constitutional isomers.[21]

1.3. AZOBENZENES AS PHOTOSWITCHES

Azobenzenes have been used in photopharmacological applications since the discovery of their photochemical properties, more specifically, their ability to undergo reversible isomerization (Figure 2).[10] Depending on the substitution pattern of the benzene rings, these molecules are able to absorb light of different frequencies (most on the ultraviolet, UV range), which enables the isomerization to their *cis* configuration.[11] The high degree of reversibility of this change and its implications on the molecule's three-dimensional arrangement make them very interesting to harmlessly modulate the pharmacological activity of drugs, as well as being robust, stable molecules.[9]

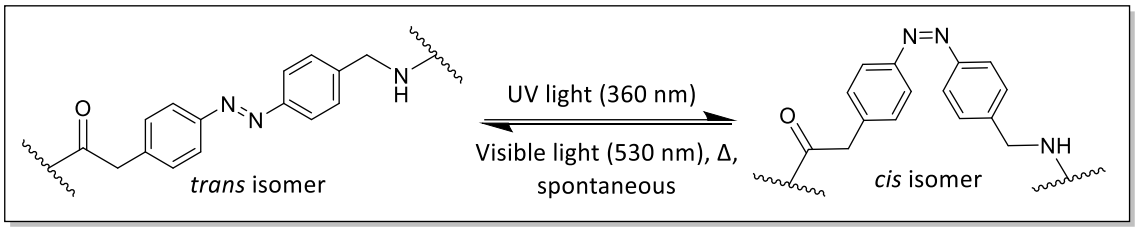

Figure 2: trans-cis isomerization of azobenzenes

Azobenzenes have distinct physical properties in their *trans* and *cis* configurations: unsubstituted *trans* azobenzenes have coplanar rings, have a lower dipolar moment and display fewer steric clashes between substituents; in their *cis* configuration, the planes of both rings become tilted with respect to one another, they acquire a dipole moment owing to the asymmetrical distribution of the benzene groups around the azo bond within the molecule and the rings impose a decent amount of steric hindrance to each other, which is only increased if they are substituted. Finally, as this family of compounds has been extensively used as dyes in amenities such as food and clothing, their safety and biological profile is well understood and documented when compared to other photoswitches, which make them an attractive option for medicinal applications. [12]

1.4. TETRA-*ORTHO*-SUBSTITUTED AZOBENZENES

Visible light offers an advantage over UV light which is typically needed to isomerize azobenzenes, and that is its overall harmless and clean properties that make it safe to use on biological organisms.[9,14] The use of visible light photopharmacology to modulate antibiotic activity has only been recently documented.[15]

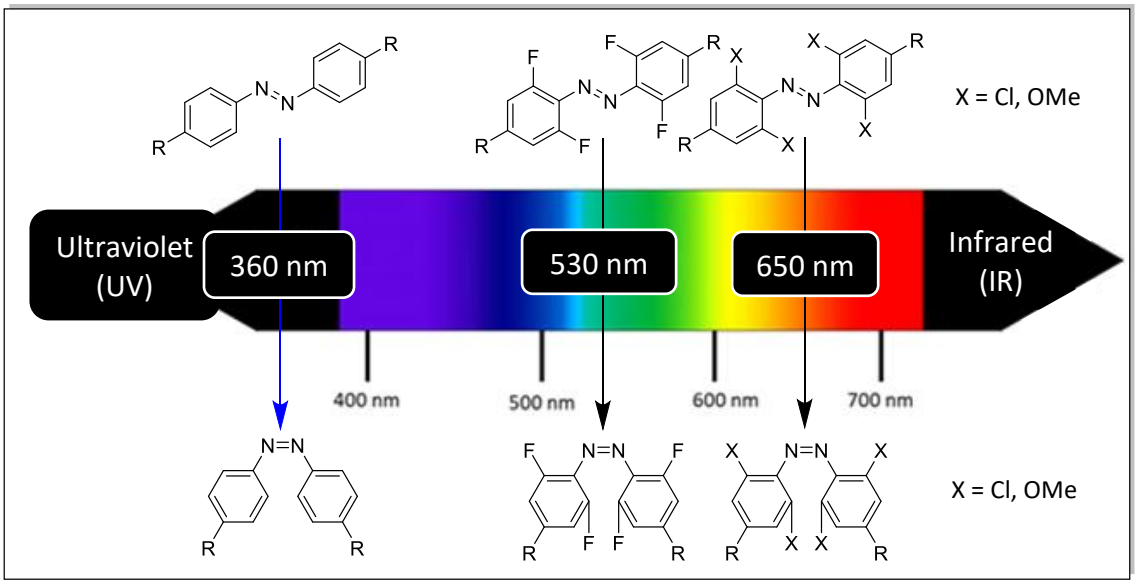

Figure 3: Azobenzenes with different substitution patterns and their respective wavelengths for trans→cis isomerisation. Modified from ref 13.

In this context, tetra-*ortho*-chlorinated, tetra-*ortho*-methoxylated and tetra-*ortho*-fluorinated azobenzenes offer an advantage over other azobenzene analogues as they allow the use of harmless green and even red light to isomerize and modify the structure of the azobenzene, and potentially that of the entire molecule to which they are bonded to (Figure 3).[9,14,16] In particular, the former two allow for the use of low energy red light comprised in the so called "biological optical window" or "near-infrared window (NIR)" to isomerize its structure, which in the context of photopharmacology also proves useful in increasing the depth at which we can achieve photo-activation, as light within the NIR exhibits lower scattering in anatomical tissue and thus is capable of penetrating deeper into tissue than UV light.[9,17,18]

In the context of Solid Phase Peptide Synthesis (SPPS), tetra-*ortho*-chlorinated azobenzenes were used to attempt the synthesis of antimicrobial peptides taking advantage of the aforementioned properties. However, the same study documented that these azobenzenes were unstable under the basic conditions used to deprotect 9-Fluorenylmethyloxycarbonyl (Fmoc) from each coupled amino acid, which severely

limited the range of synthetically accessible peptides using this azobenzene in SPPS. It was hypothesized that the aromatic rings in the tetra-*ortho*-chloro-diethylelene-azobenzene amino acid (CEBA) (Figure 4) were susceptible to aromatic nucleophilic substitution of the chlorine substituents with piperidine.

Therefore, the chemical structure and properties of tetra-*ortho*-methoxylated azobenzenes would theoretically make it more resistant to this kind of reaction owing to the electron-donating nature of its substituents, though there are no previous studies on this hypothesis up until the research described in this thesis.[19,20]

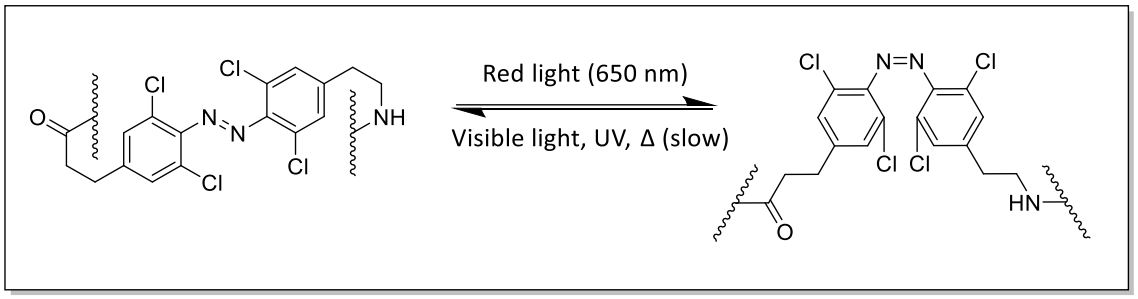

Figure 4: trans-cis isomerization of CEBA. Red light is used to isomerize these azobenzenes instead of UV light, but it is sensitive to ordinary Fmoc deprotection conditions.

Tetra-*ortho*-substituted azobenzenes also offer an extremely important photochemical feature of interest in preventing the appearance of further AMR: its spontaneous and rapid reversion to the *trans* isomer upon white light (including sunlight) irradiation, which could prevent the accumulation of a pharmacologically active drug in the environment and thus reduce the appearance of AMR.[15,17,18,20,21]

All in all, this allows tetra-*ortho*-methoxylated azobenzenes to be safe photoswitches for *in situ* activation of the drug, as well as preventing the antibiotics they are bonded to from exerting the evolutionary pressure that would lead to an increased rate of appearance of AMR once released into the environment. It also possesses the structural and chemical features that make it an ideal candidate to design antimicrobial peptides using SPPS. With this in mind, an amino acid analogue of tetra-*ortho*-methoxylated azobenzene (MEBA) (Figure 5) was developed (Figure 6) for the SPPS applications described in this work.[22]

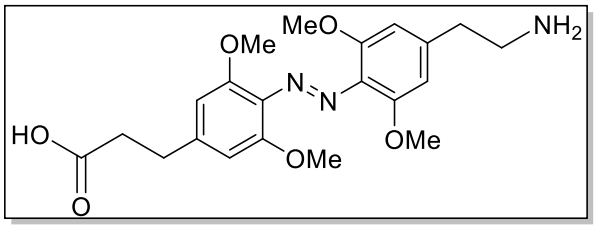

Figure 5: MEBA

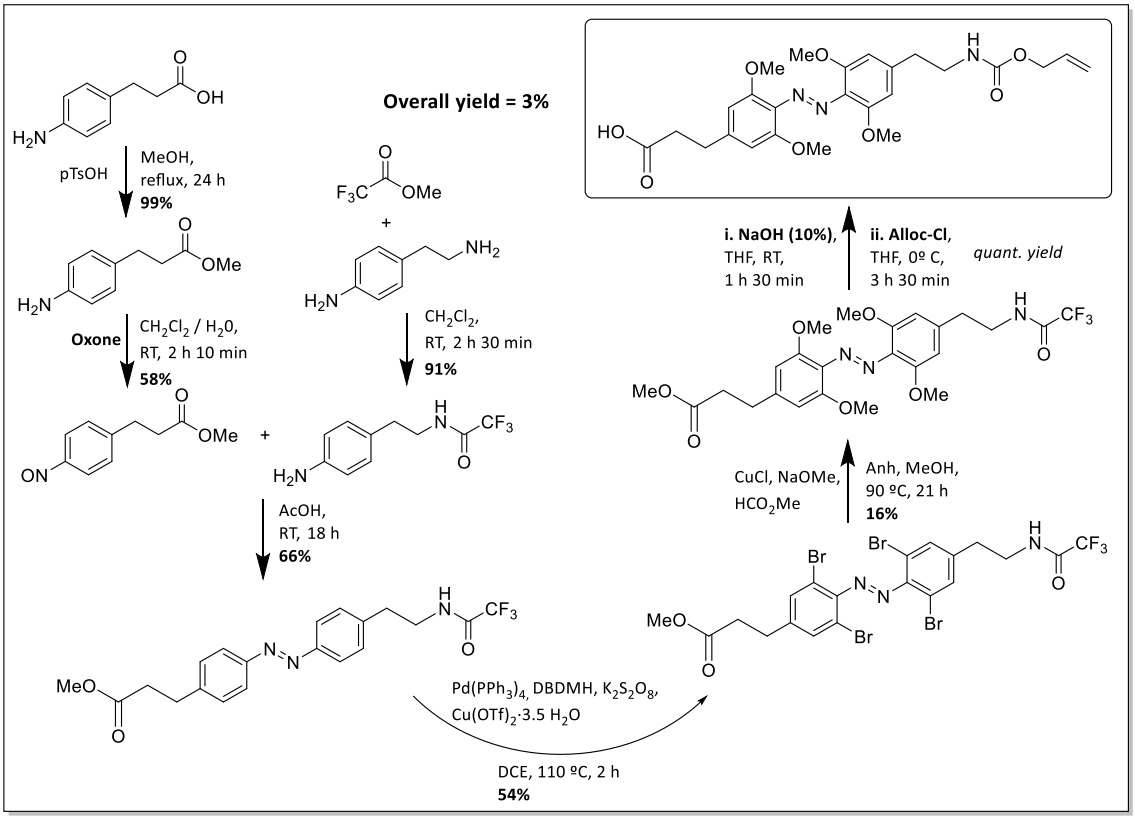

Figure 6: Synthesis of Alloc-MEBA used in this work, as described in ref 22

1.5. PHOTOSWITCHABLE ANTIMICROBIAL PEPTIDES

Antimicrobial peptides, though they are not the overwhelming majority class of antibiotics used in clinical practice today, are nevertheless the most widespread type of antibiotic substance found in nature, produced by microorganisms and pluricellular organisms, such as humans, alike. They usually have unspecific mechanisms of action that allow them to be effective against a wide range of pathogens and bypass many resistance mechanisms, and as such usually serve as a first line of defence.[23,24]

Moreover, they are an interesting class of antibiotics with which to research and study antibiotic modulation through the addition of a photoswitchable moiety sensitive to light. Their “modular” structure in the form of a sequence of amino acids allows for easy

substitution of one or more of these to incorporate a photoswitchable building block without much disturbance to the overall structure. In addition, because many peptide antibiotics exert their action specifically through the acquisition of a particular three dimensional structure, and because the activity modulation sought when designing azobenzene photoswitchable antibiotics relies mainly on the activation/deactivation of the drug through configurational change of the photoswitch upon light irradiation, it is expected that this class of antibiotics would be especially susceptible to biological activity modulation through azobenzene configuration control.[25,26] One of the most employed, naturally occurring peptide antibiotics used for photopharmacology research has been gramicidin S, which is primarily active against Gram-positive bacteria. [26]

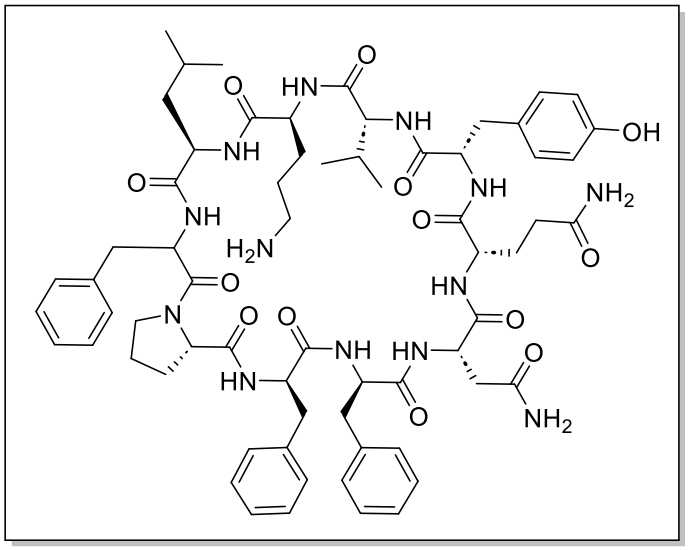

Figure 7: Tyrocidine A

In this context, Tyrocidine A (Figure 7), a cyclic decapeptide found alongside gramicidin S in tyrothricin, a mixture of peptide antibiotics secreted by *Brevibacillus brevis*, is also primarily active against Gram-positive bacteria and has been previously used in photopharmacological research, with its activity being well characterized. A linear analogue of Tyrocidine A was chosen as a framework with which to research the photopharmacological modulation capabilities of tetra-*ortho*-methoxylated azobenzenes in this work due to the research group's previous experience and research on it with tetra-*ortho*-chloro-azobenzene photoswitches, as the linear analogues were the best performing in terms of their antimicrobial properties as well as their photochemical modulation capabilities in that same study.[22]

1.6. SOLID PHASE PEPTIDE SYNTHESIS

Solid Phase Peptide Synthesis (SPPS) stands as the current method of choice for peptide preparation in organic synthesis. It involves a step-by-step elongation of a peptide linked to a solid support, which consists of the iteration of coupling steps and N-terminus deprotection steps as many times as residues are desired in the final peptide, and the solid support onto which the peptide is anchored serves to easily and quickly clean, filter and manipulate reaction mixtures without having to carry out any additional separation steps, easily retaining and isolating the peptide product linked to the resin after each step and allowing the use of excess reagents to achieve high yields that can be promptly filtered out. This is followed by a final cleavage step to remove the peptide from the resin and bring it into solution prior to its final purification. Overall, this method achieves higher yields in less time that would not be otherwise possible in solution methods.[27]

There are multiple strategies to tackle SPPS, and the chosen method must prevent most cross-reactions (through the use of so called orthogonal groups) and result in the highest possible yields: one of the most common, and the one employed in this work is the use of Fmoc-protected amino acids on the *N*-terminus to ensure that only their carboxylic acid group reacts with the free amino group of either the linker attached to the resin or the last added amino acid on the peptide chain. This reaction is performed with coupling reagents such as carbodiimides and other additives, which act by reacting with the carboxylic acid of the incoming amino acid and activating it, increasing its electrophilicity and priming it for rapid reaction with the free amine on the peptide chain (Figure 8).[27,28,29.30]

In between these coupling steps a ninhydrin test is performed, which uses the compound ninhydrin that can react with any free primary amines in solution to yield Ruhemann's purple, a striking purple-coloured compound. If the latter is obtained, it can hint at the coupling reaction not being complete or not having been performed correctly, as it would indicate that the peptide's amino terminus is free and unprotected, and thus that no additional amino acid has been coupled.[31,32]

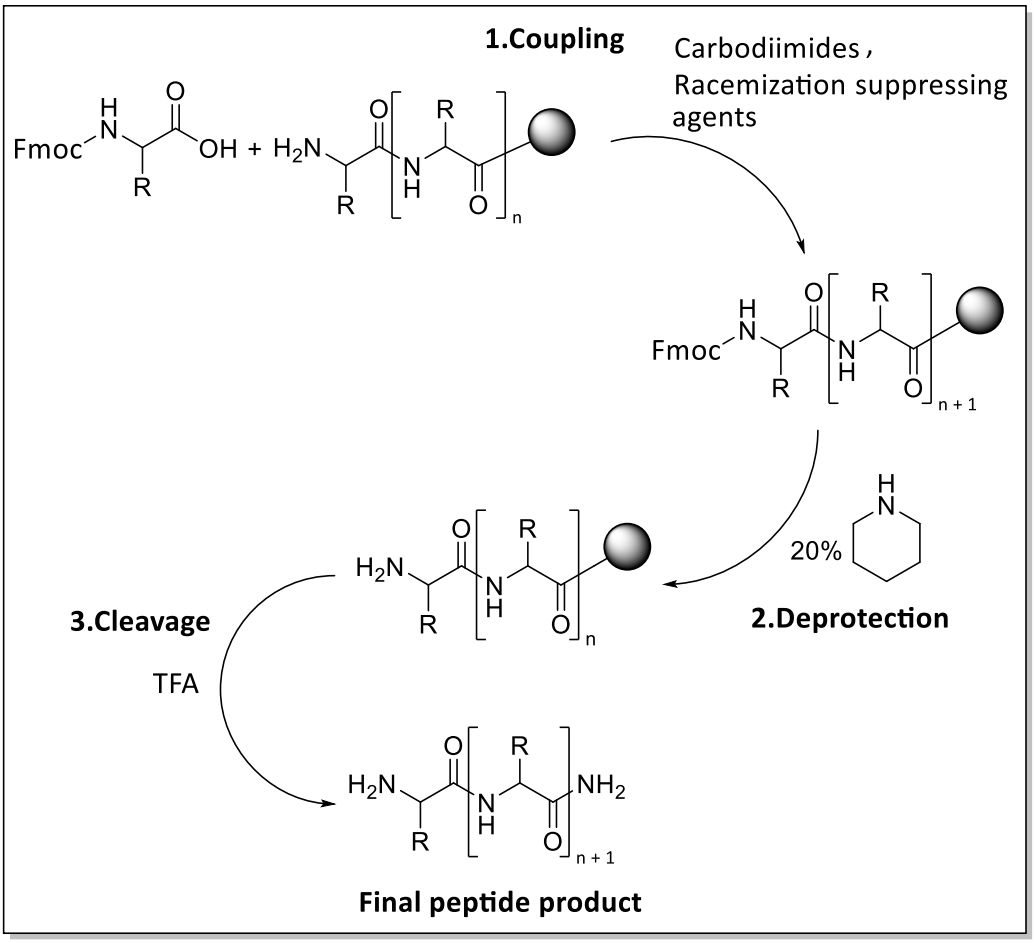

Figure 8: General scheme of SPPS. Note that the protecting group may vary, and then so will the conditions used for deprotection and cleavage

2. OBJECTIVES

The main goal of this Degree's Final Year Project is the synthesis, isolation and procurement of visible-light-operated photoswitchable Tyrocidine A analogues that contain a tetra-*ortho*-methoxylated azobenzene amino acid (MEBA) substituted in different positions. In order to achieve it, we have established the following objectives:

- Test the compatibility of MEBA with conventional SPPS methods by making sure that ordinary SPPS steps such as coupling, deprotection of protecting groups, and cleavage of the peptide from the resin work as expected.

  This was done through conventional "checkpoint" methods such as a ninhydrin test after each coupling, as well as by studying the structure of the final peptide obtained by HPLC-MS analysis.
- Synthesize a small collection of linear tyrocidine A analogues incorporating MEBA as a substitute of different pairs of amino acids through conventional SPPS, optimizing conditions to improve yields when appropriate.
- Purify and characterise the photoswitchable peptides obtained by SPPS, which also implies the determination of, and optimization of the conditions needed to cleave the peptides from the resin so as to produce the cleanest and highest yielding crude peptide possible, as well as for the conditions used in reverse phase column chromatography to isolate and purify the peptide.

With these objectives in mind the hypotheses that are sought to be proven in this work and future studies conducted on these peptides are:

- The synthesis of Tyrocidine A-MEBA-modified analogues as described in this work is plausible and yields the desired product in sufficient amounts.
- The conditions used for cleavage, isolation and purification are effective and reproduceable, and are a useful standard for these types of peptides' isolation and purification

Additionally, the following hypotheses were also initially proposed in the context of this project; however, due to time constraints their exploration will be carried out at a later date by the research group within which this project was conducted:

- Tyrocidine A-MEBA-modified analogues can be isomerized quantitatively and without compromising the structural integrity of the peptide, and the reversal to its trans isomer is plausible and rapid under visible light.
- Tyrocidine A can be modified to obtain pharmacologically active MEBA-analogues.
- The antibiotic activity of the Tyrocidine A analogue can be modulated by controlling its configuration, which in turn can be effectively controlled through cis-trans isomerization of the tetra-ortho-methoxylated azobenzene photoswitch promoted by red light irradiation.

Nevertheless, the procurement of a collection of photoswitchable linear Tyrocidine A analogues, which was set as an objective of this project and accomplished, will allow these objectives to be pursued in future studies.

3. RESULTS AND DISCUSSION

The synthesis and isolation of MEBA-Tyrocidine A analogues using a modified linear Tyrocidine A as a framework (Figure 9) was sought out using and achieved through SPPS. The substitution of two pairs of adjacent Tyrocidine A amino acids with MEBA at a time for each of the peptides synthesized was planned for each peptide. As natural occurring Tyrocidine A has 10 amino acids, the different possible substitution combinations of two amino acids for our MEBA photoswitchable building block yield a total of 9 different possible peptide combinations, one for each MEBA position within the peptide. Adopting conventional IUPAC *N*-terminal to *C*-terminal sequence order for peptides, the different combination peptides were named **1-MEBA**, the peptide that has MEBA in its *N*-terminal position; **2-MEBA**; **3-MEBA**; **4-MEBA**; **5-MEBA**; **6-MEBA**; **7-MEBA**; **8-MEBA**; and **9-MEBA**, which would have MEBA in its *C*-terminal position (Table 1). As of the time this work is being written, peptides in bold in the next table have been synthesized, while peptides in red were personally synthesized.

Linear Tyrocidine A analogue	H2N-D-Phe-Pro-L-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-OH
1-MEBA	H2N-MEBA-L-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-OH
2-MEBA	H2N-D-Phe-MEBA-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-OH
3-MEBA	**H2N-D-Phe-Pro-MEBA-Asn-Gln-Tyr-Val-Orn-Leu-OH**
4-MEBA	**H2N-D-Phe-Pro-L-Phe-MEBA-Gln-Tyr-Val-Orn-Leu-OH**
5-MEBA	**H2N-D-Phe-Pro-L-Phe-D-Phe-MEBA-Tyr-Val-Orn-Leu-OH**
6-MEBA	**H2N-D-Phe-Pro-L-Phe-D-Phe-Asn-MEBA-Val-Orn-Leu-OH**
7-MEBA	**H2N-D-Phe-Pro-L-Phe-D-Phe-Asn-Gln-MEBA-Orn-Leu-OH**
8-MEBA	H2N-D-Phe-Pro-L-Phe-D-Phe-Asn-Gln-Tyr-MEBA-Leu-OH
9-MEBA	**H2N-D-Phe-Pro-L-Phe-D-Phe-Asn-Gln-Tyr-Val-MEBA-OH**

Table 1: Amino acid sequence of linear Tyrocidine A and its MEBA derivatives. Note that elongation was performed from right to left, while name of the peptide was attributed for its MEBA position from left to right, following conventional IUPAC rules. Peptides in bold have been synthesized, and those in red are included in this thesis.

Linear peptide analogues were synthetised owing to a more interesting pharmacological profile than cyclic photoswitchable analogues of Tyrocidine A synthesized previously by the research group in which this work was carried out and as previously discussed in the introduction section of this thesis.[21] Cyclic analogues incorporating a photoswitch

(CEBA, in this case) did not show a significant difference in activity compared to natural Tyrocidine A, whereas linear analogues not only showed different activity, but it was also significantly different between the trans and cis isomers, and thus capable of being modulated through light.

The synthesis of **7-MEBA**, **5-MEBA** and **4-MEBA** substituted tyrocidine A described in this thesis were personally prepared, purified, and characterised, while all other peptides were synthetised by other members of the group. Additionally, **Tyr7 6-MEBA** substituted Tyrocidine A (in which the Val residue in seventh position that was originally planned for 6-MEBA's design was exchanged for a Tyr residue) was originally planned to have the ordinary **6-MEBA** structure, but owing to a mistake in the planification of its synthesis, it ended up with an exchanged amino acid.

Finally, the synthesis of **8-MEBA** substituted tyrocidine A was also attempted, but HPLC-MS analysis revealed that it was lacking the L-Phe amino acid, making it shorter than it should have been, and its purification was not pursued.

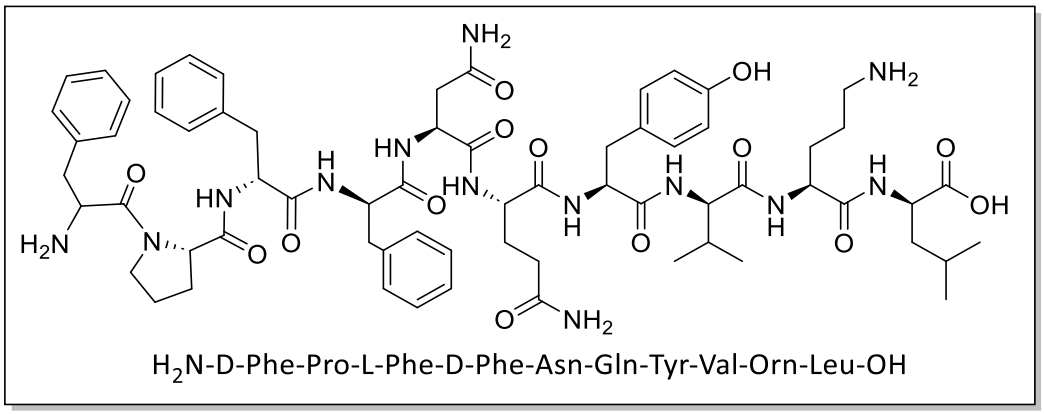

Figure 9: Linear Tyrocidine A

Once synthesized, all peptides were analysed by HPLC-MS to determine the outcome of the synthesis and decide on optimal purification conditions with a *Biotage® Isolera One,* using a reverse phase column chromatography in order to isolate the product. After purification, HPLC-MS was also employed to assess the resulting purity.

HPLC-MS analysis showed the presence of the two expected isomers, indicating that these could be separated under the used conditions. However, irradiation studies to study the isomerisation have not been performed yet.

3.1. SPPS, PURIFICATION AND CHARACTERIZATION OF MEBA SUBSTITUTED TYROCIDINE A ANALOGUES

To prepare the peptides, SPPS was used as described in the materials and methods section. The Rink-Amide linker allows the isolation of the corresponding C-terminal amide after cleavage under acidic conditions. Moreover, the ChemMatrix resin is based on a polyethylene glycol polymer with excellent swelling properties that facilitates high yielding reactions.

With the first synthesized peptide (**7-MEBA**), two different cleavage cocktails were tested: one with a mixture of TFA-TES-H_2O (95 : 2.5 : 2.5) and another with a TFA-DCM (95:5) mixture. It was hypothesized that the addition of water and TES in the first cleavage cocktail would act as scavengers, intercepting reactive species formed under the acidic conditions of peptide cleavage and consequently preventing unwanted side reactions, which generally lead to a "dirtier" reaction crude and decrease the yield.

As mentioned above, the first peptide synthesized was **7-MEBA** substituted tyrocidine A, where MEBA was the third amino acid introduced, replacing Val and Tyr in the natural peptide sequence (Figure 10), and for which elongation of the peptide was performed uneventfully.

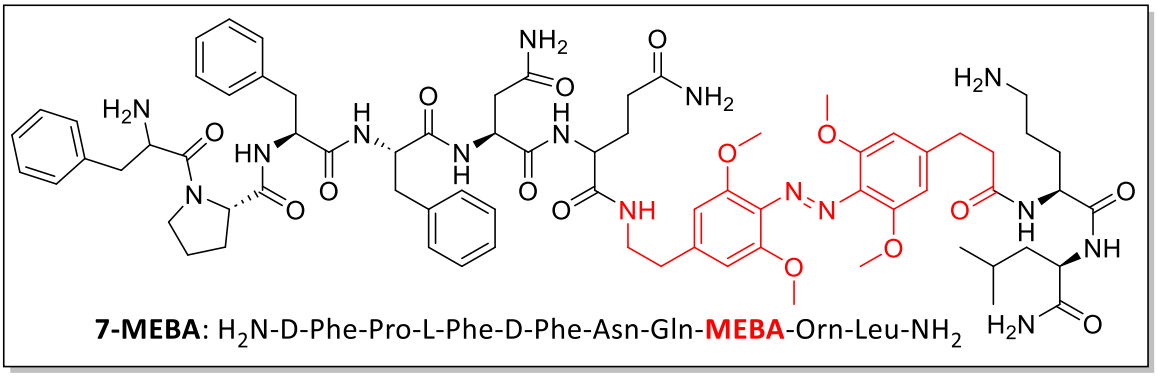

Figure 10: ***7-MEBA substituted tyrocidine A***

Once this peptide was synthesized, both pilot "mini-cleavages" mentioned above were carried out to assess the success of the synthesis, as well as to evaluate which cleavage cocktail yielded the best results, considering a cleaner cleavage crude as indicative of such. Two small samples of the peptide-bound resin were taken, and a different cleavage cocktail was used on each.

The obtained MS spectra confirmed the presence of the desired product in both samples, and the chromatograms for the TFA-TES-H_20 "mini-cleavage", when compared to the TES-DCM "mini-cleavage", showed decreased background noise in each of them relative to the absorption peaks associated with the peptide, which is especially significant in the 450 nm chromatogram. This wavelength corresponds to the isosbestic point of MEBA and the higher relative absorption indicates a lower proportion of peptide degradation subproducts and a higher relative proportion of the intact, target peptide. Simultaneously, an increased number of peaks were observed in the 254 nm chromatogram, which generally corresponds to the frequency at which many aromatic compounds show increased absorption; this could be indicative of a higher presence of scavenger subproducts resulting from the prevention of side reactions. In addition, the chromatograms also displayed two prominent adjacent peaks of absorption associated with the desired peptide product, especially in the 450 nm one, which hint at the presence of both isomers of the peptide, meaning these peptides are able to isomerize under visible light as intended; these paired peaks were observed in this and all other analysed peptides.

The TFA-TES- H_20 cleavage methodology was then standardised for the cleavage of this and the rest of the peptides going forward.

The entire peptide product was then obtained using the most effective cleavage method (TFA-TES-H_2O), and another analysis by HPLC-MS was carried out that revealed it to be more impure than originally expected. Reverse phase column chromatography was performed, and two fractions were isolated, from which the product was obtained in one while the other only contained MEBA-derived impurities along with other unidentified substances and was promptly discarded.

Another purification was carried out, and after evaporation of the solvent two fractions were retrieved and combined after finding through HPLC-MS analysis that both contained the product. The peptide was considered of sufficient purity, and no further steps were taken.

The third peptide synthesized was **5-MEBA** substituted tyrocidine A where MEBA was the fifth amino acid introduced, replacing Gln and Asn in the natural peptide (Figure 11).

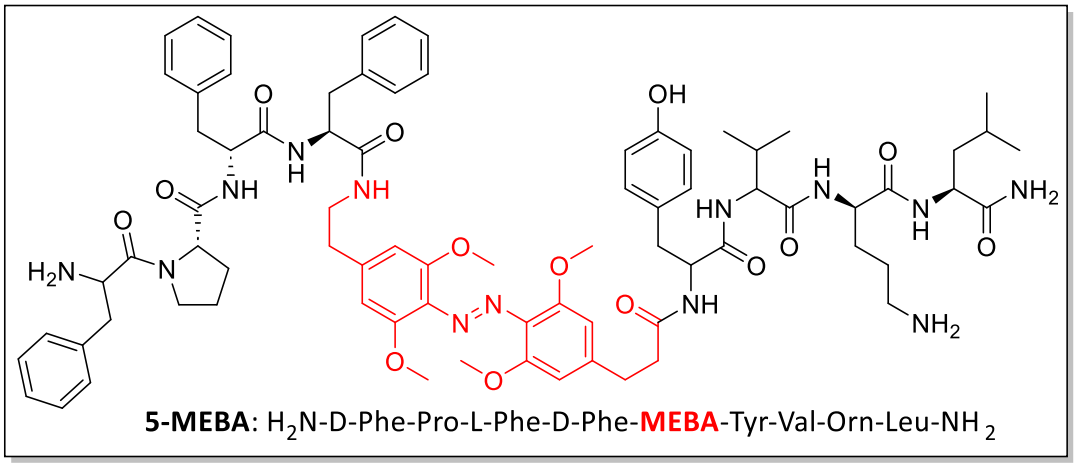

Figure 11: ***5-MEBA substituted tyrocidine A***

After confirmation of the desired product's presence through the pilot "mini-cleavage" the entire peptide product was cleaved and this product was then analysed by HPLC-MS, though this step was performed by the research group with which this project was carried out of this work due to time constraints. Purification was then carried out and the peptide was found in one of the two fractions isolated. However, the product obtained was too little to continue with its purification and characterization, as what little was left would be lost thereafter, and this was the final product obtained. The researchers with whom this project was carried out subsequently synthesised more of this peptide for future purification and characterization.

The fourth peptide synthesized was **4-MEBA** substituted tyrocidine A where MEBA was the sixth amino acid introduced, replacing Gln and 4,D-Phe in the natural peptide (Figure 12).

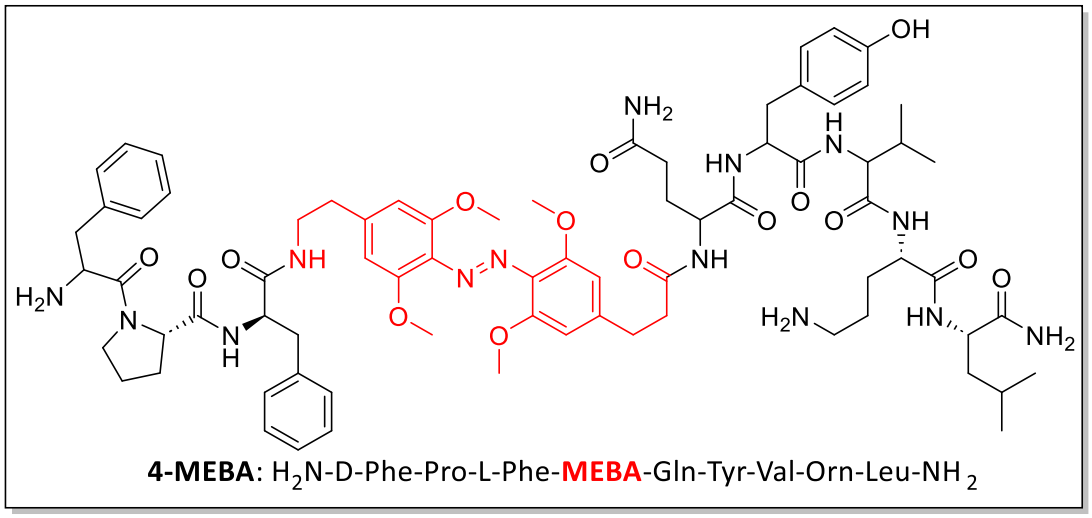

Figure 12: ***4-MEBA substituted tyrocidine A***

Confirmation of the successful synthesis of the desired product was obtained through a "mini-cleavage", though it was also observed that it was overall a significantly impure crude. Whole-peptide cleavage was conducted and re-analysed, and the aforementioned degree of impurity was confirmed.

Subsequent purifications were not carried out personally due to time constraints, but the research team continued with this task.

Finally, the second peptide synthesized was the **Tyr7 6-MEBA** analogue of tyrocidine A (Figure 13). This peptide was the product of the erroneous introduction of a tyrosine residue in seventh position instead of the valine residue that the natural peptide would have had in this position, and was originally intended to be the ordinary **6-MEBA** substituted tyrocidine A planned. MEBA was the fourth amino acid introduced, replacing Gln and erroneously replacing Val.

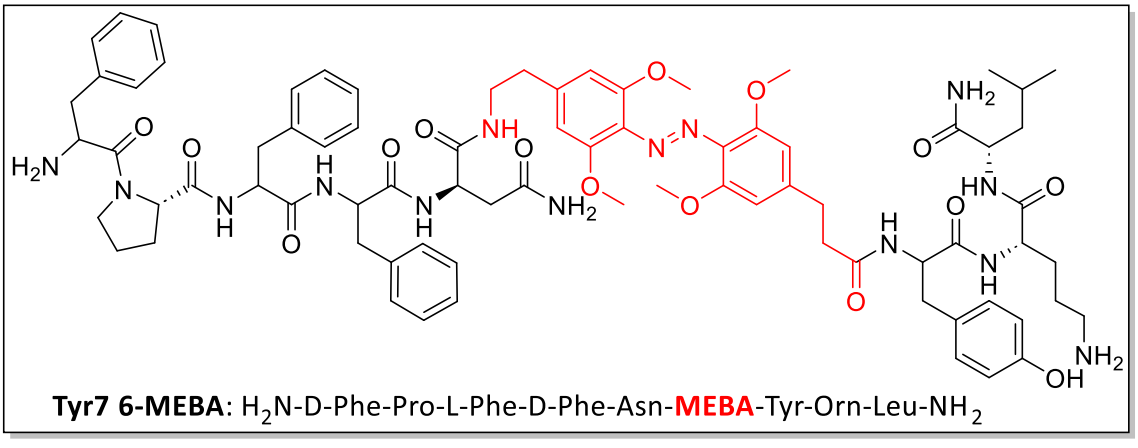

Figure 13: ***Tyr7 6-MEBA substituted tyrocidine A***

After the final coupling was performed, a “mini-cleavage” revealed that the peptide obtained did not match the expected sequence. After careful analysis, it was determined that Val7 had been accidentally replaced by tyrosine. Moreover, the final D-Phe1 residue was missing, indicating that Fmoc deprotection after the coupling of Pro had not been performed. Thus, the missing D-Phe at the N-terminus was introduced, yielding an unplanned analogue (Tyr7 6-MEBA, Figure 13). In any case, since one of the main objectives of this project is to obtain a library of photoswitchable peptide antibiotics, full peptide cleavage, one purification and HPLC-MS characterization of the purification product were carried out. Subsequent purifications were not carried out personally due to time constraints.

4. CONCLUSIONS

Four visible-light-operated photoswitchable Tyrocidine A analogues that contain a MEBA moiety were synthesized within the framework of this project, which was the main aim of this thesis. In this process, the compatibility of MEBA with conventional SPPS methods was verified and it is expected that MEBA will be compatible with a wide range of peptide products obtained using similar SPPS conditions; a small library of tyrocidine A-MEBA analogues was also prepared for future studies, achieving another of the originally set objectives for this project. However, the complete purification was only able to be carried out with **7-MEBA** substituted Tyrocidine A due to time constraints.

Most importantly, this is the first case of a visible light operated photoswitchable amino acid fully compatible with standard SPPS methods. Additionally, conditions for peptide cleavage were improved compared to previous methods used, yielding cleaner cleavage crudes that ease purification efforts.

With these completed objectives in mind, it can be asserted that SPPS is an effective method in preparing Tyrocidine A-MEBA analogues which yields the desired products in synthetically useful amounts. The conditions used for cleavage are effective and reproducible, but purification and isolation proved difficult and time consuming at times, requiring many purification steps that significantly decreased the final yield. Future studies could look at optimizing conditions for purification in a more efficient manner in order to optimize the process.

Although originally set as objectives for this thesis, isomerization and *in vitro* pharmacological studies could not be conducted within this Degree's Final Year Project timeframe.

5. MATERIALS AND METHODS

All solvents and reagents were purchased from commercial suppliers and used without further purification except for MEBA, which was synthesized locally at the lab following previously established protocols designed by our research group.[21] All SPPS was carried out in plastic syringes connected to a vacuum and fitted with a Teflon tap. The 20% piperidine solution in dimethylformamide (DMF) used for Fmoc deprotection was prepared *in situ*.

Resin loading was determined after Fmoc deprotection of the first amino acid by UV-Vis spectrophotometry of the combined deprotection and washing solutions at 301 nm.

A qualitative analysis of the efficiency of each coupling was performed with ninhydrin (Kaiser test), adding the corresponding solutions and incubating at 105 °C for 5 min.

HPLC analysis was performed on a Waters Alliance 2695 separation module coupled to a Waters 2996 photodiode detector (PDA) and to an electrospray ionization source Waters ACQUITY QDa detector, using the MassLynx 4.1 software for data acquisition and a XSelect CSH C18 OBD column. The flow rate was 0.6 mL·min−1, and MeCN (0.1% formic acid) and H_2O (0.1% formic acid) were used as solvents. The elution runtime was 3.5 min at 50 °C.

Purification of cleavage crudes and non-pure peptide mixtures was performed using *Biotage® Isolera One* automated chromatography purification system with MeCN (0.1% formic acid) and H_2O (0.1% formic acid) as solvents.

ChemDraw software was used to draw and display the different compounds and reactions shown in this work.

5.1. SOLID PHASE PEPTIDE SYNTHESIS

5.1.1. RESIN CONDITIONING/PRIMING

Resin priming was performed in the same polypropylene syringe later used for SPPS. 0.025 mmol of Rink-Amide ChemMatrix resin (46.3 mg; functionalisation: 0.54 mmol/g) was loaded into the SPPS syringe and washed once using methanol, followed by DMF (× 5) and dichloromethane (DCM) (× 5).

5.1.2. COUPLING

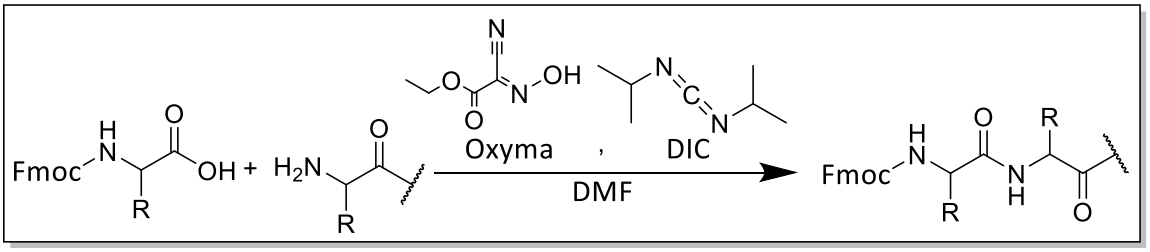

Figure 14: General scheme of a coupling reaction employed in this work

Manual peptide elongation was performed in polypropylene syringes fitted with a frit for filtration and a Teflon stirring rod using the Fmoc strategy. RinkAmide ChemMatrix resin (functionalisation 0.54 mmol/g) was used to obtain C-terminal amides upon cleavage. The first amino acid was introduced by treating the resin with the appropriate commercially available Fmoc-amino acid (3.0 equiv), Oxyma (3.3 equiv) and DIC (3.3 equiv) in DMF (0.42 mL, 0.20 M solution of amino acid) during overnight gentle shaking. Subsequent couplings were performed treating the resin with the appropriate Fmoc-amino acids (2.3 equiv.), Oxyma (2.5 equiv) and DIC (2.5 equiv) in DMF (0.32 mL, 0.19 M solution of amino acid) for 1 h 30 min (Figure 14).

Alloc-MEBA (Figure 15) was introduced as regular amino acids using the same reaction conditions but performing the coupling overnight.

While the reactions were taking place, the syringe was sealed with parafilm.

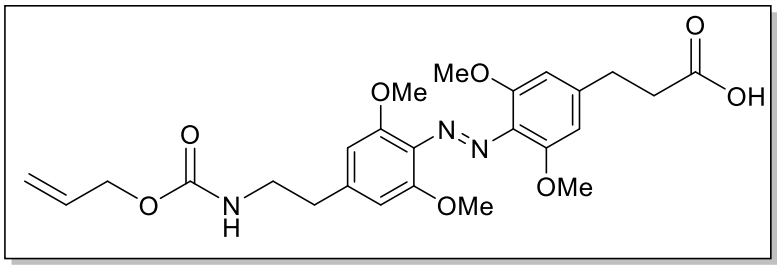

Figure 15: Alloc-MEBA-OH

5.1.3. Fmoc DEPROTECTION

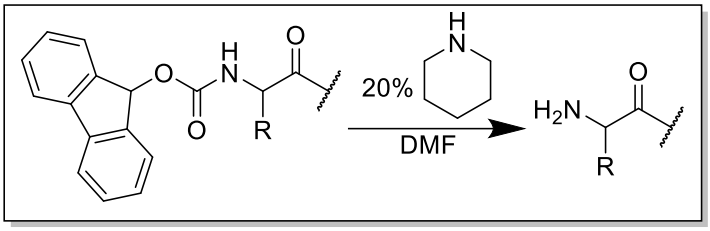

Figure 16: Deprotection of Fmoc-protected amino acids

In accordance with established literature,[24] 20% piperidine in DMF was used to deprotect the temporary Fmoc protecting groups (2 x 1 min + 2 x 5 min treatments) after each successful coupling (Figure 16). The success of each coupling was assessed performing the ninhydrin (Kaiser) test.

After every elongation and deprotection the resin was washed with DMF (x 5) and DCM (x 5).

5.1.4. Alloc DEPROTECTION OF MEBA

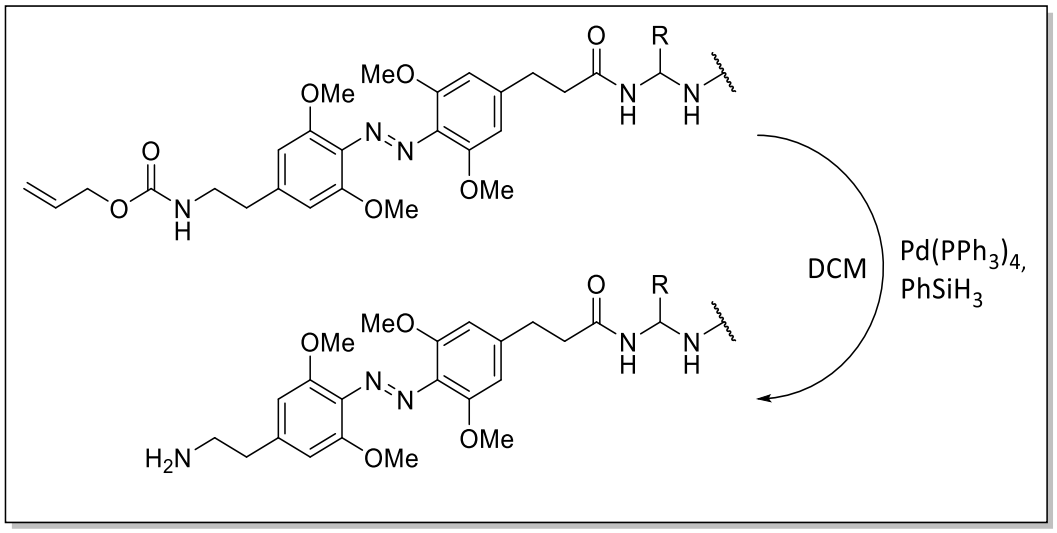

Figure 17: Alloc-deprotection of MEBA

A solution of $Pd(PPh_3)_4$ (3 mg, 2.60 μmol), $PhSiH_3$ (62 μL, 0.653 mmol) in DCM (0.5 mL) was added to the SPPS syringe after each successful MEBA coupling and left to react for 1 h (Figure 17). This was performed twice, and the resin was washed with DMF (× 5) and DCM (× 5) after each treatment.

5.1.5. PEPTIDE CLEAVAGE

A mini cleavage was first conducted on all finished peptides with a sample of the resin, which was introduced into an Eppendorf tube along with 150 μL of TFA/H20/TES (95 :

2.5 : 2.5). The mini-cleavage was performed for 30 min and the cleavage crude was then evaporated under a stream of N_2. The residue was dissolved in methanol and then prepared for HPLC-MS analysis.

In order to cleave the entire peptide product, resins were treated with the above solution (5 x 45 min treatments), then washed with DCM (x 5), unless indicated otherwise.

The cleavage crude was collected in a flask and was evaporated under a stream of $N_{2(g)}$. When isolating the peptide after purification with *Biotage® Isolera One* solvents were removed through to vacuum distillation with dry ice in a rotatory evaporator to evaporate volatiles. Toluene was added as needed in order to fully evaporate all volatiles. When only a red solid was left in the flask, its contents were transferred into a vial and put into the freezer until further use.

6. BIBLIOGRAPHY

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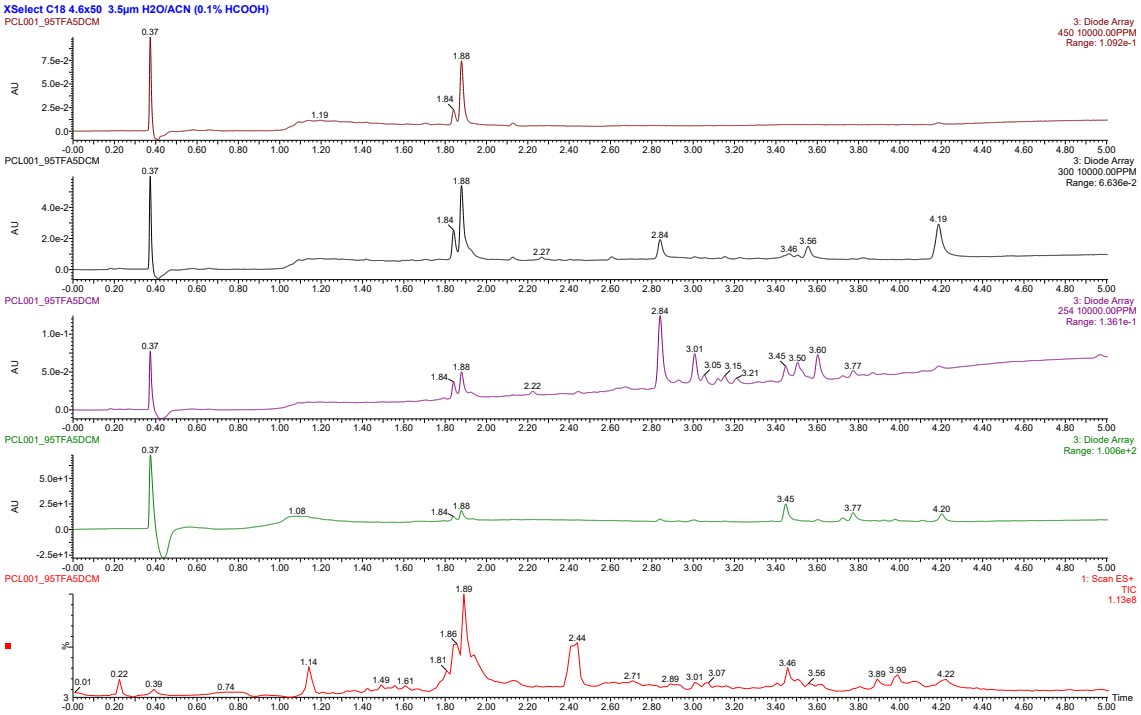
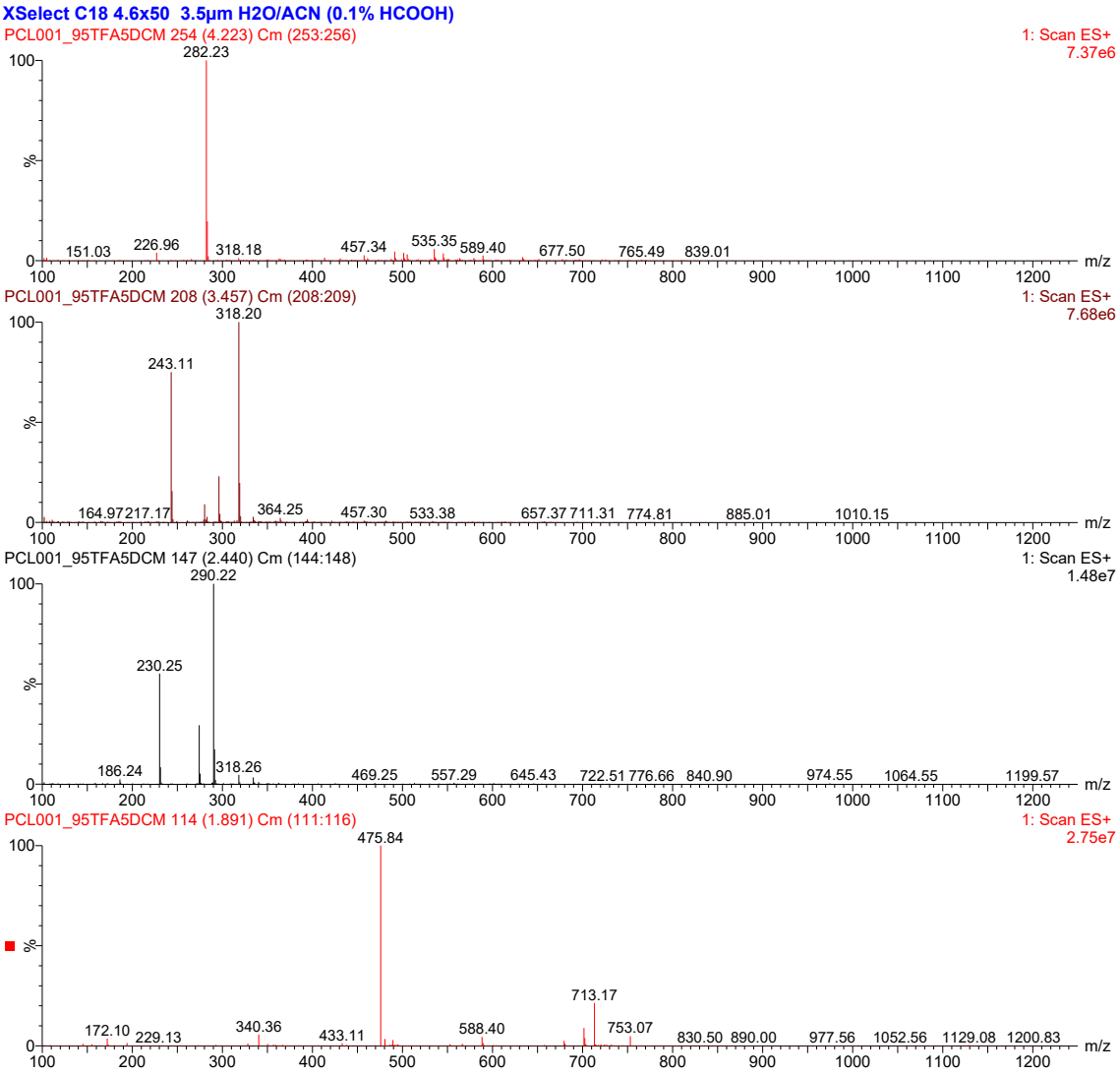
7.ANNEX

7.1. HPLC-MS SPECTRA FOR 7-MEBA SUBSTITUTED TYROCIDINE A

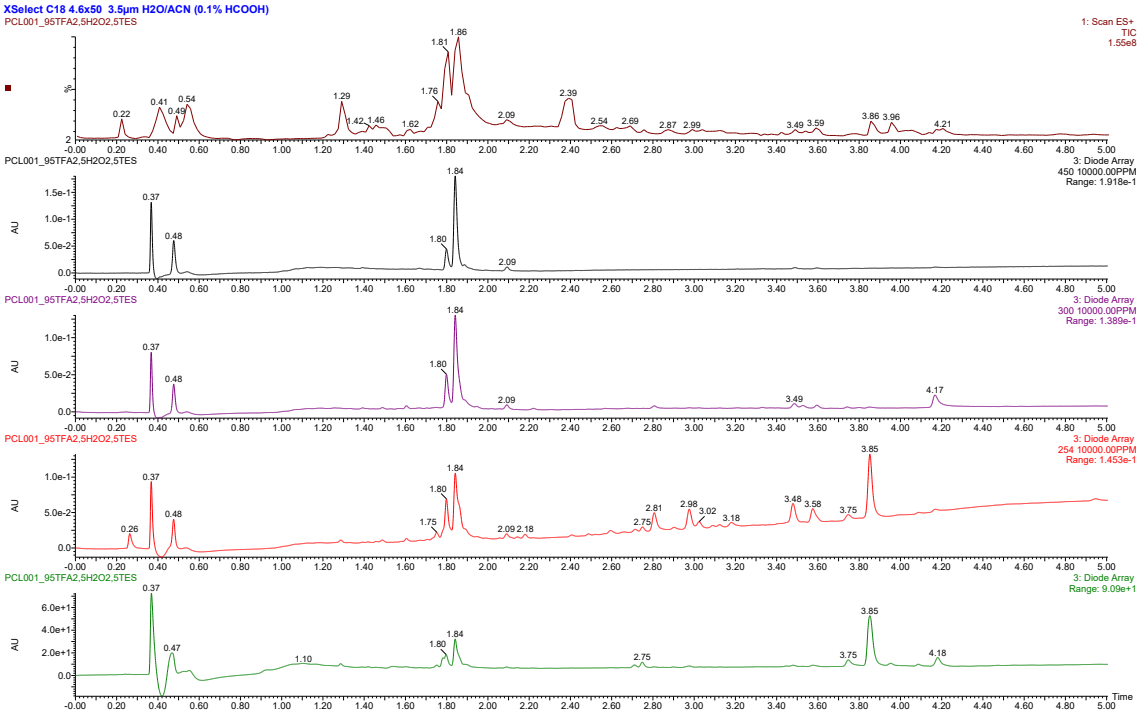
7.1.1. PCL001 PILOT CLEAVAGE COCKTAIL

7.1.1.1. TFA/DCM (95:5)





7.1.1.2. TFA/TES/$H_2$0 (95:2,5:2,5)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PCL001_95TFA2,5H2O2,5TES
1: Scan ES+
TIC
1.55e8
3: Diode Array
450 10000.00PPM
Range: 1.918e-1
3: Diode Array
300 10000.00PPM
Range: 1.389e-1
3: Diode Array
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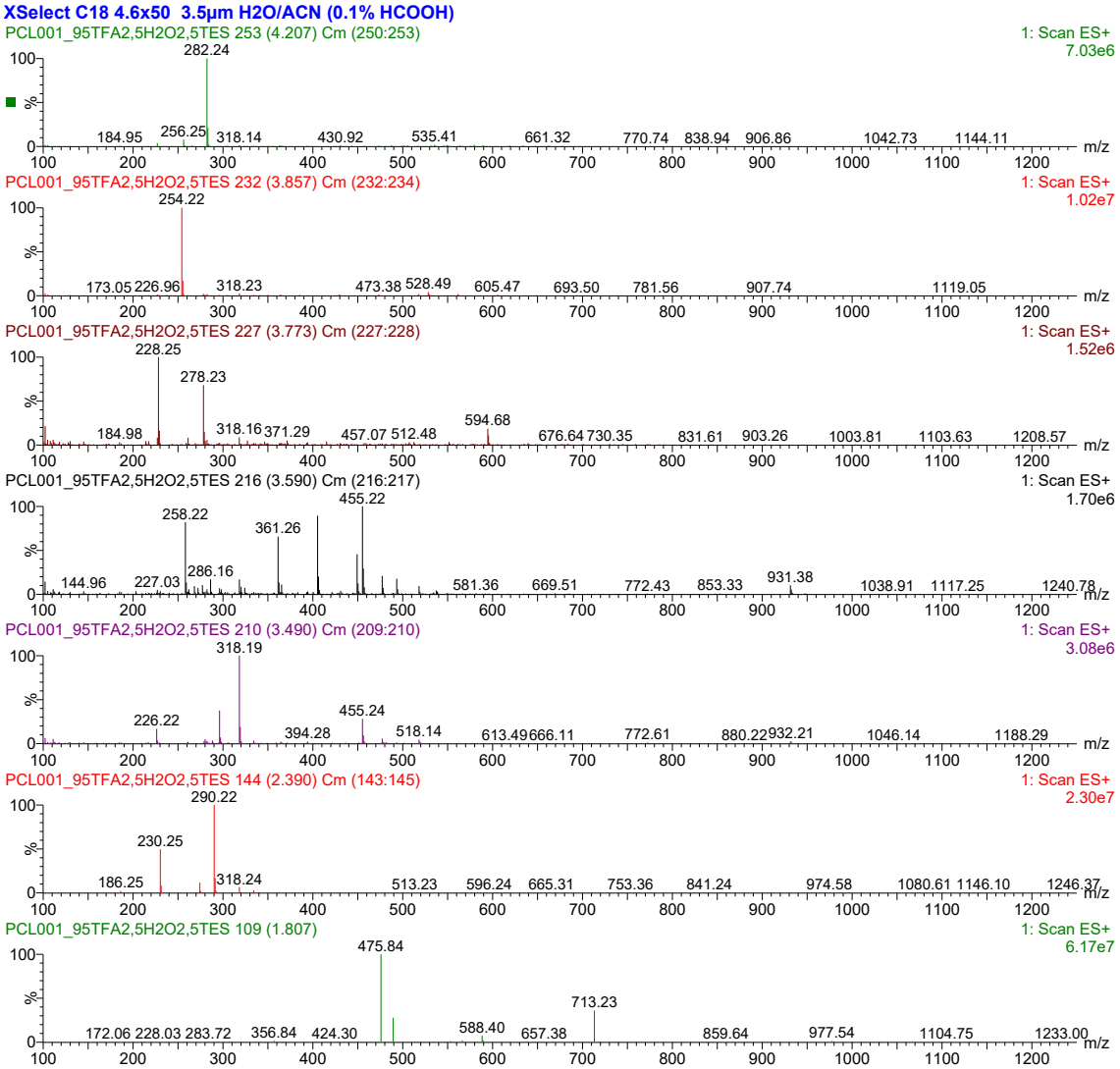
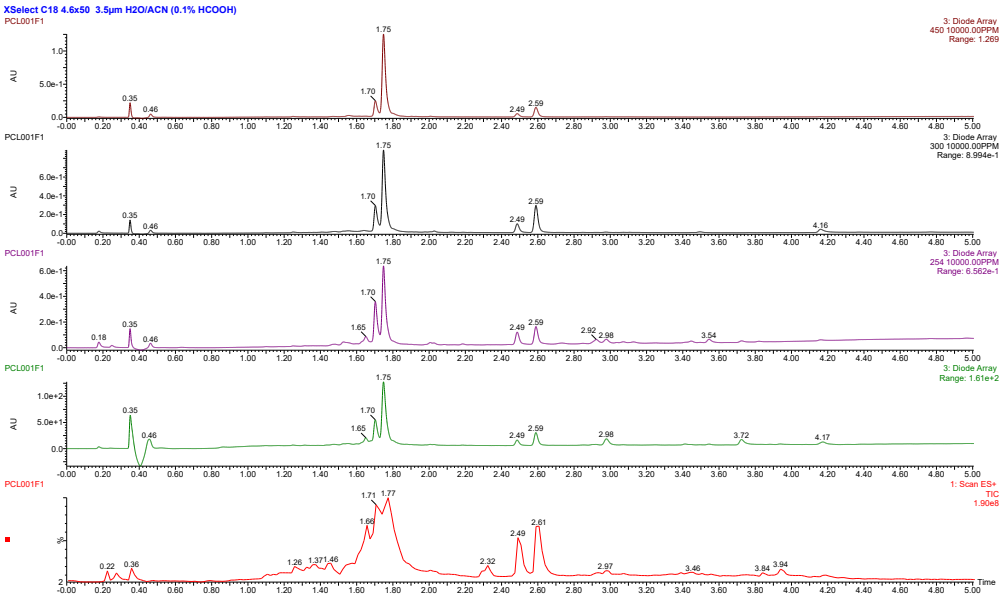
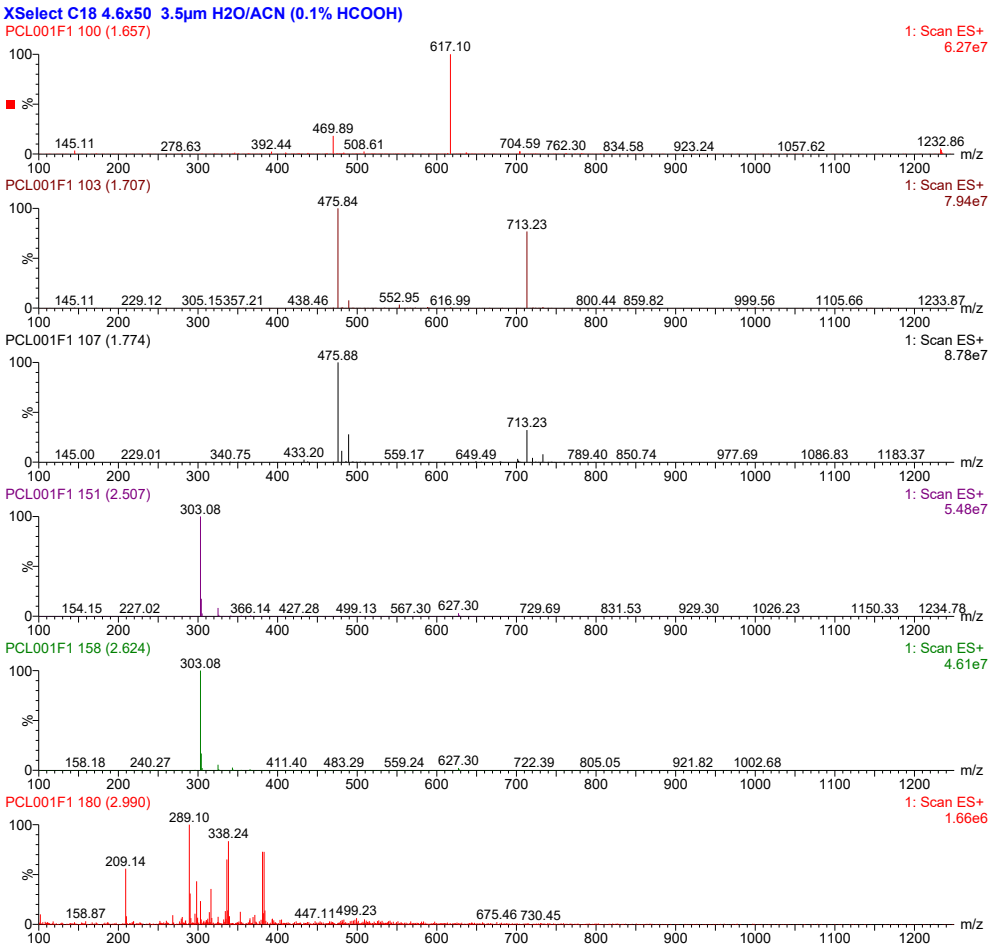
Range: 9.09e+1
AU
Time


XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
PCL001_95TFA2,5H2O2,5TES 253 (4.207) Cm (250:253)
1: Scan ES+
7.03e6
PCL001_95TFA2,5H2O2,5TES 232 (3.857) Cm (232:234)
1: Scan ES+
1.02e7
PCL001_95TFA2,5H2O2,5TES 227 (3.773) Cm (227:228)
1: Scan ES+
1.52e6
PCL001_95TFA2,5H2O2,5TES 216 (3.590) Cm (216:217)
1: Scan ES+
1.70e6
PCL001_95TFA2,5H2O2,5TES 210 (3.490) Cm (209:210)
1: Scan ES+
3.08e6
PCL001_95TFA2,5H2O2,5TES 144 (2.390) Cm (143:145)
1: Scan ES+
2.30e7
PCL001_95TFA2,5H2O2,5TES 109 (1.807)
1: Scan ES+
6.17e7
m/z

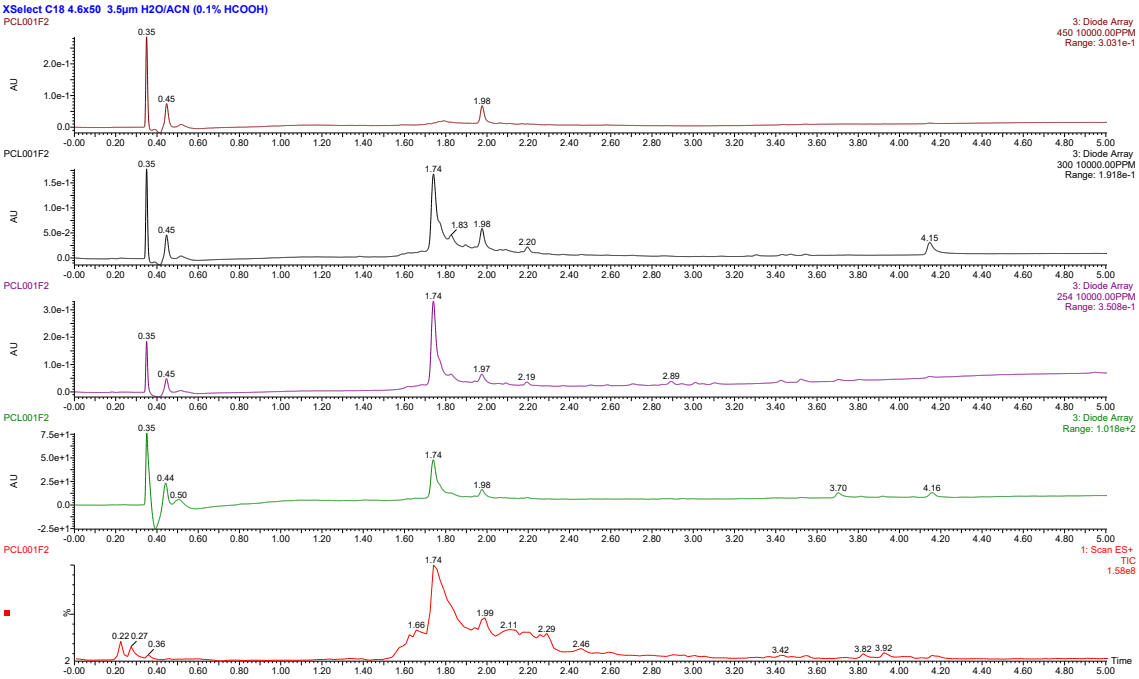
7.1.2. PCL-001 FIRST PURIFICATION

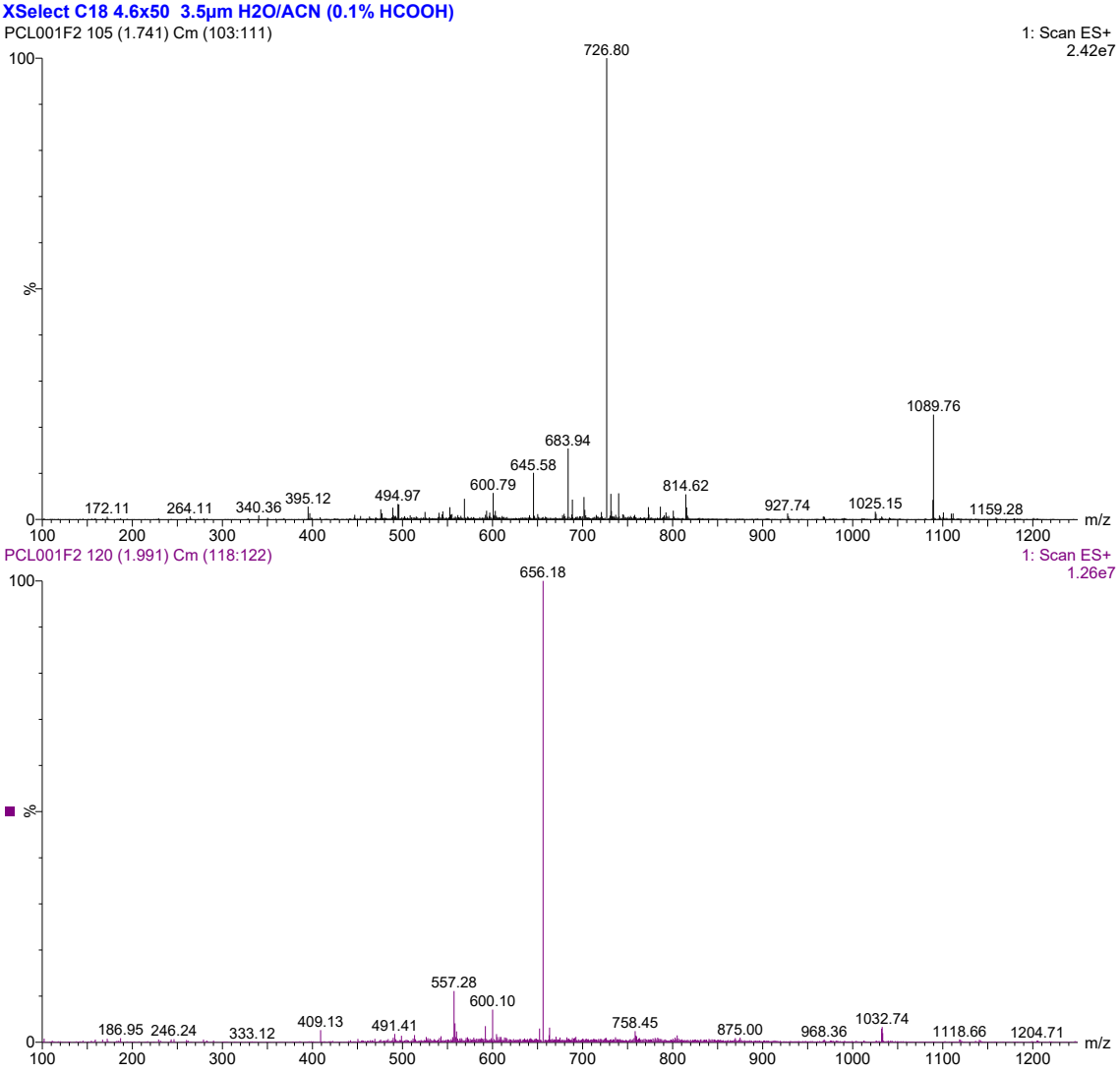
7.1.2.1 PCL-001F1 (FRACTION 1)





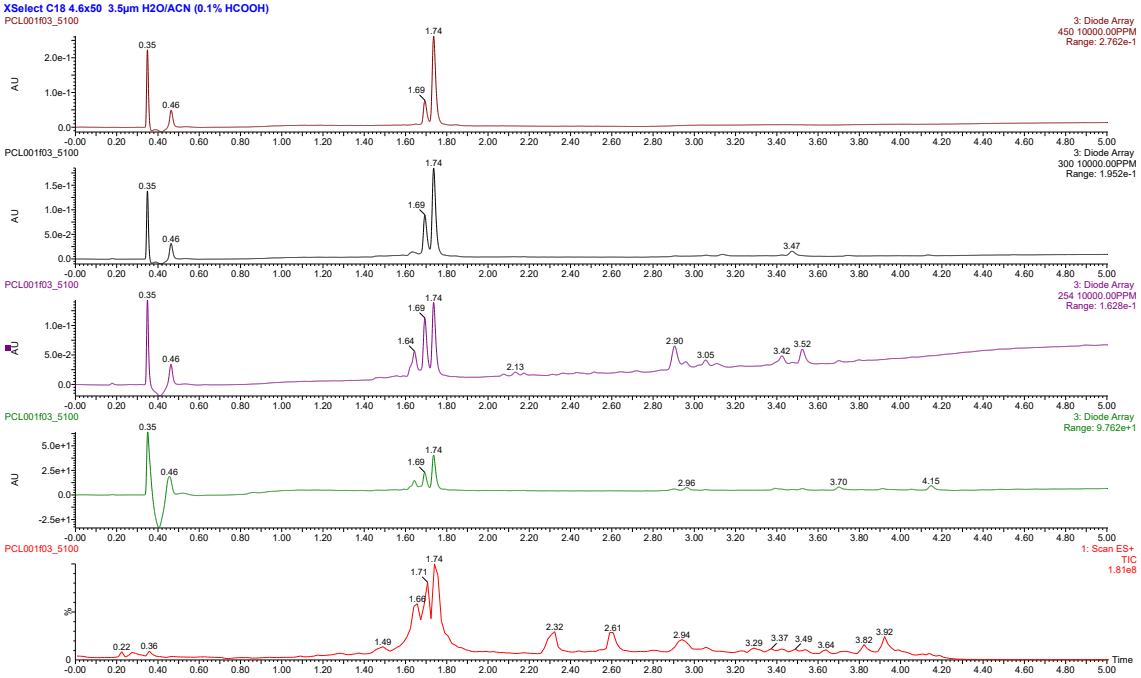
7.1.2.2. PCL-001F2 (FRACTION 2)

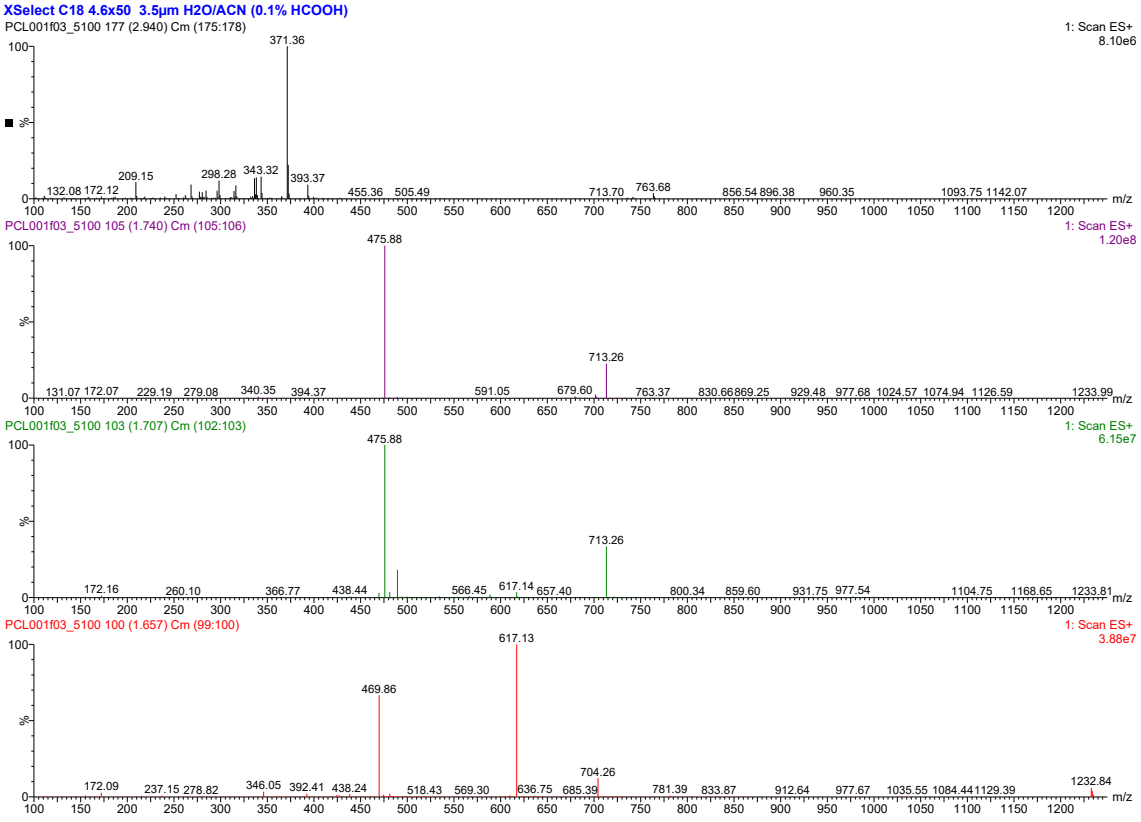




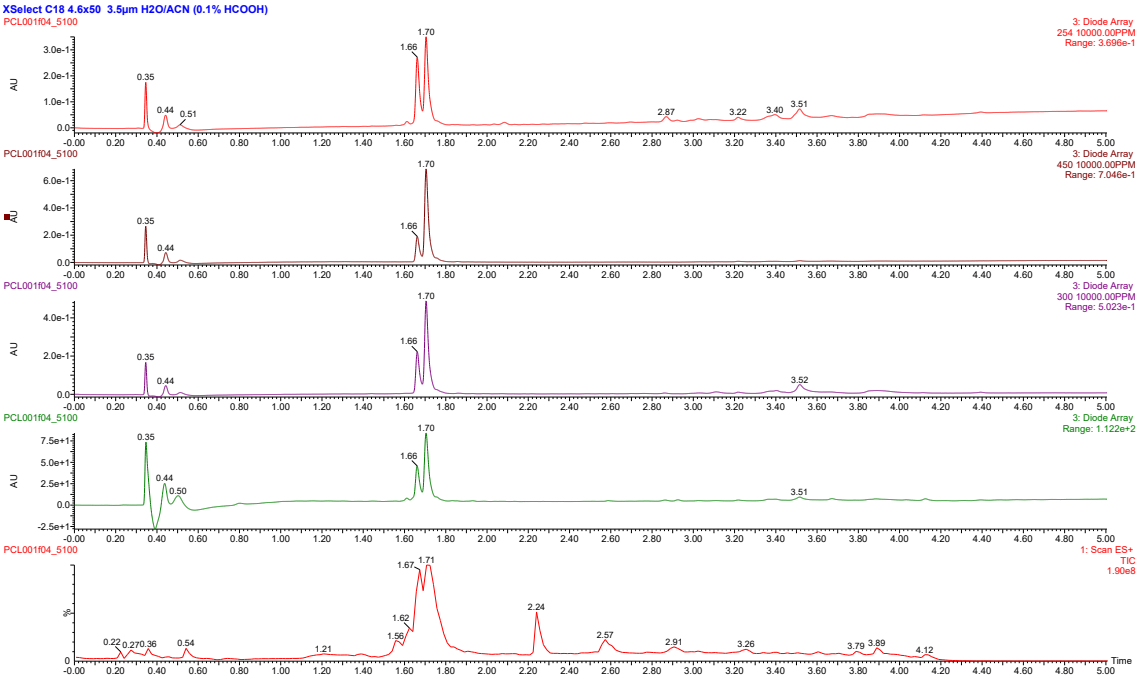
7.1.3. PCL-001 SECOND PURIFICATION

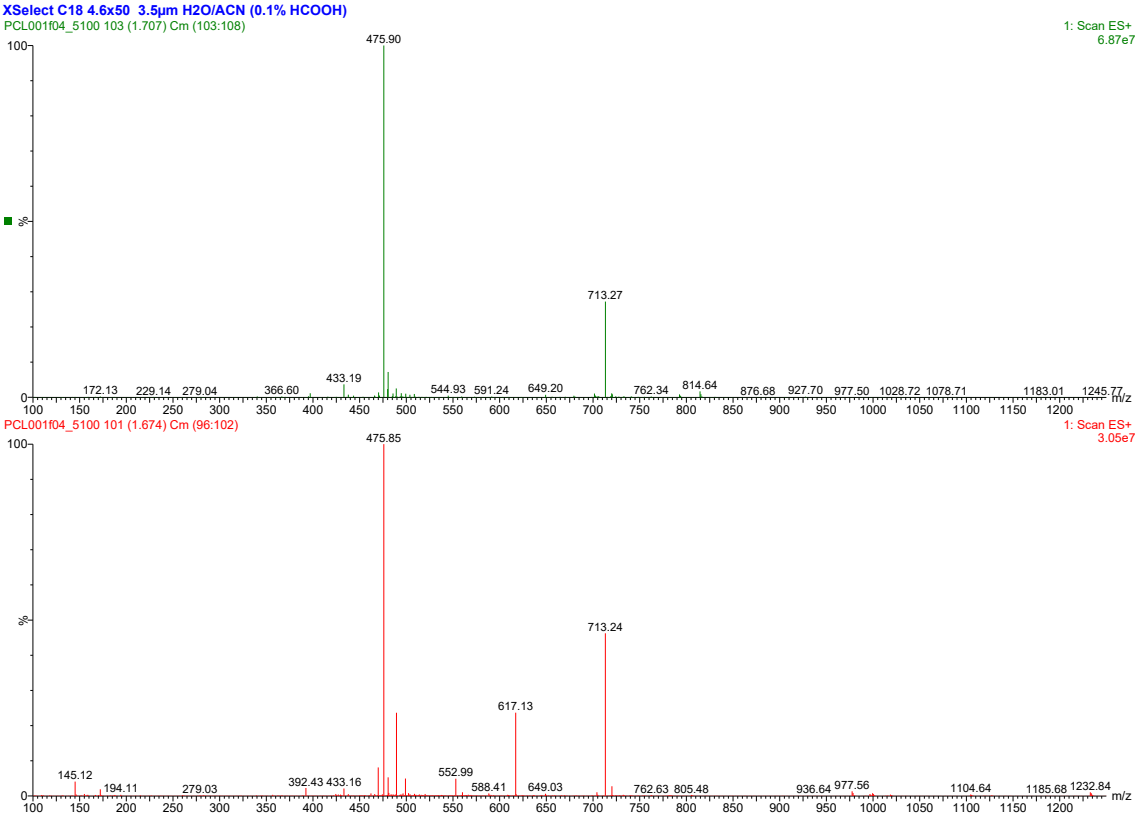
7.1.3.1. PCL-001F3 (FRACTION 3)





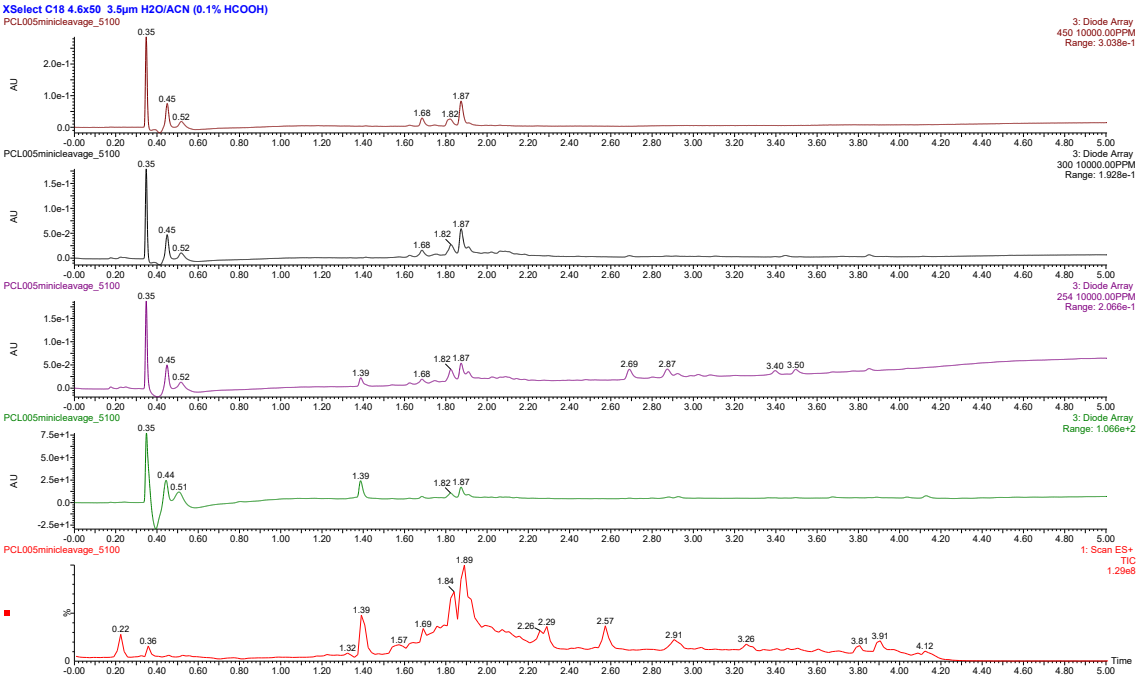
7.1.3.2. PCL-001F4 (FRACTION 4)

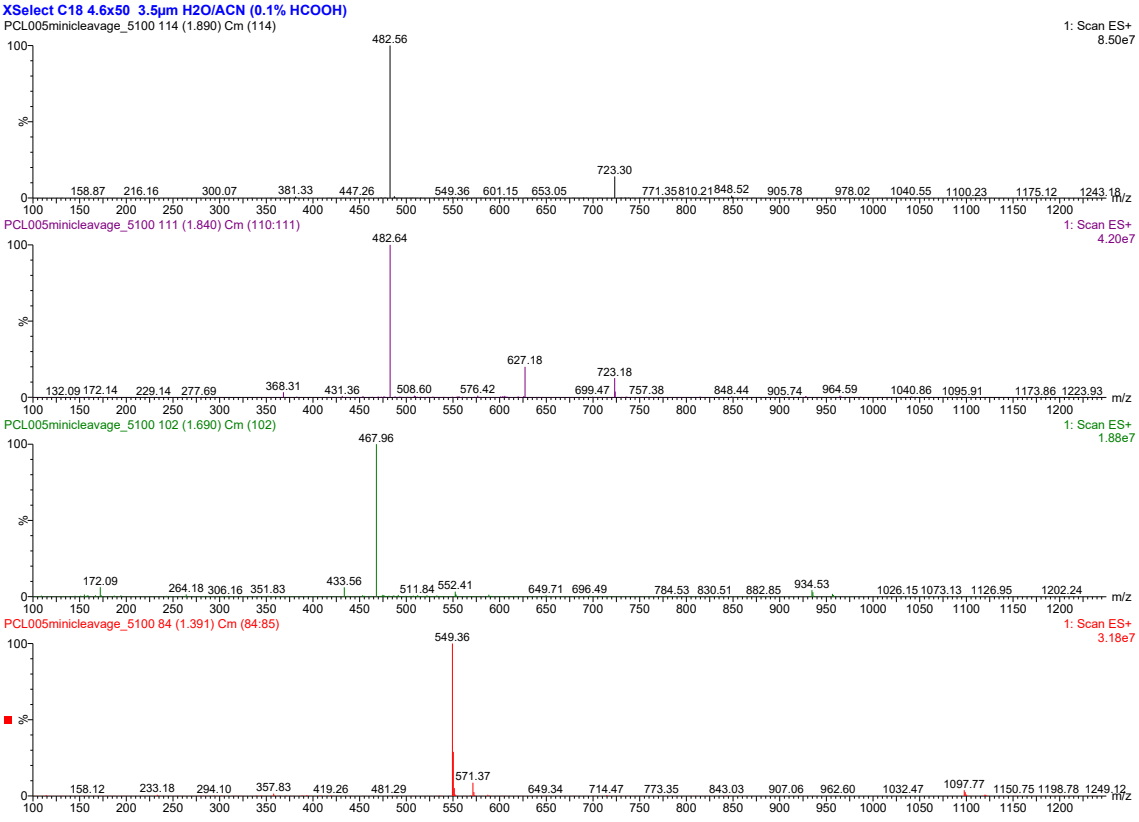


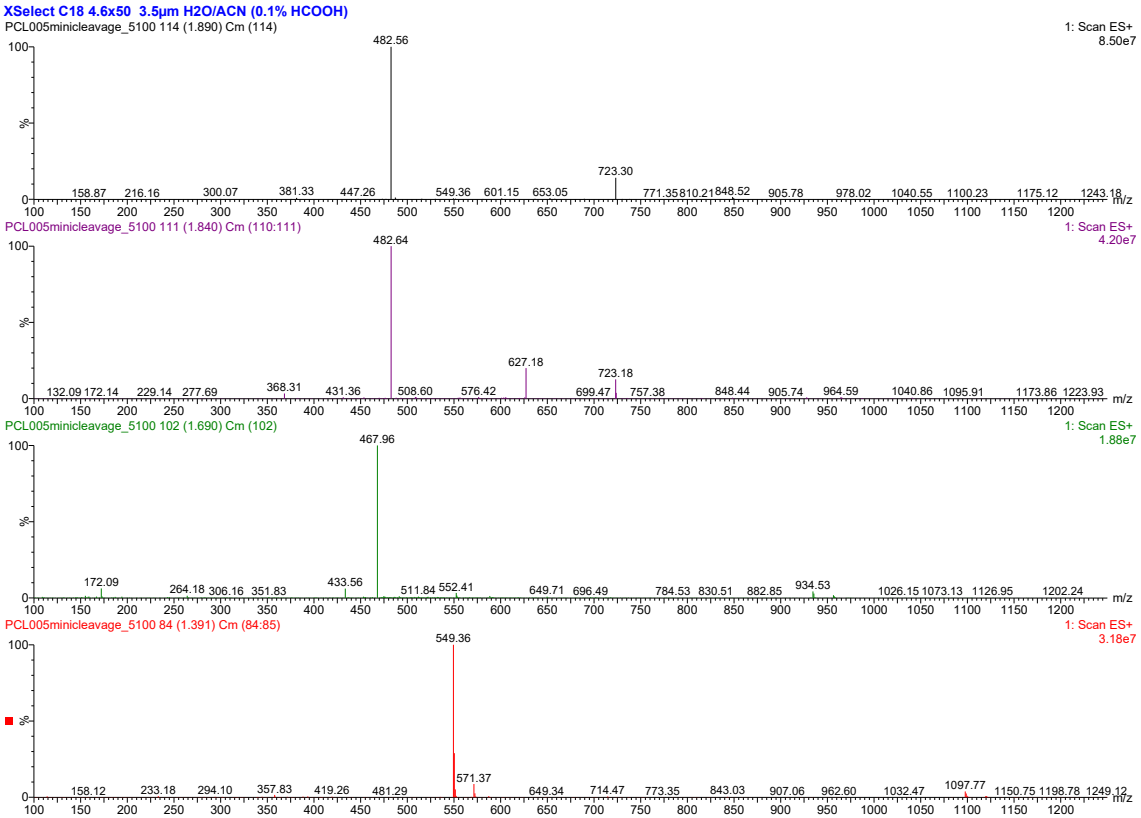


7.2. HPLC-MS SPECTRA FOR 5-MEBA SUBSTITUTED TYROCIDINE A

7.2.1. PCL005 MINI CLEAVAGE

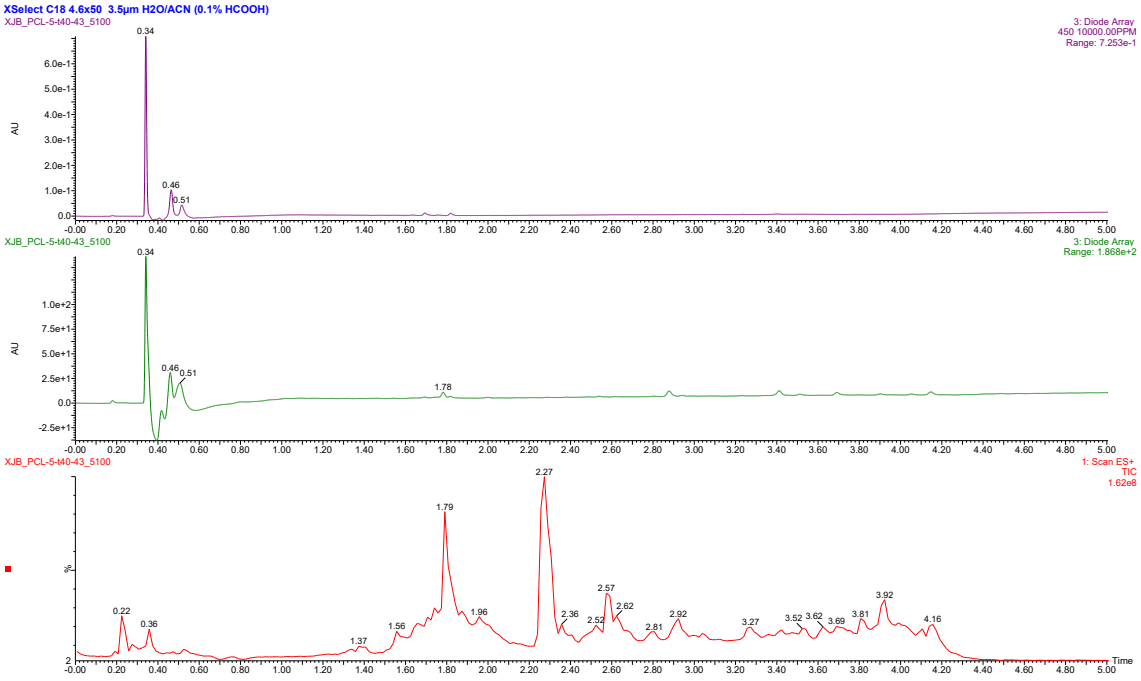

XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
PCL005minicleavage_5100
3: Diode Array
450 10000.00PPM
Range: 3.038e-1
PCL005minicleavage_5100
3: Diode Array
300 10000.00PPM
Range: 1.928e-1
PCL005minicleavage_5100
3: Diode Array
254 10000.00PPM
Range: 2.066e-1
PCL005minicleavage_5100
3: Diode Array
Range: 1.066e+2
PCL005minicleavage_5100
1: Scan ES+
TIC
1.29e8
AU
%
Time

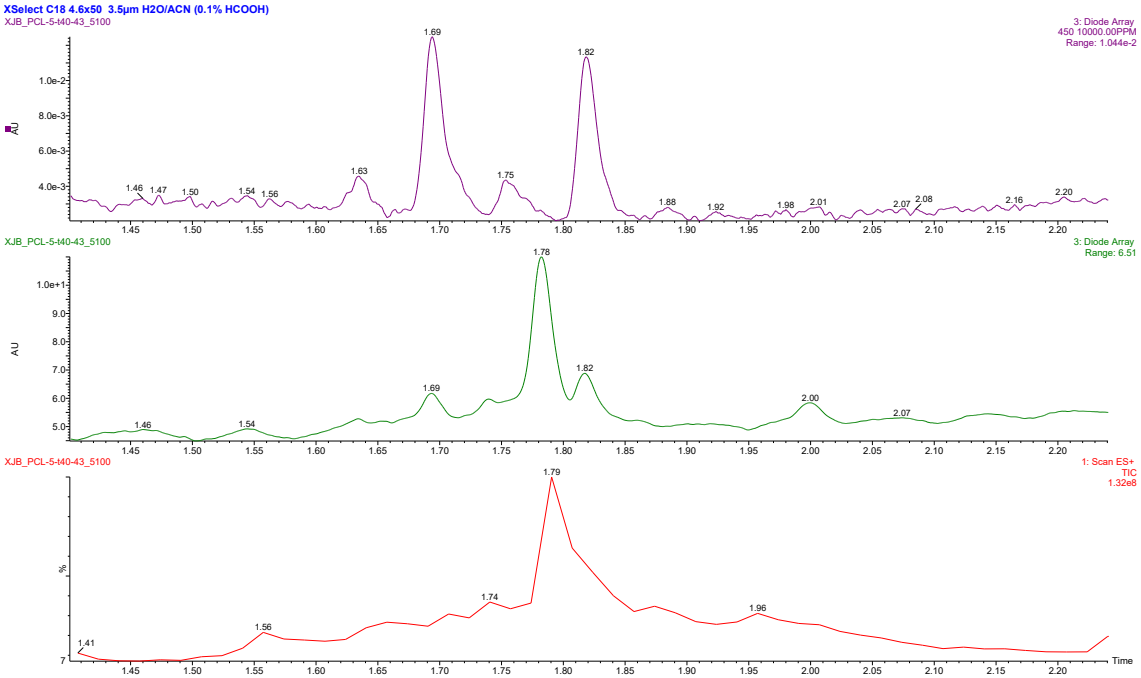

XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
PCL005minicleavage_5100 114 (1.890) Cm (114)
1: Scan ES+
8.50e7
PCL005minicleavage_5100 111 (1.840) Cm (110:111)
1: Scan ES+
4.20e7
PCL005minicleavage_5100 102 (1.690) Cm (102)
1: Scan ES+
1.88e7
PCL005minicleavage_5100 84 (1.391) Cm (84:85)
1: Scan ES+
3.18e7
%
m/z

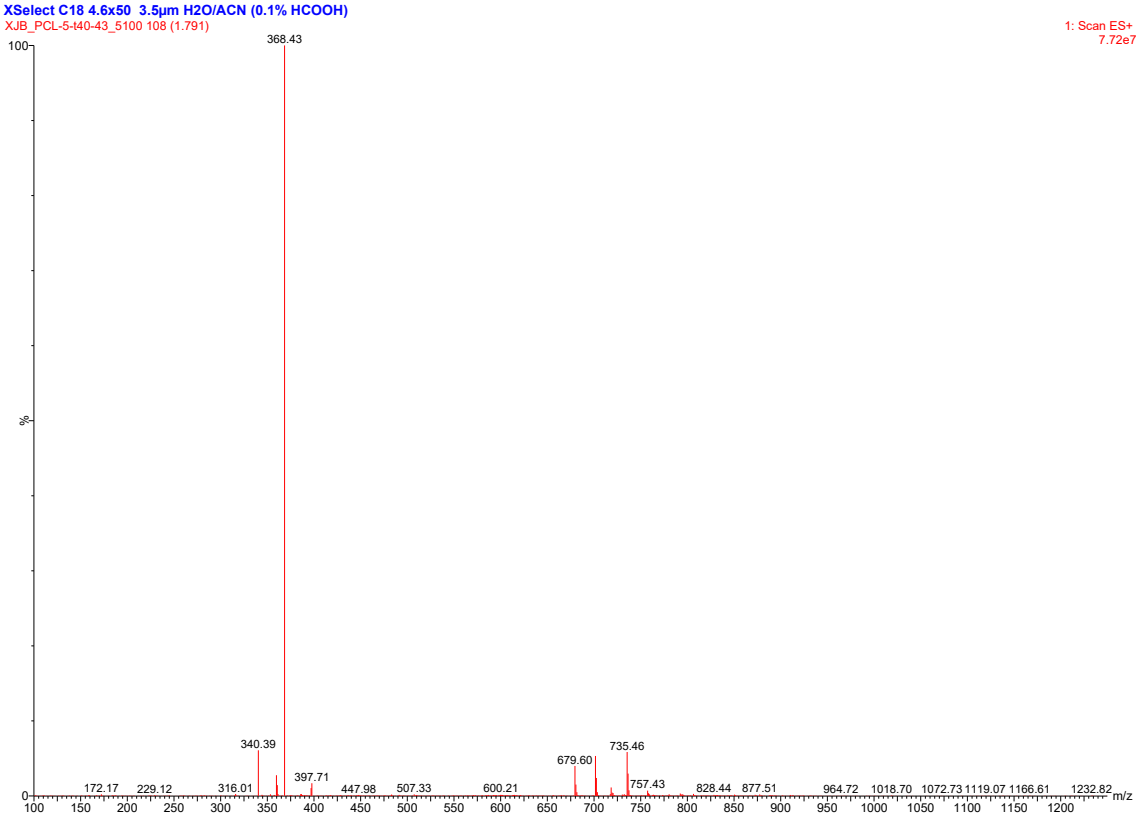


7.2.2. PCL005 PURIFICATION

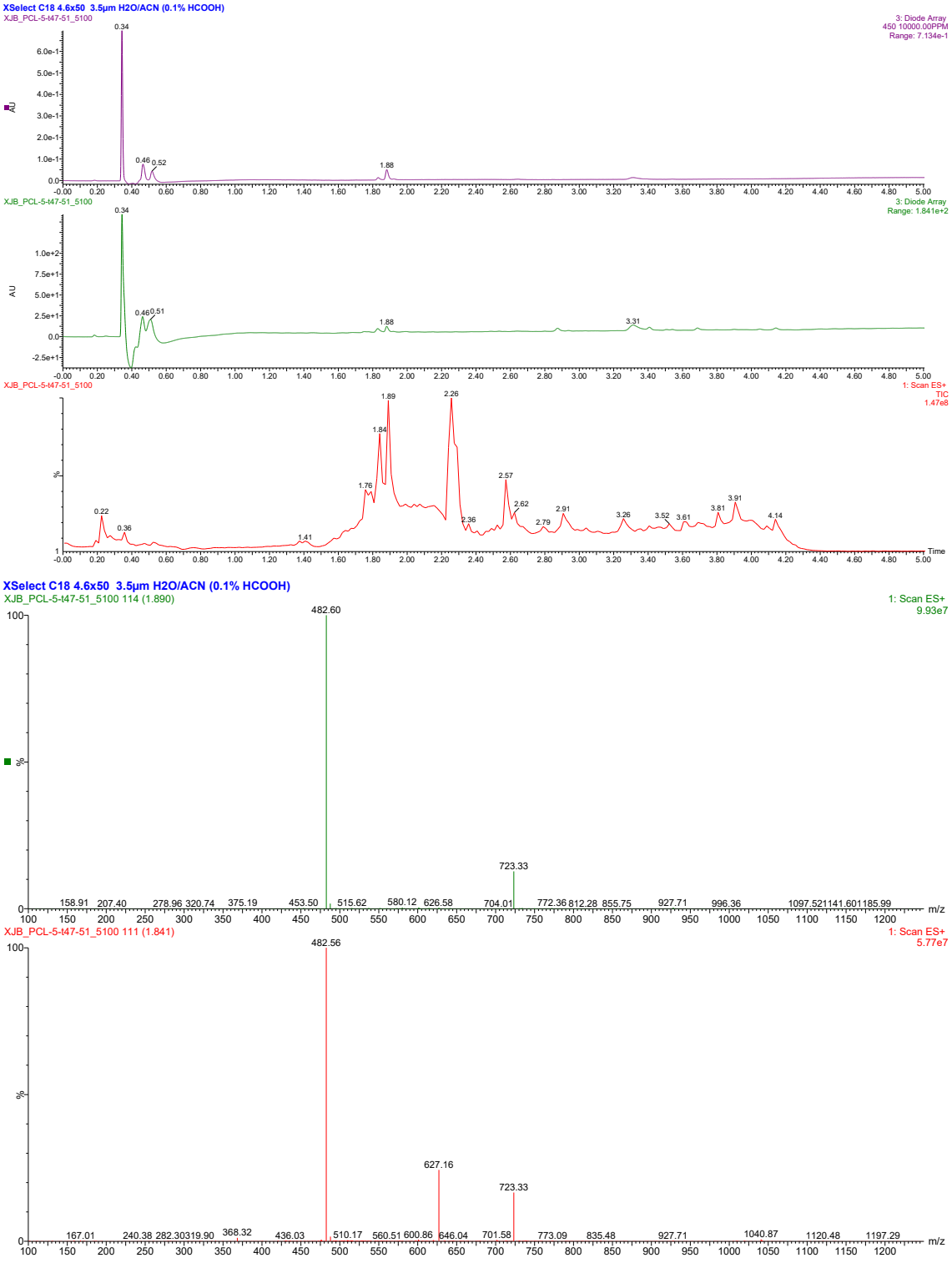
7.2.2.1. PCL005F1





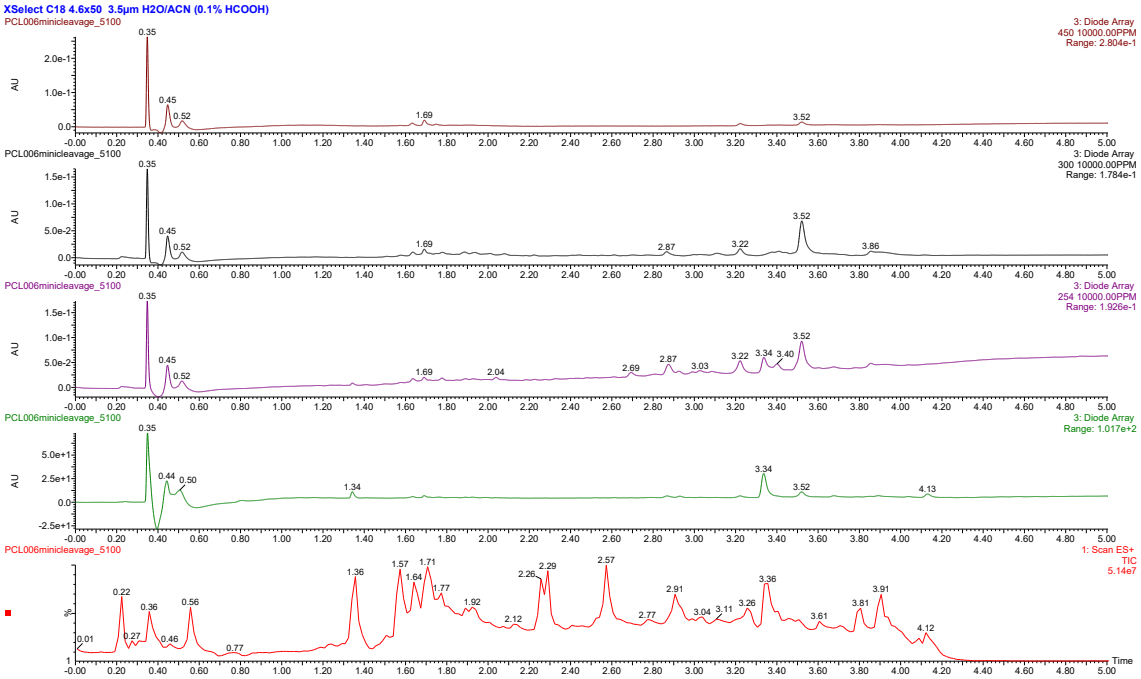


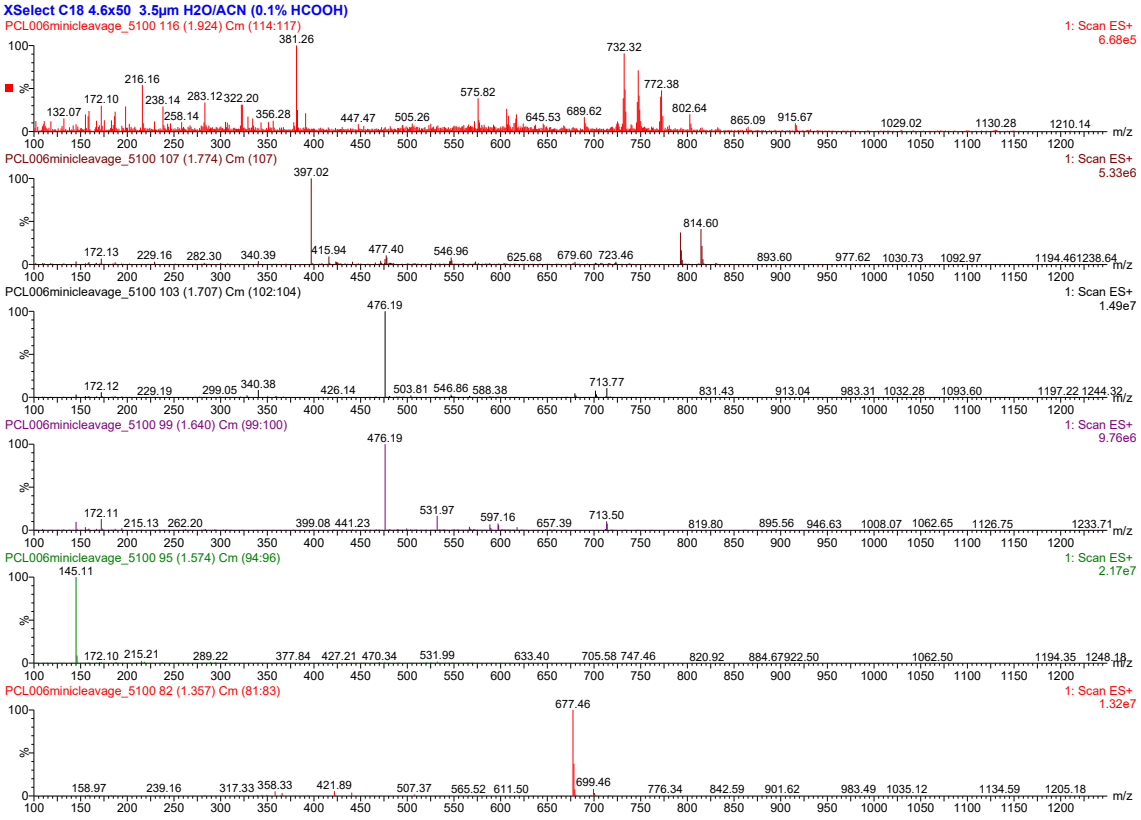
7.2.2.2. PCL005F2

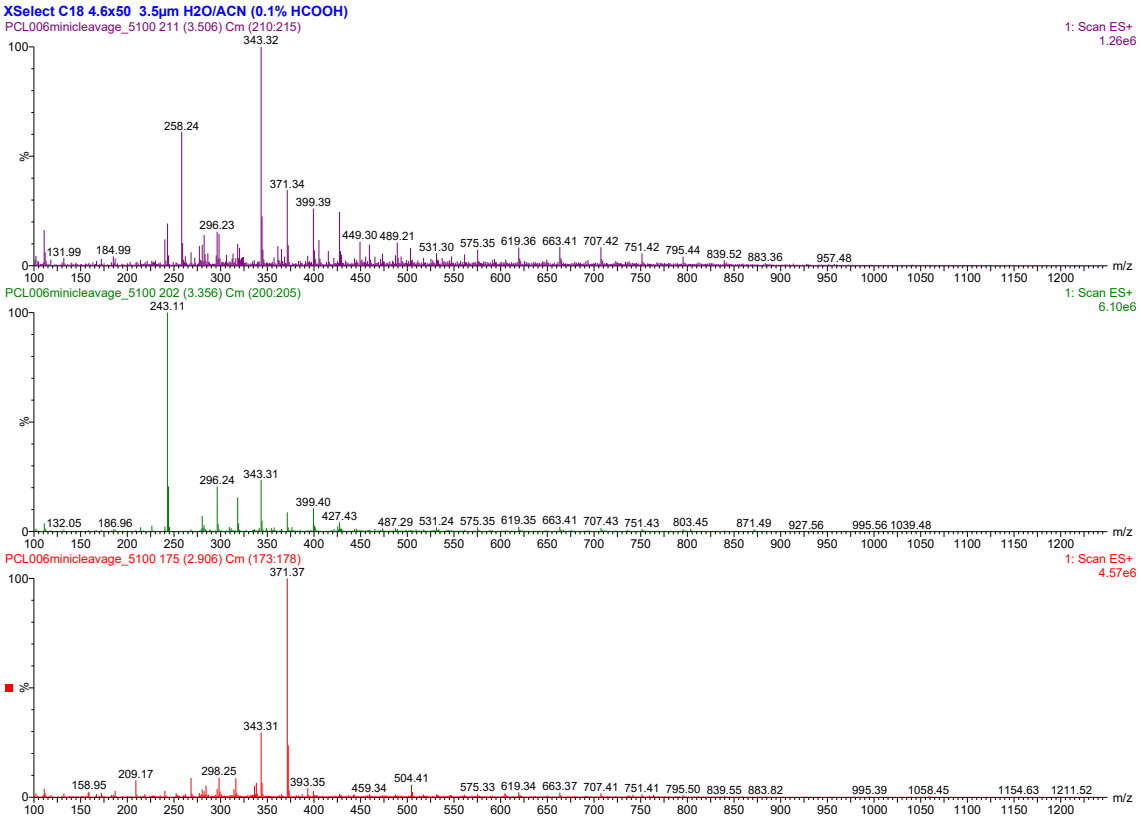


7.3. HPLC-MS SPECTRA FOR 4-MEBA SUBSTITUTED TYROCIDINE A

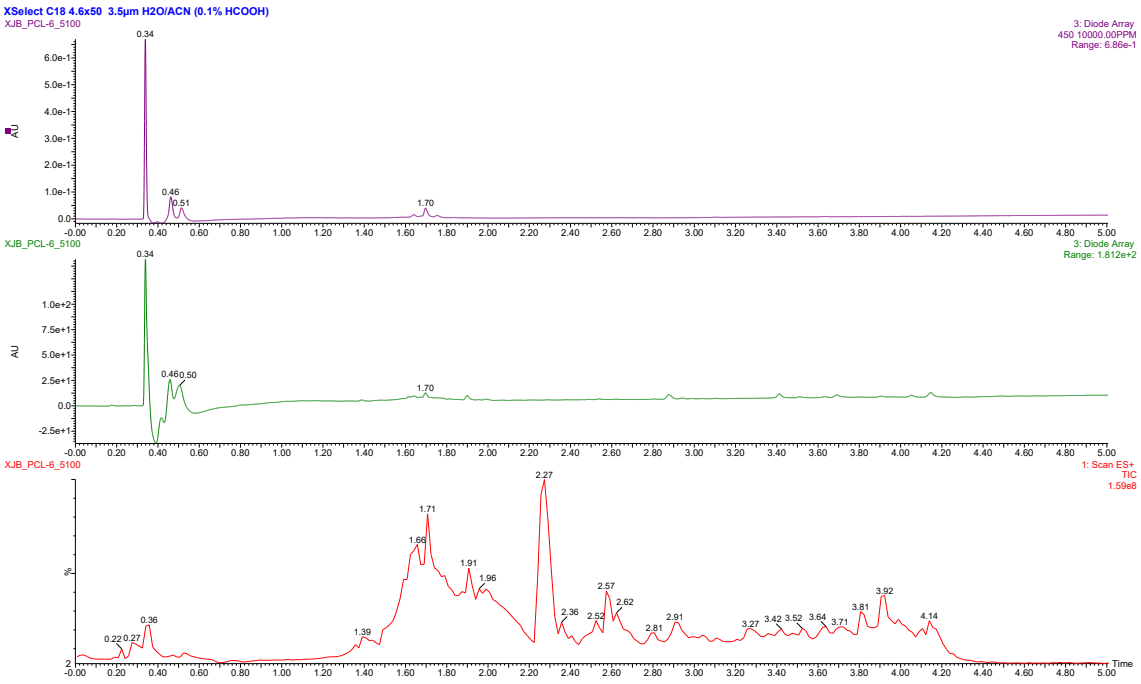
7.3.1. PCL006 MINI CLEAVAGE


XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
PCL006minicleavage_5100
3: Diode Array
450 10000.00PPM
Range: 2.804e-1
PCL006minicleavage_5100
3: Diode Array
300 10000.00PPM
Range: 1.784e-1
PCL006minicleavage_5100
3: Diode Array
254 10000.00PPM
Range: 1.926e-1
PCL006minicleavage_5100
3: Diode Array
Range: 1.017e+2
PCL006minicleavage_5100
1: Scan ES+
TIC
5.14e7
AU
%
Time


XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
PCL006minicleavage_5100 116 (1.924) Cm (114:117)
1: Scan ES+
6.68e5
PCL006minicleavage_5100 107 (1.774) Cm (107)
1: Scan ES+
5.33e6
PCL006minicleavage_5100 103 (1.707) Cm (102:104)
1: Scan ES+
1.49e7
PCL006minicleavage_5100 99 (1.640) Cm (99:100)
1: Scan ES+
9.76e6
PCL006minicleavage_5100 95 (1.574) Cm (94:96)
1: Scan ES+
2.17e7
PCL006minicleavage_5100 82 (1.357) Cm (81:83)
1: Scan ES+
1.32e7
%
m/z

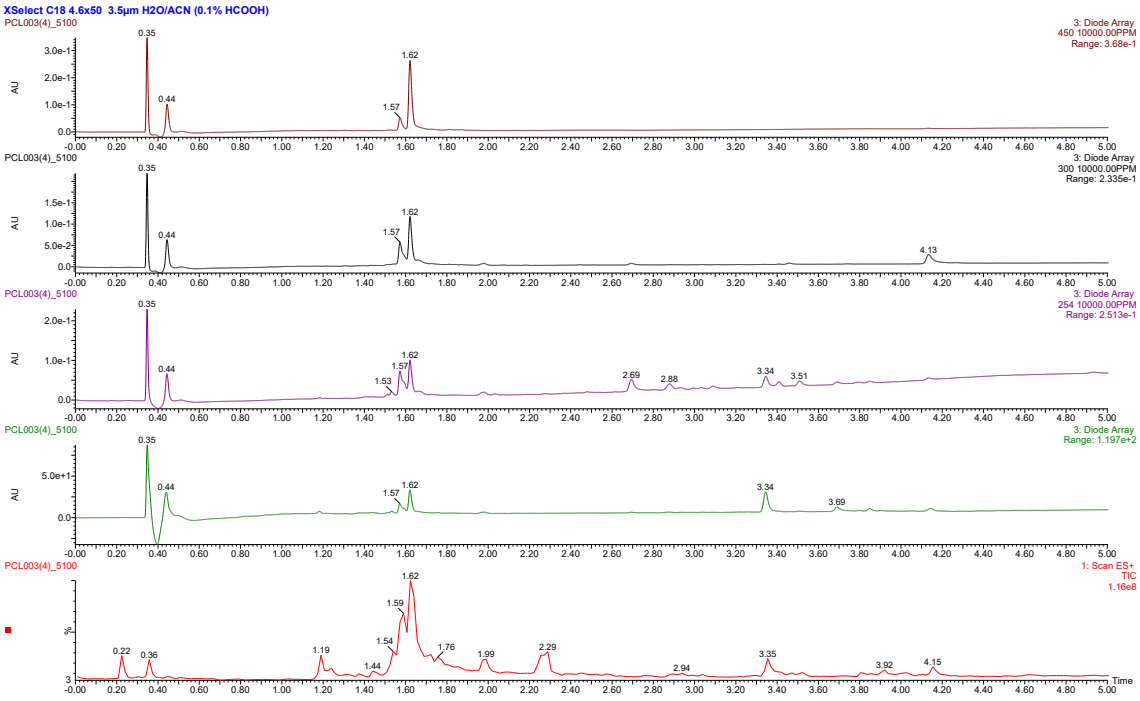


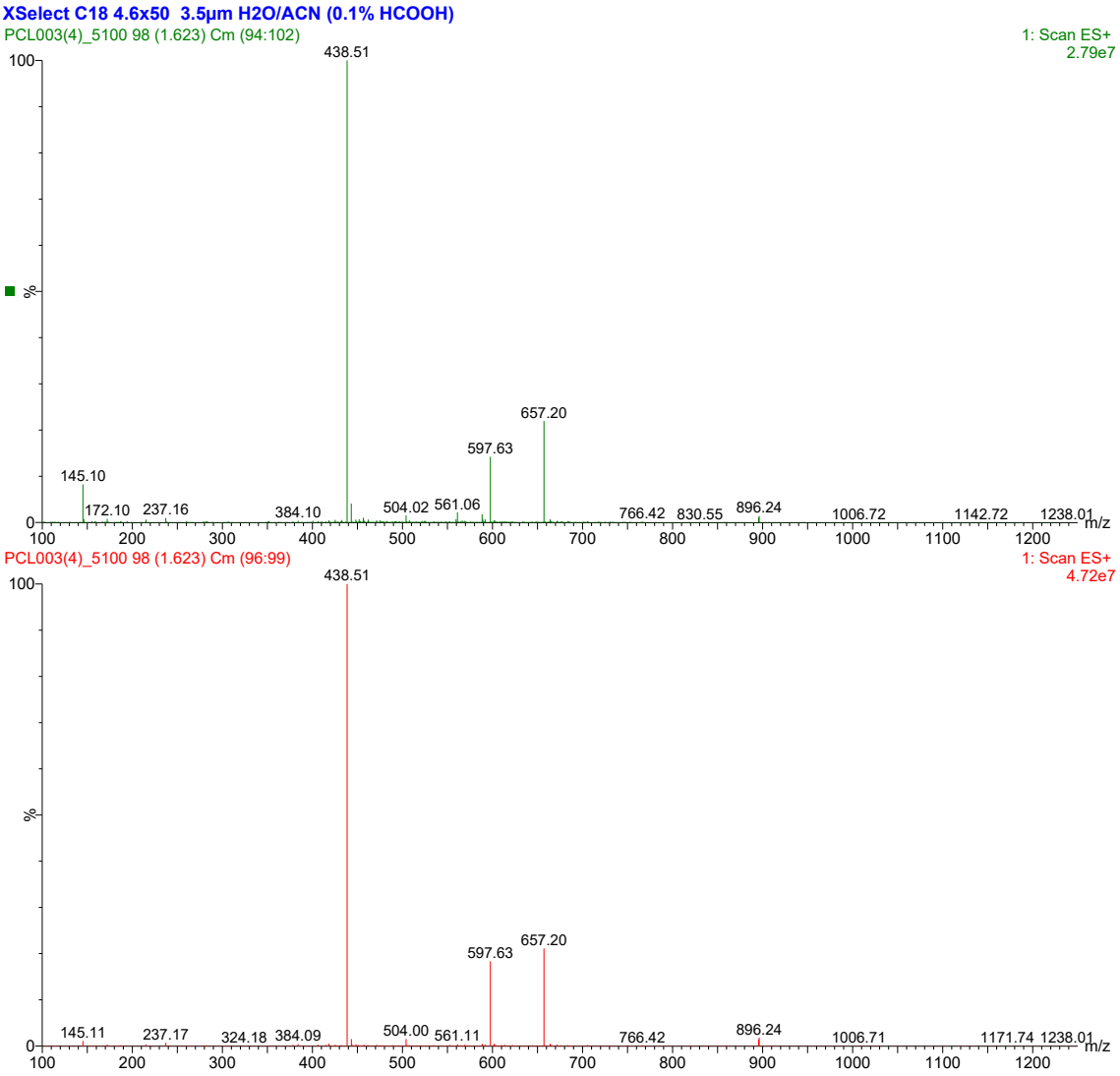
7.3.2. PCL006 PURIFICATION



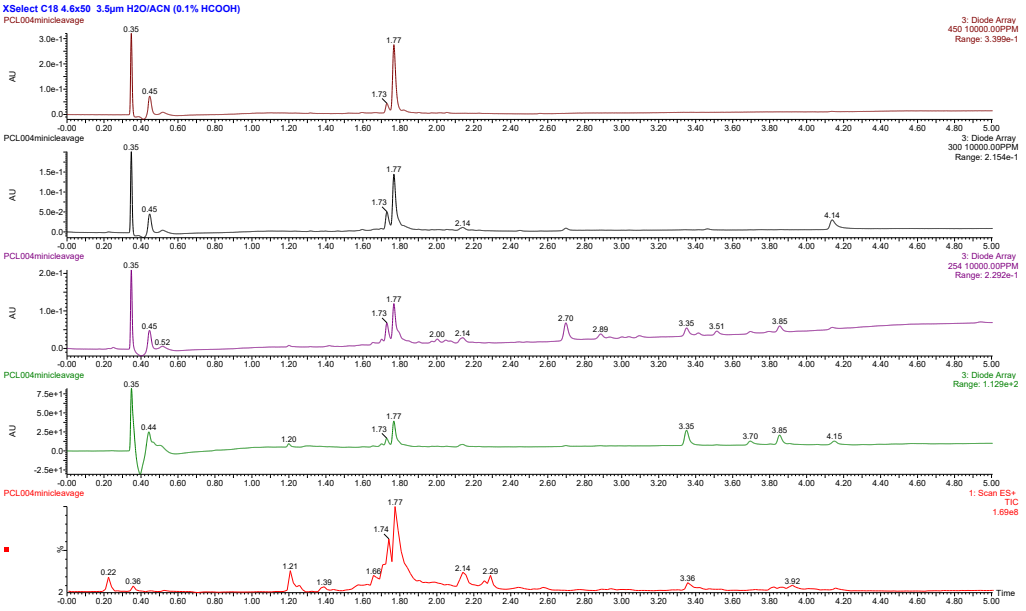
7.4. HPLC-MS SPECTRA FOR Tyr7 6-MEBA SUBSTITUTED TYROCIDINE A

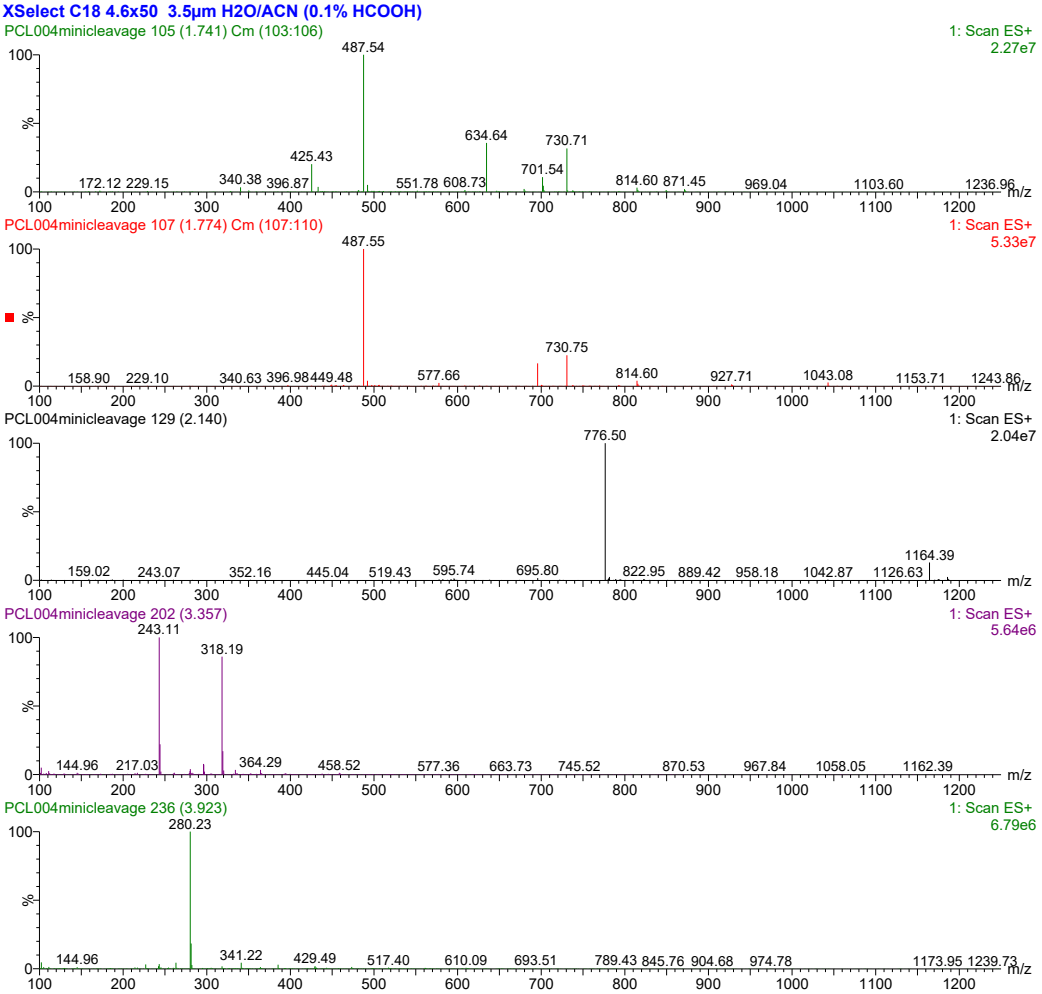
7.4.1. PCL003 INITIAL MINI CLEAVAGE



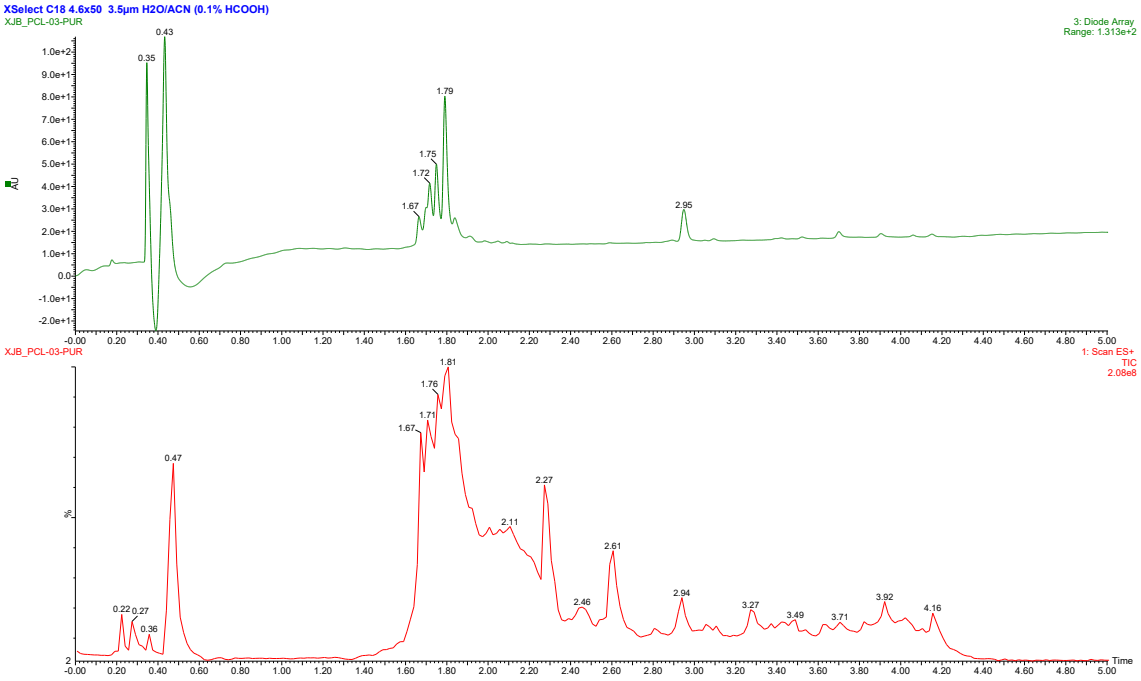


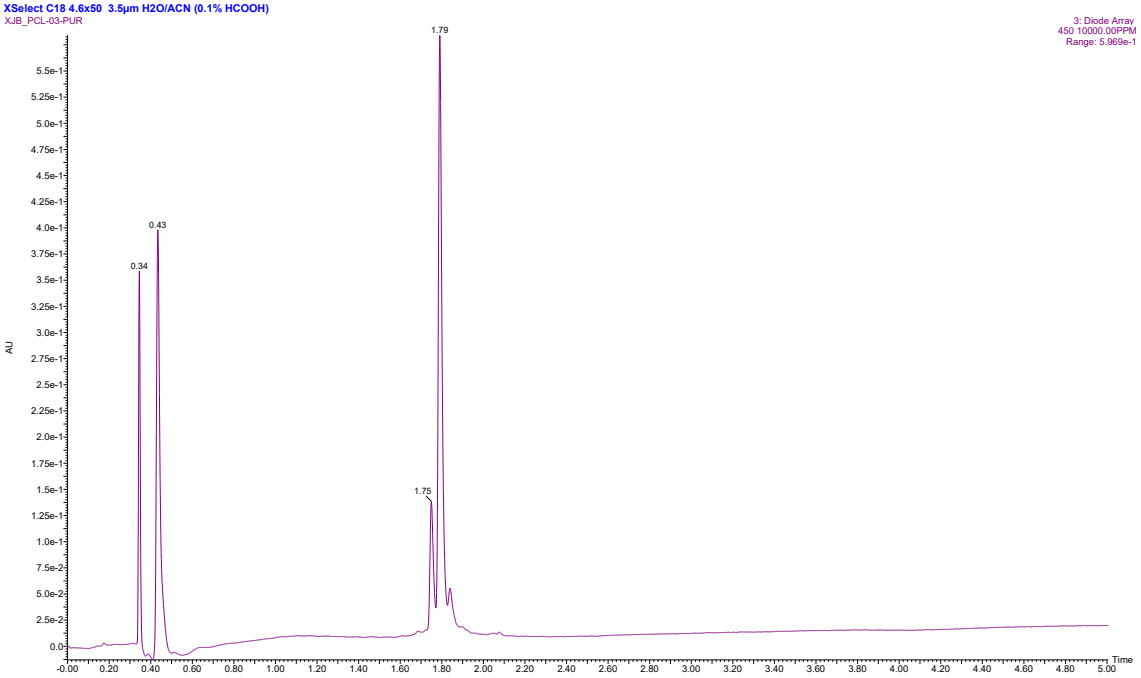
7.4.2. PCL003 LAST AMINO ACID RECOUPLED MINICLEAVAGE

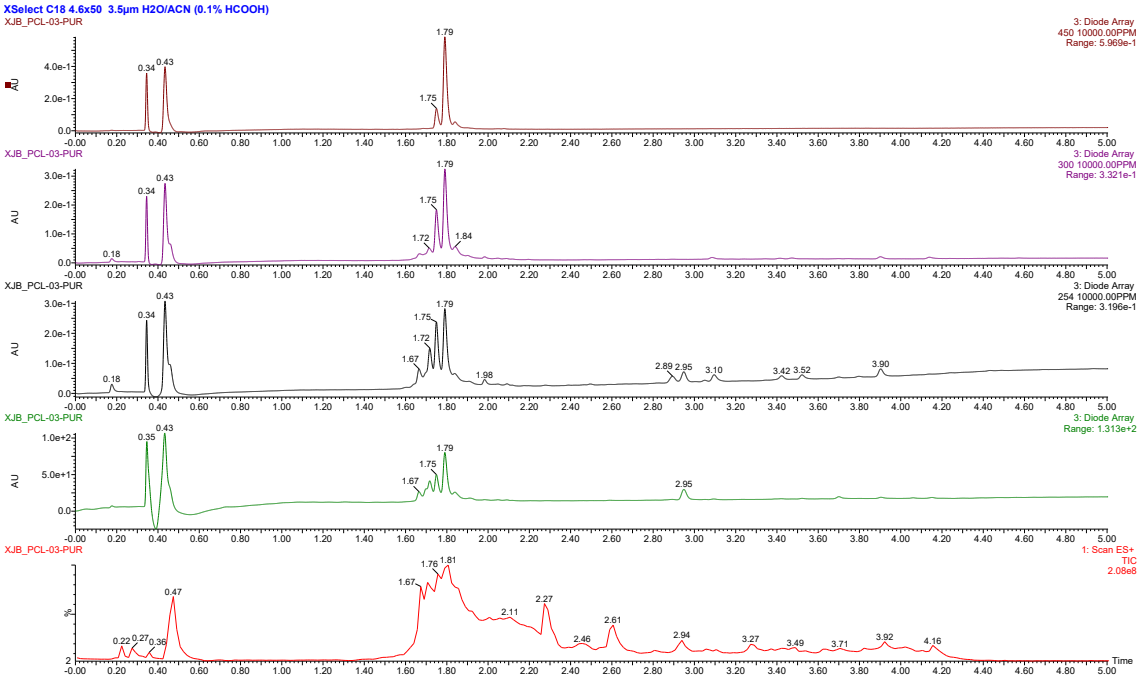


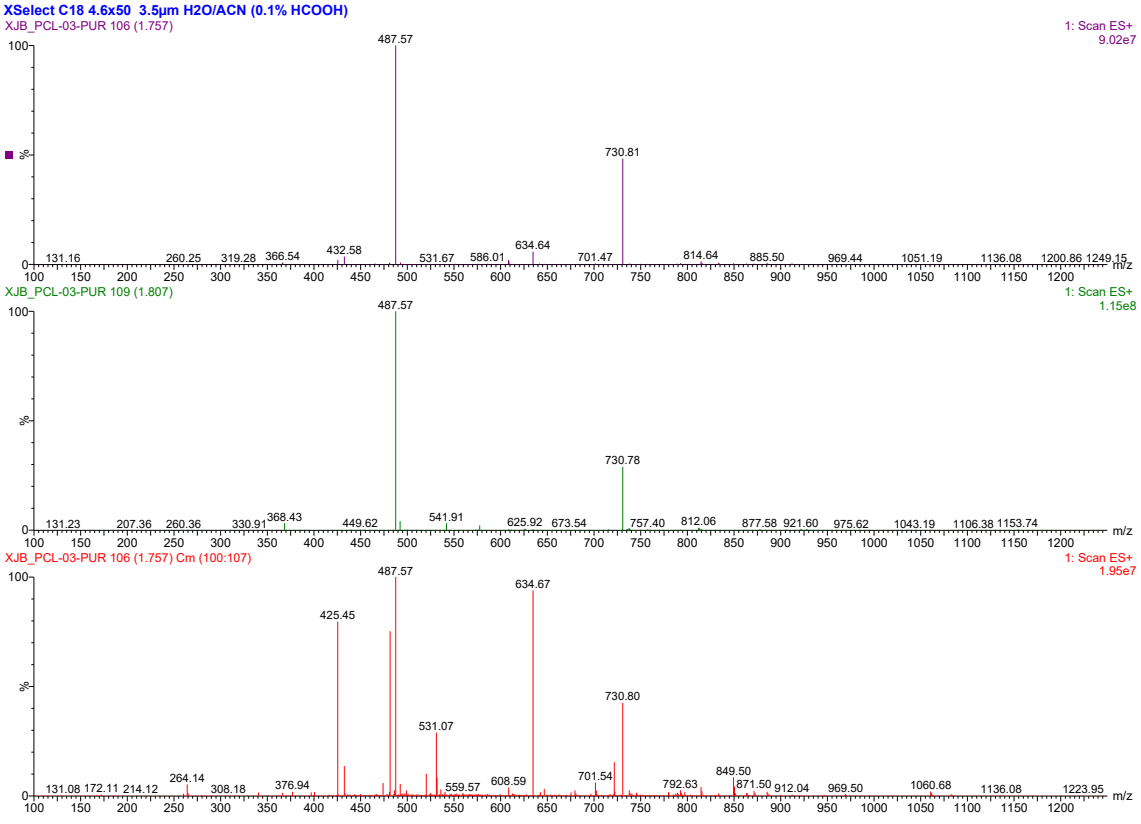


7.4.3. PCL003 PURIFICATION


XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
XJB_PCL-03-PUR
3: Diode Array
Range: 1.313e+2
AU
0.43
0.35
1.79
1.75
1.72
1.67
2.95
XJB_PCL-03-PUR
1: Scan ES+
TIC
2.08e8
%
1.81
1.78
1.71
1.67
0.47
2.27
2.11
2.61
2.46
2.94
3.27
3.49
3.71
3.92
4.16
0.22
0.27
0.36
Time


XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
XJB_PCL-03-PUR
3: Diode Array
450 10000.00FPM
Range: 5.969e-1
AU
1.79
0.43
0.34
1.75
Time


XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
XJB_PCL-03-PUR
3: Diode Array
450 10000.00PPM
Range: 5.969e-1
AU
0.34
0.43
1.75
1.79
XJB_PCL-03-PUR
3: Diode Array
300 10000.00PPM
Range: 3.321e-1
AU
0.18
0.34
0.43
1.72
1.75
1.79
1.84
XJB_PCL-03-PUR
3: Diode Array
254 10000.00PPM
Range: 3.196e-1
AU
0.18
0.34
0.43
1.67
1.72
1.75
1.79
1.98
2.89
2.95
3.10
3.42
3.52
3.90
XJB_PCL-03-PUR
3: Diode Array
Range: 1.313e+2
AU
0.35
0.43
1.67
1.75
1.79
2.95
XJB_PCL-03-PUR
1: Scan ES+
TIC
2.08e8
%
0.22
0.27
0.36
0.47
1.67
1.76
1.81
2.11
2.27
2.46
2.61
2.94
3.27
3.49
3.71
3.92
4.16
Time


XSelect C18 4.6x50 3.5µm H2O/ACN (0.1% HCOOH)
XJB_PCL-03-PUR 106 (1.757)
1: Scan ES+
9.02e7
%
487.57
730.81
131.16
260.25
319.28
366.54
432.58
531.67
586.01
634.64
701.47
814.64
885.50
969.44
1051.19
1136.08
1200.86
1249.15
m/z
XJB_PCL-03-PUR 109 (1.807)
1: Scan ES+
1.15e8
%
487.57
730.78
131.23
207.36
260.36
330.91
368.43
449.62
541.91
625.92
673.54
757.40
812.06
877.58
921.60
975.62
1043.19
1106.38
1153.74
m/z
XJB_PCL-03-PUR 106 (1.757) Cm (100:107)
1: Scan ES+
1.95e7
%
487.57
425.45
634.67
730.80
531.07
849.50
131.08
172.11
214.12
264.14
308.18
376.94
559.57
608.59
701.54
792.63
871.50
912.04
969.50
1060.68
1136.08
1223.95
m/z