

Discovery and development of new antibiotics based on the Trojan Horse strategy

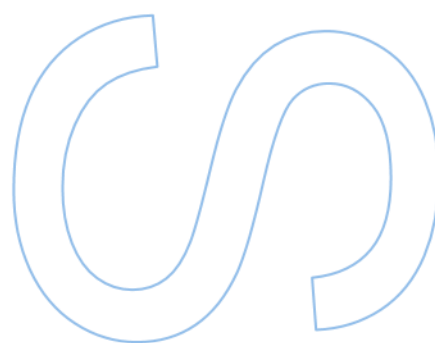
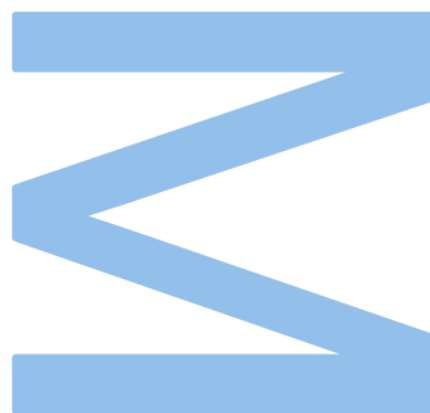
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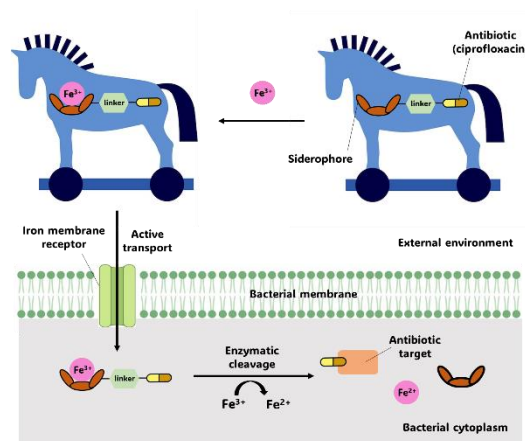
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Discovery and development of new antibiotics based on the Trojan Horse strategy

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Dedicated to my father and my sister

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Resumo

A resistência aos antimicrobianos (RAM) constitui uma crescente ameaça para a saúde pública, a nível mundial, uma vez que se tem observado um escalar do número de bactérias que desenvolvem resistência aos antibióticos existentes. Deste modo, torna-se premente a descoberta e desenvolvimento de novos antibacterianos com novos modos de ação para combater a RAM. Como tal, diversas estratégias têm sido estudadas, nomeadamente a exploração dos processos metabólicos das bactérias como potenciais alvos antibacterianos. Uma destas abordagens, denominada Estratégia do Cavalo de Troia, explora os sistemas bacterianos de absorção de ferro através da produção de sideróforos (quelantes orgânicos de ferro produzidos pelas bactérias). Esta estratégia consiste em ludibriar estes sistemas bacterianos de aquisição de ferro, através da utilização de conjugados antibiótico-sideróforo que mimetizem o ligando natural. Esta é uma abordagem promissora para incorporar o antibiótico no citoplasma bacteriano, uma vez que permite contornar alguns mecanismos de RAM, nomeadamente a reduzida permeabilidade membranar e a ação das bombas de efluxo.

Este projeto baseia-se no desenho racional e síntese de conjugados ciprofloxacina-sideróforo, nos quais miméticos de sideróforos foram ligados, diretamente ou através de espaçadores cliváveis (polietilenoglicóis e dióis), ao grupo carboxilo da ciprofloxacina. Este antibiótico foi ligado a derivados da deferiprona (miméticos das 3-hidroxipiridin-4-onas) e a um derivado do ácido kójico (mimético da 3-hidroxipirona). Estes acoplamentos foram realizados, recorrendo a reações de esterificação que, teoricamente, permitem a libertação e separação do antibiótico do agente quelante. Tentou-se, ainda que sem sucesso, sintetizar conjugados correspondentes à ciprofloxacina acoplada a derivados dos ácidos protocatecuico e cafeico (miméticos de catecóis).

Através das técnicas de ressonância magnética nuclear uni e bidimensionais (RMN de 1D e 2D) foi possível realizar uma elucidação estrutural dos compostos sintetizados. Uma pequena biblioteca de conjugados ciprofloxacina-sideróforo (6 compostos finais) foi obtida com êxito. A sua atividade quelante de ferro foi avaliada utilizando o método colorimétrico da ferrozina. Os resultados mostraram que os conjugados que contêm derivados da deferiprona apresentaram melhores propriedades quelantes de ferro do que os que possuem o ácido kójico. Por fim, foram realizados ensaios preliminares de screening à atividade antibacteriana e perfil toxicológico. Dois conjugados ciprofloxacina-mimético de sideróforo destacaram-se pela sua atividade antibacteriana, quer contra bactérias Gram-positivas quer contra as bactérias Gram-

negativas, bem como pelo sua segurança, a nível toxicológico. Alguns resultados também demonstraram que o sistema bacteriano de aquisição de ferro está envolvido no transporte e incorporação dos conjugados no citoplasma bacteriano .

Palavras-chave: Resistência aos antimicrobianos (RAM), Antibacterianos, Ciprofloxacina, Sideróforo, Conjugados antibiótico-sideróforo

Abstract

Antimicrobial resistance (AMR) is a growing worldwide health threat, as we witness a growing number of bacteria becoming resistant to the existing antibiotics. Within this emergence, discovering and developing new antibacterial drugs with novel modes of action (MoA) are crucial to tackle AMR. Bacteria metabolic processes are being explored to help in this demand, and the Trojan Horse strategy, which exploits bacteria's iron uptake pathways via the production of siderophores (organic iron chelators produced by bacteria), is an example. This strategy consists of hijacking the bacteria iron uptake machinery through the use of antibiotic-siderophore conjugates, which can mimic the cognate ligand. This is a promising strategy to deliver the antibiotic into the bacterial cytoplasm, since it allows the bypass of some AMR mechanisms, namely the reduced membrane permeability and the action of efflux pumps.

This project is based on the design and synthesis of siderophore-ciprofloxacin conjugates, in which siderophore mimetics were linked, directly or through cleavable linkers (polyethylene glycols and diols), to the carboxyl group of ciprofloxacin. This antibiotic was attached with deferiprone derivatives (3-hydroxypyridin-4-ones mimetics) and kojic acid derivative (3-hydroxypyruone mimetic). These ciprofloxacin-siderophore mimetics coupling was performed using esterification reactions, which theoretically allow the antibiotic release from the chelating agent. Attempts were also carried out to synthesize conjugates corresponding to ciprofloxacin linked to protocatechuic and caffeic acid derivatives (catechol mimetics), however they were ineffective.

Structural elucidation of the synthesized compounds was attained by one- and two- dimensional nuclear magnetic resonance (1D and 2D NMR) techniques. A small library of ciprofloxacin-siderophore conjugates (six final compounds) was successfully obtained. Their iron chelating activity was assessed using colorimetric ferrozine assay. The results showed that the conjugates containing deferiprone derivatives presented better iron chelating properties than the ones possessing kojic acid moiety. Finally, preliminary antibacterial and toxicological screening assays were performed. Two ciprofloxacin-siderophore mimetic conjugates stood out due to their antibacterial activity against both Gram-positive and Gram-negative bacteria and safety toxicity window. Some results also proved that the delivery of the conjugates into bacterial cytoplasm is carried out through siderophore-dependent iron uptake system.

Keywords: Antimicrobial Resistance (AMR), Antibacterial, Ciprofloxacin, Siderophore, Siderophore-Antibiotic Conjugates.

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List of Abbreviations

- Ab** - *Acinetobacter baumannii* bacteria
- Abs** - Absorbance
- AMR** - Antimicrobial resistance
- Boc** - tert-butyloxycarbonyl protecting group
- Boc anhydride (Boc₂O)** - Di-tert-butyl dicarbonate
- Ca** - *Candida albicans* fungi
- CDCl₃** - Deuterated chloroform
- CEMUP** - Materials Centre of the University of Porto
- CFU** - Colony forming unit
- CLSI** - Clinical & Laboratory Standards Institute
- CO-ADD** - Community for Open Antimicrobial Drug Discovery
- COSY** – ¹H-¹H Homonuclear Correlated Spectroscopy
- COVID-19** - Coronavirus disease 2019
- DCM** – Dichloromethane
- 1D/2D** - One- and two-dimensional
- DEPT** - Distortionless Enhancement by Polarization Transfer
- DFO** - Desferrioxamine
- DFX** – Deferasirox
- DIPEA** - N,N-Diisopropylethylamine
- DMAP** - 4-(Dimethylamino)pyridine
- DMEM** - Dulbecco's Modified Eagle Medium
- DMF** - Dimethylformamide
- DMSO** - Dimethyl sulfoxide-d₆
- DNA** - Deoxyribonucleic acid
- EDTA** - Ethylenediamine tetraacetic acid
- Ec** - *Escherichia coli* bacteria
- EMA** - European Medicines Agency
- EtOAc** - Ethyl acetate
- FAO** - Food and Agriculture Organization of the United Nations
- FBS** – Fetal Bovine Serum
- FDA** - U.S. Food and Drug Administration
- Ferrozine** - 3-(2-Pyridyl)-5,6-bis(4-sulfophenyl)-1,2,4-triazine disodium salt)
- HBTU** - Hexafluorophosphate Benzotriazole Tetramethyl Uronium

H_k – Cytotoxicity on human embryonic kidney cells

H_m - Haemolytic Activity on human red blood cells

HMBC - ¹H-¹³C Heteronuclear Multiple Bond Correlation

HSQC - ¹H-¹³C Heteronuclear Simple Quantum Correlation

HTS - High-throughput screening

ID – Iron-depleted media

IM – Inner membrane

Kp - *Klebsiella pneumoniae* bacteria

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy

log β - Cumulative affinity constants for iron

MBC - Minimum bactericidal concentration

MDR bacteria - Multidrug-resistant bacteria

MECAM - 1,3,5-N,N',N''-tris-(2,3-dihydroxybenzoyl)-triaminomethylbenzene

MeOD - Deuterated methanol

MeOH – Methanol

MHB – Mueller-Hinton Broth media

MIC - Minimum inhibitory concentration

MoA - Mode of action

mRNA - Messenger ribonucleic acid

MRSA - Methicillin-resistant *Staphylococcus aureus*

MW – Molecular weight or microwave

NMR - Nuclear magnetic resonance

¹³C NMR – Carbon-13 nuclear magnetic resonance

¹H NMR - Proton nuclear magnetic resonance

OD – Optical density

OD405 – Absorbance at 405nm

OD600 - Absorbance at 600 nm

OMP – Outer membrane porins

OMR – Outer membrane receptors

p.a – Pro analysis

Pa - *Pseudomonas aeruginosa* bacteria

PBP - Penicillin binding proteins

PCA - Plate count agar

Pd/C - Palladium on carbon

PEG – Polyethylene glycols

PTSA - p-Toluenesulfonic acid

RNA - Ribonucleic acid
RT – Room temperature
R&D - Research and development
Sa - *Staphylococcus aureus* bacteria
SAC – Siderophore-Antibiotic conjugate
SAR – Structure-Antibacterial Activity Relationship
TEA - Triethylamine
TFA - Trifluoroacetic acid
THF - Tetrahydrofuran
TLC – Thin layer chromatography
TMS - Tetramethylsilane
tRNA - Transfer ribonucleic acid
UNEP - United Nations Environment Programme
VRE - Vancomycin-resistant *E. faecium*
VRSA - Vancomycin-resistant *S. aureus* isolates
WHO - World Health Organization
WOAH - World Organization for Animal Health
XDR – Extremely resistant bacteria

Symbols and physical quantities

J – Coupling constant

δ – Chemical shift

Da – Dalton

d – Doublet

dd - Doublet of doublets

Hz – Hertz

MHz - MegaHertz

μL – Microlitre

μg – Microgram

mg - Milligram

mL – Millilitre

mm – Millimetre

mM - Millimolar (mmol/L)

m – Multiplet

nm – Nanometre

ppm - Parts per million

η – Reaction Yield

R_f – Retention factor

s – Singlet

t - Triplet

Chapter 1 – Introduction

1.1. Antibiotics

1.1.1. Overview

Antibiotics are therapeutic molecules used to prevent and treat bacterial infectious diseases, and consequently, diminish the mortality and morbidity rates and improve life expectancy and quality of life of people and populations. They act by perturbing vital biochemical processes of bacteria, which leads to the inhibition of bacterial cell growth or even bacterial cell apoptosis in the presence of bacteriostatic or bactericidal agents, respectively [1].

Paul Ehrlich, a pioneer in the discovery of new antimicrobials, started his work in this field by exploring the therapeutic properties of many dyes. In 1910, Ehrlich discovered the first antibiotic – Salvarsan - an organic arsenic compound used in the treatment of syphilis disease. Gerhard Domagk, following the same research line as Paul Ehrlich, discovered the first sulfonamide – Prontosil - an azo dye that displayed antibacterial activity against a wide range of bacterial strains. In 1928, Alexander Fleming discovered penicillin by serendipity, noticing that a mould was able to inhibit *Staphylococcus* bacteria growth in a Petri (Figure 1) [1-4].

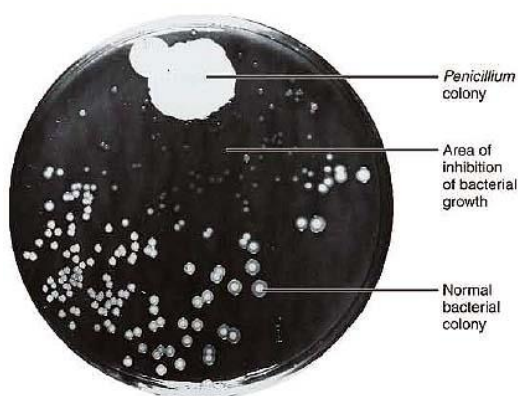


Figure 1 - Photograph of the Petri dish showing the inhibition of *Staphylococcus* colonies in the neighbourhood of mold (penicillium) colony [4]

At the beginning of the 20th century, it was found that microbes produced stable compounds with antibacterial properties. For example, pyocyanase, a natural extract of *Pseudomonas aeruginosa* bacteria, displayed antibacterial activity against a wide range of bacterial strains. In the late 1930s, Selman Waksman studied multiple secondary metabolites secreted by bacteria and fungi that exhibited antibacterial properties [1, 2, 5].

Waksman's work was instrumental in propelling the golden era of antibiotics from the 1930s to the 1960s. During this period, multiple classes of antibiotics were discovered by screening natural products produced by microorganisms. Subsequently, chemical modifications were made to these natural antibiotic structures to widen their antimicrobial range, increase their effectiveness, and prevent the development of microbial resistance [1, 2, 5].

The majority of the current antibiotics are derived from natural products and generally target bacteria cell wall, genetic material (DNA or RNA – Deoxyribonucleic and Ribonucleic Acids), essential enzymes, and ribosomes – Figure 2 [1, 6].

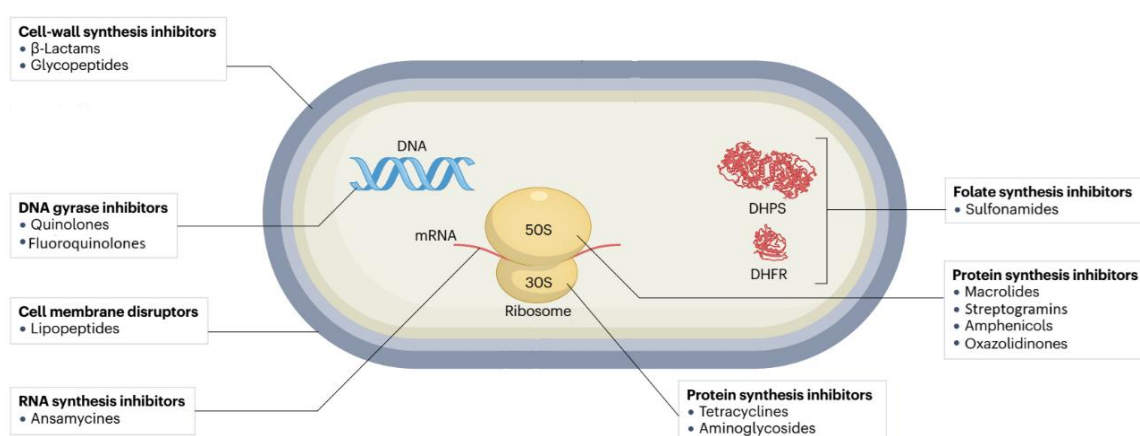


Figure 2 - Overview of the mechanisms of action of known antibiotics. Adapted from [6]

Antibiotics can be classified according to their molecular structures, mode of action and spectrum of activity. Based on their molecular structure, the antibiotics can be divided into classes, being the most common ones: β-lactams, glycopeptides, lipopeptides, aminoglycosides, tetracyclines, chloramphenicols, macrolides, oxazolidinones, streptogramins, ansamycins, quinolones/fluoroquinolones and sulfonamides, outlined in Table 1.

According with their mode of action, most of antibiotics classes follow into 5 main groups: inhibition of cell wall synthesis, depolarization of cell membrane, inhibition of protein synthesis, inhibition of nucleic acid synthesis and inhibition of bacteria's metabolic processes – Table 2 [1, 6-9].

Table 1 - Chemical structures of the most common classes of antibiotics

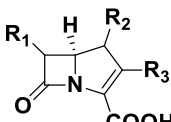
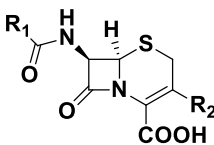
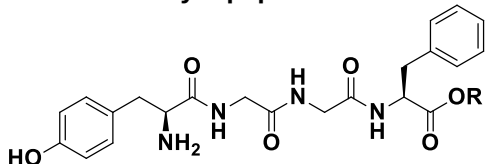
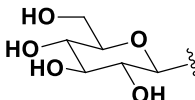
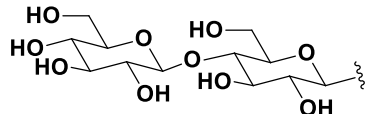
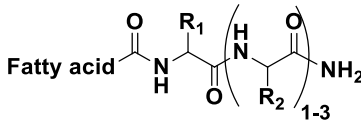
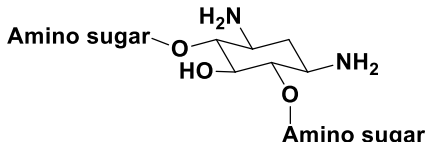
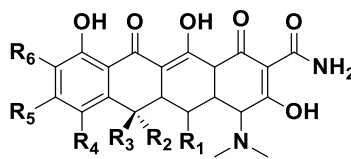
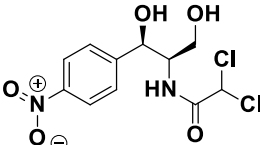
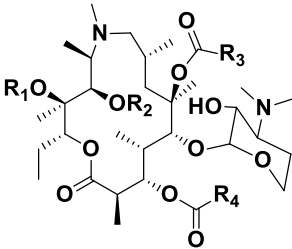
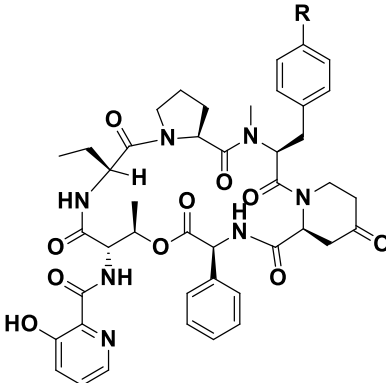
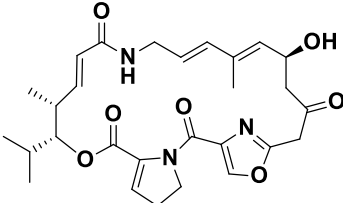
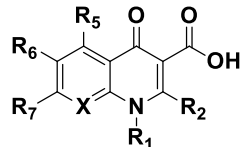
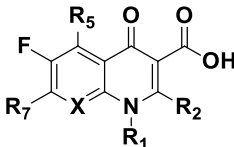
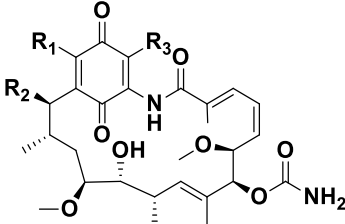
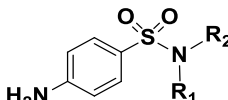
β-Lactams		Glycopeptides	
 Carbapenems	 Cephalosporins	 R =  	
Lipopeptides		Aminoglycosides	
 Fatty acid		 Amino sugar Amino sugar	
Tetracyclines	Chloramphenicols	Macrolides	
			
Oxazolidinones	Streptogramins		
	 Group A streptogramins  Group B streptogramins		
Quinolones	Fluoroquinolones	Ansamycins	Sulfonamides
 X = N or CH	 X = N or CH		

Table 2 - The main classes of clinically prescribed antibiotics.

Antibacterial Groups		Target	Mode of action	Examples
β-Lactams	Carbapenems	Inhibition of bacterial cell wall biosynthesis	Bactericidal (covalent modification of penicillin binding proteins (PBPs))	Meropenem
	Cephalosporins			Ceftriaxone, cefepime, ceftaroline, ceftobiprole
	Monobactams			Aztreonam
	Penicillins			Piperacillin, ampicillin, amoxicillin
Glycopeptides			Bactericidal (inhibits peptidoglycan synthesis)	Vancomycin Teicoplanin
Lipopeptides		Depolarization of Cell Membrane	Bactericidal	Daptomycin
Aminoglycosides		Inhibition of protein synthesis	Bactericidal (Irreversible bind to 30S Ribosomal Subunit)	Spectinomycin Kanamycin Neomycin
Tetracyclines			Bacteriostatic (Reversible bind to 30S Ribosomal Subunit)	Tetracycline, Doxycycline
Chloramphenicols			Bacteriostatic (Bind to 50S Ribosomal subunit – inhibits the action of peptidyl transferase)	Chloramphenicol
Macrolides			Bacteriostatic (Bind to 50S Ribosomal subunit- bind at the nascent peptide exit tunnel and partially occlude it)	Erythromycin Clarithromycin
Oxazolidinones			Bacteriostatic (Bind to 50S Ribosomal subunit at the P site)	Linezolid

Streptogramins		Bactericidal (Bind to 50S Ribosomal subunit -inhibition of aminoacyl tRNA ribosomal incorporation and mRNA translation.	Pristinamycin, quinupristin/dalfopristin
Ansamycins	Inhibition of Nucleic Acid Synthesis	Bactericidal (Inhibition of RNA synthesis)	Rifamycin
Quinolones and Fluoroquinolones		Bactericidal (Inhibition of DNA synthesis)	Ciprofloxacin
Sulfonamides	Inhibition of Metabolic Pathways	Bacteriostatic (inhibition of folate synthesis)	Sulfanilamide

1.2. Antimicrobial Resistance (AMR)

1.2.1. Overview

Antimicrobial resistance (AMR) refers to the ability of microorganisms to survive or multiply in the presence of antimicrobial drugs [10, 11]. This resistance occurs as the result of natural selection, following Darwin's principles. However, the overuse and misuse of antibiotics have worsened the emergence of bacterial strains resistant to several classes of antibiotics [8, 10-17].

AMR presents a serious worldwide health challenge as it weakens the ability to treat common infections, leading to prolonged sickness and an increased risk of disease transmission and complications. In 2019, the World Health Organization (WHO) declared AMR one of the top 10 threats to Public Health. This red flag gained further attention in July 2022 when the European Commission elevated AMR to the top three Health threats [8, 10-17]. In 2024, WHO updated its "Bacterial Priority Pathogens List.", which serves as a guide for research and development (R&D) in the field. In this list bacteria are categorized into three groups— critical, high, and medium —based on their level of resistance and the severity or burden of the infections they cause, representing priorities for R&D and health measures – Table 3 [18].

Table 3 - WHO Bacterial Priority Pathogens List [18]

Critical group	High group	Medium Group
✓ <i>Acinetobacter baumannii</i>; ✓ <i>Mycobacterium tuberculosis</i>; ✓ Enterobacterales pathogens.	✓ <i>Salmonella Typhi</i>; ✓ <i>Shigella spp.</i>; ✓ <i>Enterococcus faecium</i>; ✓ <i>Pseudomonas aeruginosa</i>; ✓ <i>Neisseria gonorrhoeae</i>; ✓ <i>Staphylococcus aureus</i>.	✓ Groups A and B Streptococci; ✓ <i>Streptococcus pneumoniae</i>; ✓ <i>Haemophilus influenzae</i>.

A global assessment conducted on the burden of bacterial AMR for 2019, published in 2022, revealed that 4.95 million deaths worldwide were associated with bacterial AMR. Furthermore, within this total, 1.27 million deaths were directly linked to drug resistance. This data underscores the urgent need for wide-ranging strategies to address and mitigate the impact of bacterial AMR on a global scale. It is predicted that, by 2050, AMR will lead to 10 million deaths (Figure 3) and thus become the major cause of mortality worldwide if no action is taken [6, 10, 11, 13, 16-20].

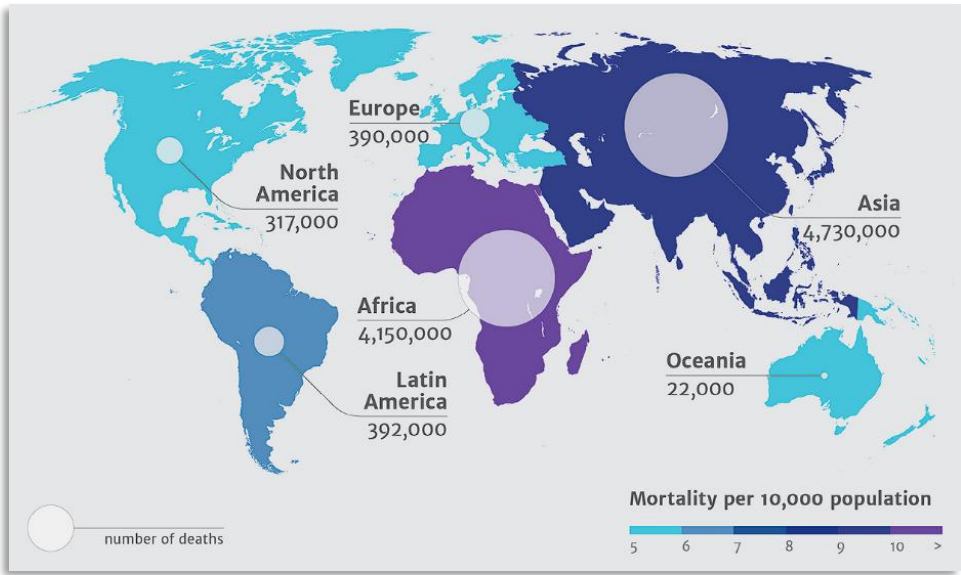


Figure 3 - Global distribution of deaths attributable to antimicrobial resistance by 2050 [17]

Along with the inherent difficulties of treating bacterial infections, bacterial AMR also compromises medical advances, such as organ transplants and cancer treatments. Additionally, this problem has a huge financial impact on the worldwide investment in healthcare systems [8, 10-17].

The imminent death toll related to AMR is even more alarming with the appearance of multidrug-resistant (MDR), extremely (XDR) and pan-resistant bacteria, which cause infections that are difficult to treat and for which it is observed a critical scarcity of effective antibiotics. This scenario is so alarming that some specialists claim that the rise of resistant bacteria may indicate the imminent end of the antibiotic era [8, 10-16, 18]. The Coronavirus disease 2019 (COVID-19) pandemic also worsened and contributed to the escalation of bacterial AMR, due to the misuse and over-prescription of antibiotics. A study in the United States reported that 72% of COVID-19 patients were prescribed antibiotics, even though it's already known that they are ineffective in the treatment of viral infections [21, 22].

Despite all that was mentioned above, the current research and development (R&D) of new antibiotics is scarce and not keeping pace with the growing of resistant bacteria strains, particularly for the treatment of Gram-negative bacteria [8, 11-14, 23]. One of the reasons for this asynchrony is the pharmaceutical industry's progressive disinvestment in this research field. Low financial return, rapid resistance rise, and extensive regulatory requirements contribute to the decline in pharmaceutical industry investment in new antibiotics [6, 8, 11, 14-20, 24].

1.2.2. Mechanisms of Antibacterial Resistance

Multiple strains of bacteria have developed resistance to several antibiotics by exchanging mobile genetic elements such as plasmids (circular DNA capable of moving between cells) and transposons (small DNA segments that can move between chromosomes and plasmids) through horizontal transfer processes, namely transformation, transposition, and conjugation – Figure 4 [16, 25]. Microorganisms also acquire resistance through the vertical transfer of resistant genes, which consists of transmitting genes from a bacterial microorganism to its offspring, at the stage of binary division during reproduction [16, 25].

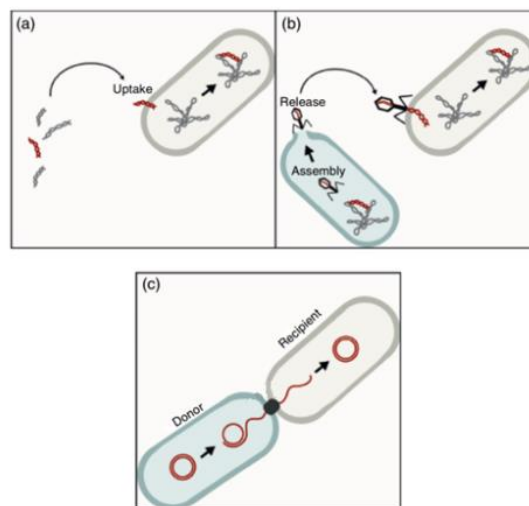


Figure 4 - Mechanisms of Horizontal Gene Transfer: (a) Transformation- uptake of free mobile genetic elements from its surrounding environment; (b) Transduction – Viral genetic material transfer via bacteriophages; (c) Conjugation – Acquisition of mobile genetic elements accomplished by direct cell-to-cell contact (intra- or interspecies transfer. Adapted from [16]

These mechanisms involve target site modification, drug inactivation and reduced intracellular accumulation owing to reduced membrane permeability and active efflux of a drug – Figure 5 [5, 7, 25, 26].

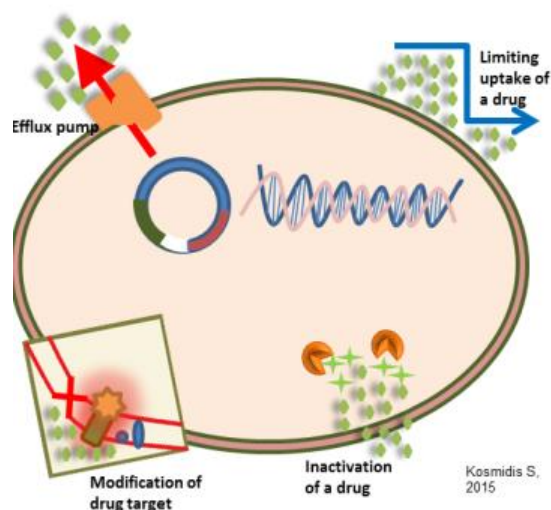


Figure 5 - Representative scheme of antimicrobial resistance mechanisms [7]

1.3. Strategies for Antibiotic Drug Discovery

It is currently recognized that the bacterial AMR problem requires a multifaceted approach based on the “One Health” concept, involving human, animal, and environmental health. Many of the antibiotics used in human medicine are also used in veterinary medicine and livestock production, and some clinically relevant resistant bacteria and/or their resistance genes can be transferred between animals and humans through the environment. Accordingly, several international organizations, namely WHO, FAO (Food and Agriculture Organization of the United Nations), UNEP (United Nations Environment Programme), and WOAH (World Organization for Animal Health), have signed a groundbreaking agreement which includes carrying on and reinforces the global action plan on AMR, launched by WHO in 2015 [27, 28].

To tackle this challenge, it is necessary to strengthen infection prevention and control measures, improve sanitation and hygiene practices, communicate risks to the public, enhance access to clean water, prioritize vaccinations to prevent infections and reduce the use of antibiotics. Moreover, it is essential to minimize antibiotics inappropriate use in agriculture, livestock, poultry, and aquaculture, avoid overprescription, establish responsible use guidelines, develop rapid diagnostic tests, invest in research for new antibacterial agents, strengthen international collaborations,

and ensure global access to second-line antibiotics, particularly in developing regions [7, 9, 10, 14, 16, 17, 21, 22, 27-29].

Among the main driving elements to tackle bacterial AMR is the urgent need for new antibacterial agents with innovative chemistry and modes of action (MoAs). The ability of innovative antibacterial agents to circumvent multidrug-resistance mechanisms may be directly correlated with a novel mode of action, which led to the exploitation and validation of new targets for the discovery and development of innovative solutions against resistant bacteria [30].

New strategies are being developed that offer a glimmer of hope on the fight against antibacterial resistance. These strategies, focusing on independent activity and innovative approaches, such as direct-acting agents, new chemical classes, new targets, and new modes of action, hold immense potential in the fight against antibiotic resistance [31]. Among these strategies, it is possible to emphasize the identification of new hit and lead compounds by designing 'biased' chemical libraries for antibacterial screening [32]. Understanding what makes a compound "antibacterial" is challenging, as the traditional guidelines for drug development may not apply well to antibiotics [33]. To overcome this, research efforts focus on creating diverse chemical compound libraries in the pursuit of novelty in the antibacterial field. These libraries include natural products sourced from diverse environments, fragment libraries for building tailored solutions, DNA-encoded libraries, and other combinatorial strategies to leverage chemical diversity [34].

Additionally, there is a growing interest in repurposing existing drugs used for other conditions as potential novel antibacterial compounds. It is often assumed that approved drugs can be repurposed quickly without extensive optimization. As an example, we can refer to statins, commonly used to lower cholesterol, which have been reported to have antibacterial activity. However, achieving therapeutic value as antibiotics with repurposed drugs may be challenging due to relatively low doses used for other health conditions compared to antibiotics [35].

Modifying an existing antibiotic by tailoring its chemical structure to circumvent the bacterial resistance mechanisms is another common strategy to find new antibacterial drugs. In 2018, Omadacycline was approved by FDA (US Food and Drug Administration) to treat community-acquired bacterial pneumonia and acute bacterial skin infections. This antibiotic is a semisynthetic tetracycline derivative, that was chemically tailored to overcome tetracycline-specific efflux pumps and ribosomal protection [36]. Another example is Cefiderocol, a cephalosporin-derived antibiotic used to treat carbapenem-resistant *Enterobacterales* and drug-resistant non-fermenting gram-positive bacterial pathogens. Approved by FDA in 2019, this new antibiotic was

synthesized by conjugating a catechol-type siderophore to the typical cephalosporin core [37]. Vancomycin is a widely used antibiotic for treating infections caused by *E. faecium* or methicillin-resistant *S. aureus* (MRSA). However, vancomycin-resistant strains have become quite common. To address this issue, the Boger laboratory at the Scripps Research Institute developed a series of vancomycin synthetic analogue molecules, also known as maxamycins, which exhibited significant bactericidal activities against vancomycin-resistant *E. faecium* (VRE) and *S. aureus* (VRSA) isolates [38]. This breakthrough work is a step toward developing next-generation glycopeptide antibiotics with improved effectiveness against vancomycin-resistant bacteria [39].

1.4. Trojan Horse Strategy

Currently, bacteria's biological pathways are being exploited for R&D of novel antibiotics. Among them, the bacterial iron acquisition system is been pursuit as a new avenue to circumvent the antibacterial resistance due to limitations on drug's uptake. For their iron supply, bacteria produce small molecules, denominated siderophores, that bind to iron and are later incorporated into bacterial cells. The Trojan Horse strategy involves hijacking the iron machinery of bacteria by conjugating natural siderophore molecules, or their mimetics, to an antibiotic, tricking the bacteria into recognizing the conjugate as a cognate siderophore, promoting antibiotic entrance into the bacterial cell – Figure 6 [6, 11-13, 15, 23, 26, 40-46].

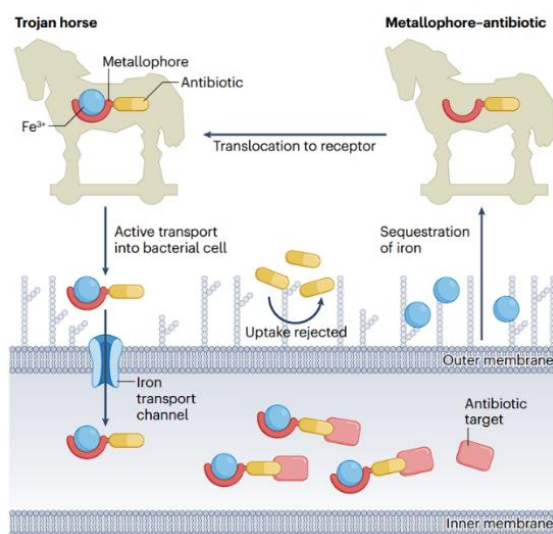


Figure 6 - Schematic of the Trojan horse strategy. The siderophore-antibiotic conjugate sequesters iron from its surrounding environment and is actively transported into the bacterial cell where the antibiotic moiety of the conjugate can reach and interact on its target [6].

Briefly, siderophores are small molecules (200-2000 Da) with a high affinity for iron (Fe(III)), expelled by bacteria and fungi under iron-depleted conditions to chelate Fe(III) ions from the surrounding environment [11, 42-45]. The resulting siderophore-Fe(III) conjugates are later recognised by bacterial-specific outer membrane receptors (OMR) and then incorporated into bacterial cytoplasm by active transport [6, 13, 41-46].

A variety of natural siderophores have already been identified, and all of them possess at least one of five of the following key iron-binding motifs: phenolate, catecholate, hydroxamate, carboxylate and α -hydroxycarboxylate, whose structures are depicted in Figure 7 [11, 24, 44, 45].

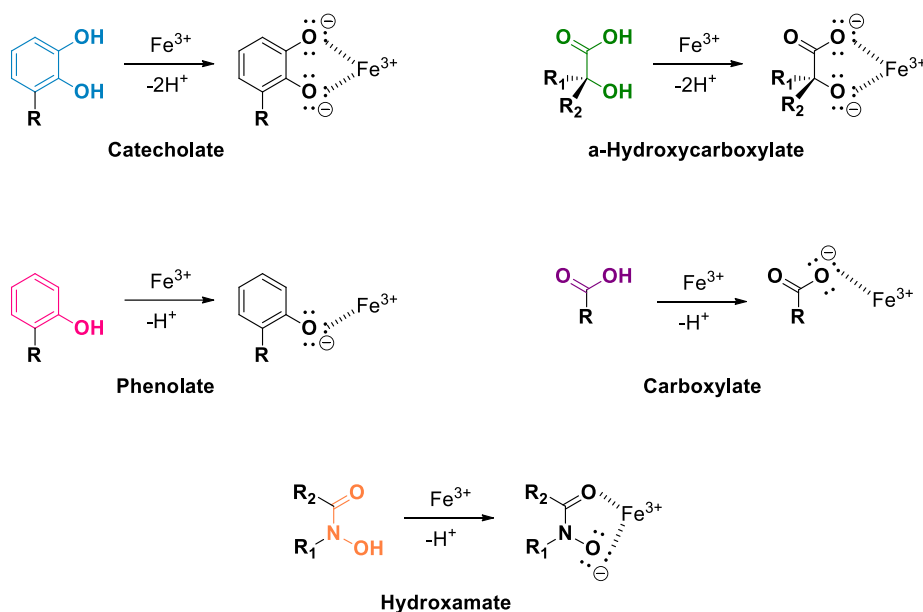


Figure 7 - Five iron binding motifs found in natural siderophores

The Trojan horse strategy concept is based on natural Siderophore-Antibiotic Conjugates (SACs) that are incorporated in bacterial cells through siderophore-iron uptake systems [13, 23, 24]. The SACs found in nature are produced by various Gram-positive and Gram-negative bacterial strains, being the most studied ones albomycin and salmycin— Figure 8 [11, 13, 42-49].

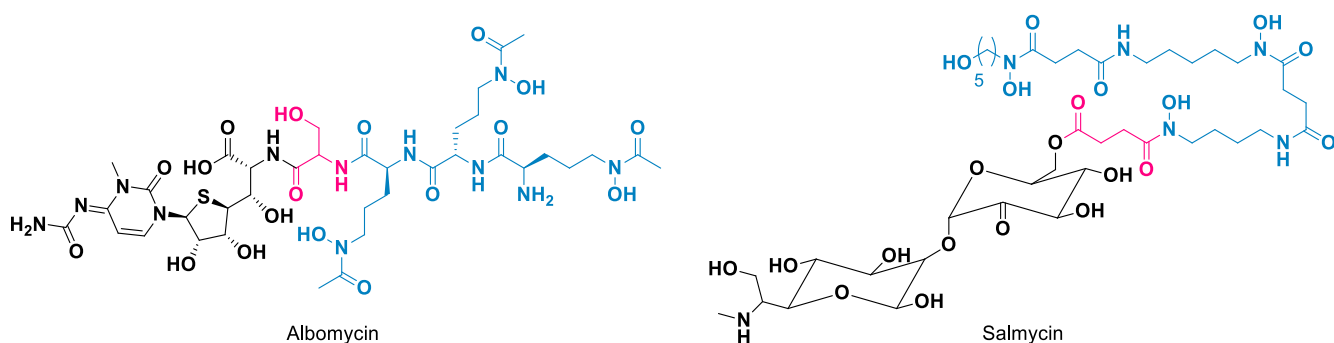


Figure 8 - Chemical structures of albomycin (left side) and salmycin (right side). Siderophore motif highlighted in blue, antibiotic component in black, linker in pink.

SACs usually are composed by three components: siderophore or its mimetic system (iron chelating moiety), an antibiotic and a linker, which connects these two components - Figure 9 [24, 46, 47, 50].



Figure 9 - Typical structure of siderophore-antibiotic conjugate

Over the years, many SACs, using as starting points several classes of antibiotics, such as β -Lactams, lipopeptides and fluoroquinolones, were synthesized and studied as potential antibiotics. Until nowadays, the most successful ones had, in their structure, antibiotics with periplasmic targets, namely β -Lactams and lipopeptides [6, 11]. Up to now the only SAC approved in therapy as an antibacterial compound is Cefiderocol (Fetroja^(R)) – Figure 10 - approved in 2019 by the FDA and EMA (European Medicines Agency) for the treatment of complex urinary tract infections. Cefiderocol contains a cephalosporin (a β -Lactam antibiotic) linked to a synthetic chlorine-substituted catecholate siderophore (Figure 10). This drug acts by inhibiting cell wall synthesis of Gram-negative bacteria, with the particularity of reaching periplasm and being relatively stable to β -lactamases bacterial enzymes action. It revealed activity against multiple carbapenem-resistant Gram-negative bacteria strains, namely *Enterobacteriaceae* (including *E. coli*, *Klebsiella spp.*, *Enterobacter spp.*, and *Serratia spp.*), *P. aeruginosa*, *A. baumannii*, *B. cepacia* and *S. maltophilia* [6, 11, 41, 44, 45, 51].

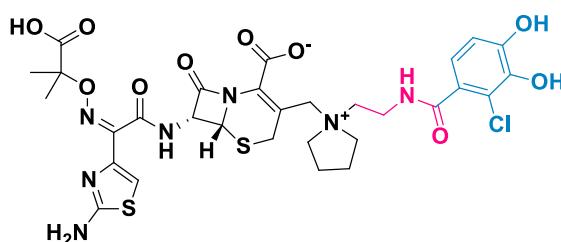


Figure 10 - Chemical structure of Cefiderocol. Siderophore motif in blue, antibiotic component in black, linker in pink

In 2021, Miller and developed some SACs, coupling MECAM siderophore (1,3,5-N,N',N''-tris-(2,3-dihydroxybenzoyl)-triaminomethylbenzene), synthetic derivative of enterobactin – highlighted in blue in Figure 11 - to ampicillin, amoxicillin (both penicillin β -lactam antibiotics) and daptomycin (lipopeptide antibiotic), using alkyl and polyethylene -(PEG)₄ - groups as linkers. The resulting ampicillin (Figures 11A and 11C) and amoxicillin (Figure 11B) conjugates showed 12-24 fold lower MICs against *E.coli* than the respective parent antibiotics. These results show that, despite the large size of SACs

(MW > 2400 Da), they can be effectively transported through specific iron bacterial membrane receptors. Furthermore, the attachment of MECAM to daptomycin (Figure 11D) allowed the conjugate to be active not only against Gram-positive pathogen but also against Gram-negative bacteria *A.baumannii*. This evidence can be extremely useful since, nowadays, it isn't known any resistance mechanisms of Gram-negative bacteria to daptomycin [11, 52].

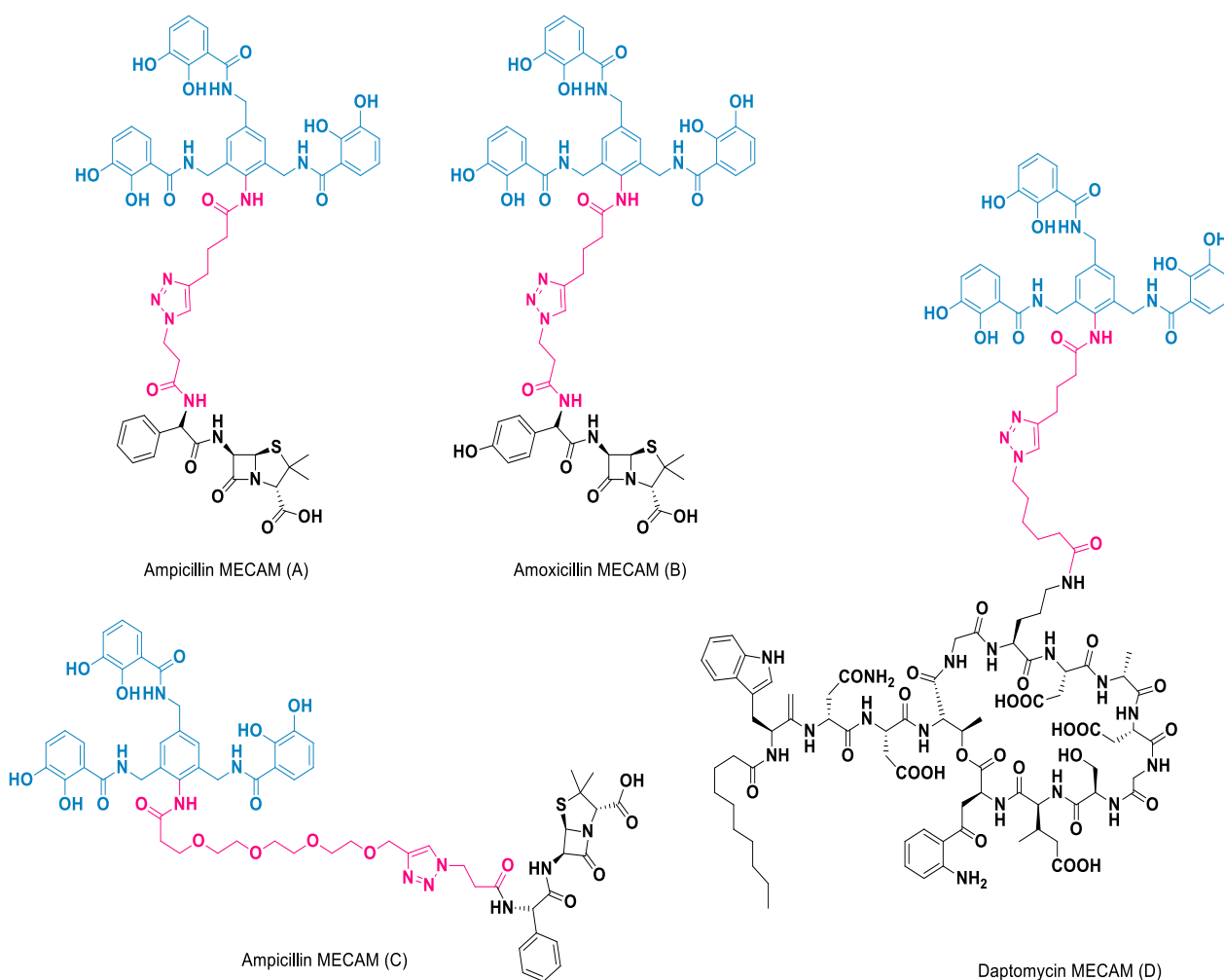


Figure 11 - SACs utilising the synthetic enterobactin derivatives - MECAM. Siderophore motif highlighted in blue, antibiotic component in black, linker in pink.

Pfizer produced a set of SACs, using as an antibiotic a lactivicin analogue (black moiety of Figures 12A, 12B and 12C), a non- β -lactam PBP (penicillin-binding proteins) inhibitor, that shows variable activity against Gram-negative bacteria and in vivo efficacy. To enhance its antibacterial properties, dihydroxypyridone (Figure 12, highlighted in blue) was connected to the oxime (Figure 11A) and α -lactone (Figure 11B) moieties of the lactivicin analogue. The α -lactone moiety of lactivicin analogue was also linked to a phthalimide-catechol siderophore (Figure 14C), resulting in the most potent antimicrobial conjugate tested in this work [11, 53].

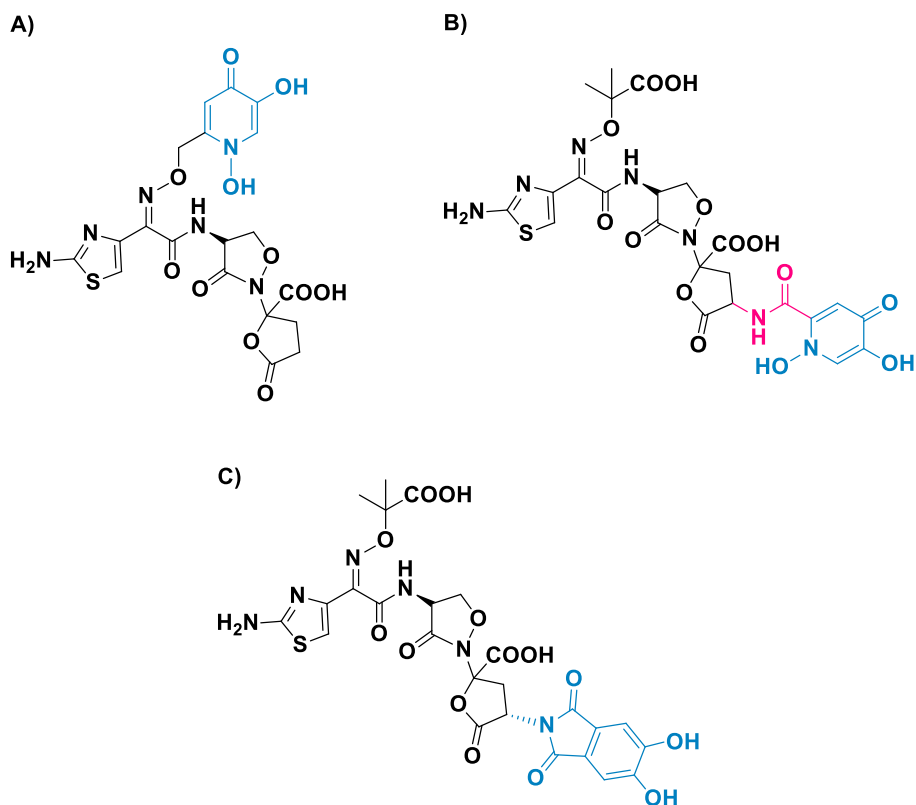


Figure 12 - Lactvicin-based SACs produced by Pfizer. Siderophore motif in blue, antibiotic moiety in black, linker in pink.

He and co-workers reported the repurposing of methotrexate, a chemotherapy agent that also presents potent antibacterial activity due to dihydrofolate reductase inhibition [11, 54]. A set of SACs were synthesized linking methotrexate to tri-hydroxamate siderophore, using multiple types of linkers namely alkyl, glycol and polyglutamate groups with different lengths. It was noticed that the most potent SAC was obtained when employing the polyglutamate group as a linker – Figure 13. This conjugate revealed strong antimicrobial properties against *Streptococcus pneumoniae* Gram-positive bacteria and Gram-negative *Yersinia enterocolitica* in iron depleted conditions. It was also seen that this conjugate presents > 2000-fold less cytotoxicity to human cells than the parent drug [11, 54].

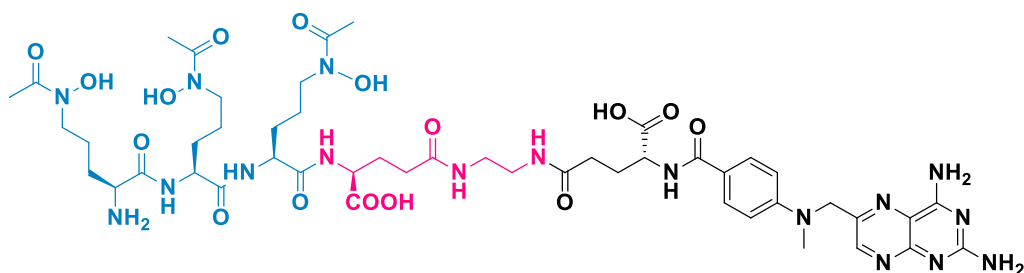


Figure 13 - Methotrexate-tri-hydroxamate SAC with non-cleavable linker. Siderophore motif highlighted in blue, linker in pink and antibiotic payload in black

In 2022, Nolan and co-workers reported a potent SAC exploring cisplatin, an anticancer drug that exhibits low antimicrobial properties and high toxicity. However, when attached to enterobactin siderophore, via a polyethyleneglycol (PEG) linker, it revealed potent antibacterial activity and low cytotoxicity - Figure 14 [11, 55].

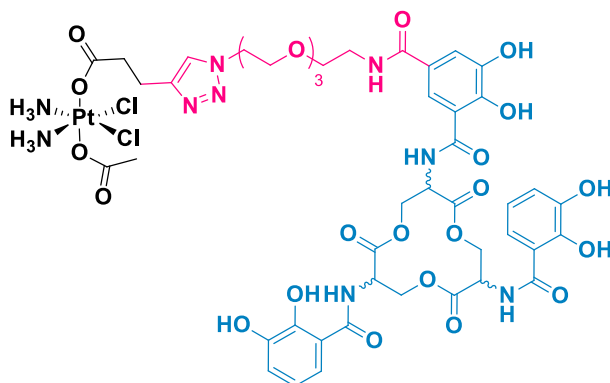


Figure 14 - Cisplatin enterobactin conjugate. Both the L- and the D-enterobactin were utilised in this study. Siderophore motif highlighted in blue, linker in pink and antibiotic payload in black.

Chapter 2 – Project

2.1. The Project

The discovery and development of siderophore-antibiotic conjugates that are able to enter the cytoplasm of bacteria is still a chimera due to their membrane complexity. Most of the research in the field uses fluoroquinolones, such as ciprofloxacin, as the starting antibiotic and cognate siderophores as the chelators [43, 56-60]. However, to date, the obtained conjugates have not proven to be more effective than the antibiotic from which they were derived [44, 61, 62]. This feature can, in some way, be explained by the retention of the conjugate in the periplasmic space and its inability to cross the inner membrane or by the non-dissociation of the antibiotic from the siderophore, not allowing its binding to the therapeutic target [12, 44, 61-63].

The project depicted herein is based on the rational design and synthesis of fluoroquinolone-siderophore conjugates. Thus, it follows a brief overview of the three built-in components of these conjugates, along with a summary of the fluoroquinolone-based SACs reported in the literature.

2.2. Overview of the fluoroquinolone antibiotic ciprofloxacin

Ciprofloxacin (Figure 15) was approved in 1987 by the FDA for the treatment of urinary tract infections, prostatitis, skin and soft tissue infections, respiratory tract infections, sexually transmitted infections, typhoid fever and gastrointestinal infections. This antibiotic presents a broad spectrum since it is effective against both Gram-negative and Gram-positive bacteria [9, 14, 60, 61, 64-68]. Ciprofloxacin bactericidal activity occurs by inhibition of type II topoisomerases, namely DNA gyrase (in Gram-negative bacteria) and DNA topoisomerase IV (in Gram-positive bacteria), enzymes that influence bacterial cell growth and division [15, 60, 61, 65-68].

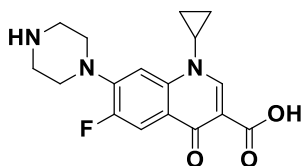


Figure 15 - Chemical structure of ciprofloxacin

The chemical backbone of ciprofloxacin consists of a benzene ring fused to a pyrid-4(1H)-one heterocyclic ring. It has been reported that the β -ketoacid group (Figure 16) is a key part of the molecule for antibacterial activity, mediating the binding to DNA-

gyrase and topoisomerase IV complexes. Studies of this antibiotic's structure-activity-relationship (SAR) also showed that bulky groups at C2 generate a high steric hindrance with the β -ketoacid moiety affecting the interaction between the molecule and the target's binding site. The cyclopropyl ring at position C1 heightens antibacterial activity against gram-negative bacteria, and the fluoride atom at position C6 confers between 5- and 100-fold higher antibacterial potency than any other halogen (Figure 16). This halogen also improves oral absorption, chemical stability, and antibacterial activity of this antibiotic against anaerobic microorganisms. The 5-membered nitrogen heterocycle (piperazine, Figure 16) at position C7 plays a pivotal role in the antibiotic's potency, oral efficacy, pharmacokinetics and spectrum of action, enhancing the antibacterial spectra and pharmacokinetics properties of ciprofloxacin against Gram-negative bacteria. It has been noticed that this bulky group may confer protection against bacterial efflux pumps [61, 64-66].

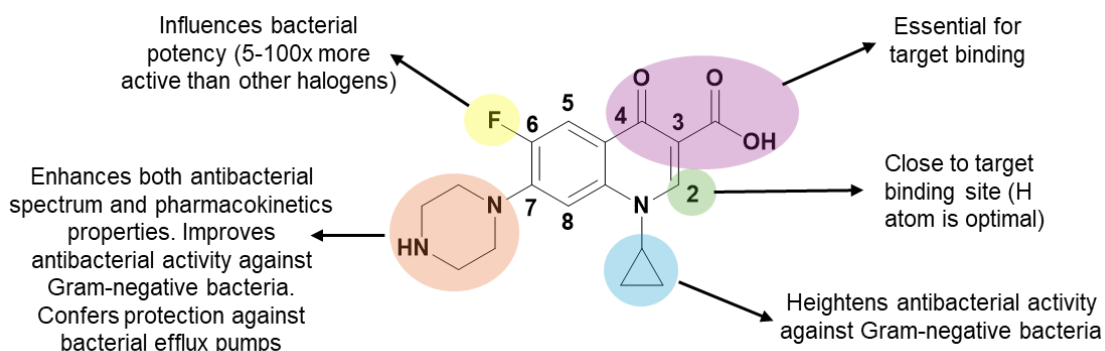


Figure 16 - Structure-Antibacterial Activity Relationship (SAR) of ciprofloxacin

The main mechanisms of antibacterial resistance to fluoroquinolones include conformational changes on the drug target, preventing the antibiotic from binding to its target, enzymatic inactivation, reduction of membrane permeability, increase of the action of efflux pumps, and down-regulation of the production of outer membrane porins (which are involved in antibiotic uptake) [7, 15, 25, 57, 59-61, 66-68].

2.3. Overview of the linkers

When designing SACs, it is crucial to choose an appropriate linker to attach an antibiotic to a siderophore. Linker rigidity, length, and attachment points to siderophore and antibiotic moieties are parameters that can have a quite significant influence on the potency of the SAC [11, 45]. Linkers can be classified as cleavable or non-cleavable.

Non-cleavable linkers are commonly employed in SACs using antibiotics with periplasmic targets (e.g., β -lactam and peptide antibiotics). These chemical structures present high stability regardless of the chemical, biological, or enzymatic external environment conditions [11, 45]. Examples of non-cleavable linkers (Figure 17) include PEG chains, alkyl groups, structures involving carbonyl groups (such as 1,3-diketones, succinic and sebacic acids), thiol-maleimides, piperazine, and dimethyl squarate [11].

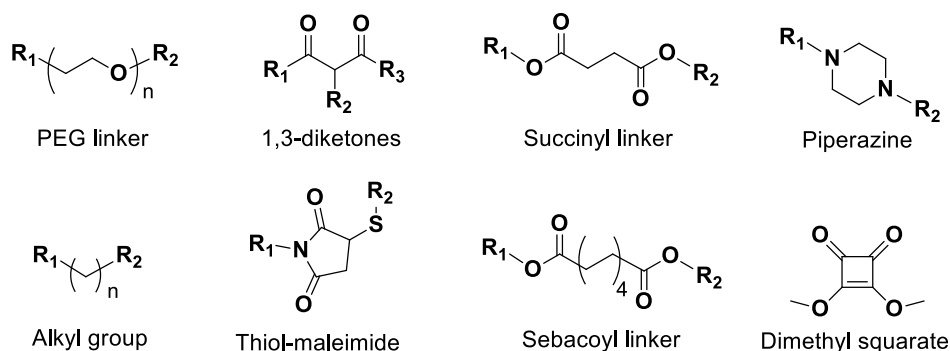


Figure 17 - Chemical structures of some non-cleavable linkers

Cleavable linkers are chemical structures with labile moieties that show instability under certain chemical and/or biological conditions [11, 45]. Usually, a cleavable linker is used when the target of antibiotics is located in the bacterial cytoplasm to ensure that the antibacterial compound reaches its site of action [11, 45]. These types of linkers are often employed when designing SACs based on the pro-drug concept to prevent the conjugate from undergoing a premature cleavage before reaching its target and ensure the intracellular release of the antibiotic by the action of endogenous enzymes and reduction. Examples of cleavable linkers include esters, acyloxyalkylesters, disulphides and trimethyl lock (an ortho-hydroxydihydrocinnamic acid derivative) – Figure 18 [11, 13, 43, 45, 69].

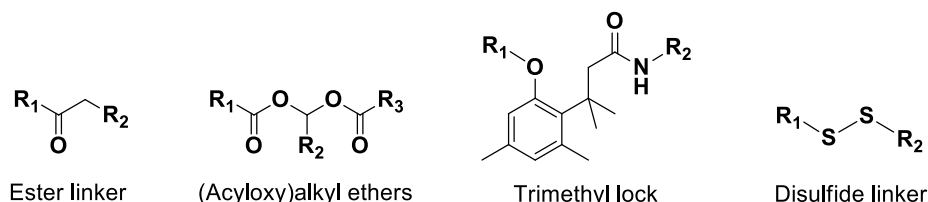


Figure 18 - Chemical structures of some cleavable linkers

Concluding, the design and synthesis of SACs involving antibiotics with cytoplasmic targets typically require the use of cleavable linkers. However, the success of a SAC consisting in 3-hydroxy-pyridin-4(1H)-one linked to ciprofloxacin, a

fluoroquinolone with intracellular target, via a non-cleavable linker, also demonstrated that potent SACs, employing antibiotics with cytoplasmic targets, can be obtained using non-cleavable [11, 13, 43, 45, 69].

2.4. The siderophores mimetics/ iron chelator moieties

Iron chelators such as deferiprone, deferasixor and desferrioxamine are used in thalassaemia therapies to remove iron excess and avoid its undesired pathological effects (p.e. heart failure, liver cirrhosis and diabetes), through the formation of water-soluble complexes and its urinary excretion. These iron chelators correspond to oxygen species, such as catecholates, carboxylates, hydroxamates and hydroxypyridinones, since they present high binding affinities for Fe(III) due to their high negative charge density [70-72].

Hydroxypyridinone derivatives, more specifically 1-hydroxypyridin-2-one, 3-hydroxypyridin-2-one and 3-hydroxypyridin-4-one (Figure 19) are known for its great iron chelating properties, in terms of selectivity and affinity, at physiological conditions. These compounds blend structural features of both catechol and hydroxamate groups. During iron chelation by the 2 vicinal oxygen atoms, 5-membered rings are formed [70-74].

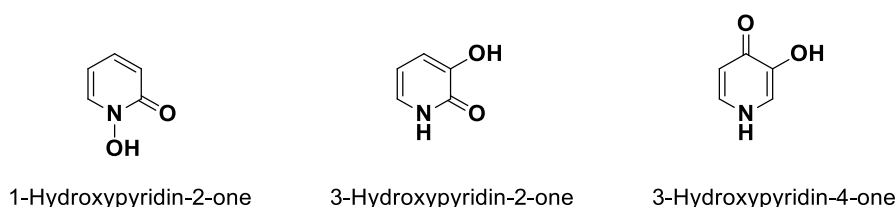


Figure 19 - Chemical structure of hydroxypyridinone ligands

Within the pH range of 6.5-10.5, hydroxypyridinones act as monoprotic acids and predominantly form a neutral complex with Fe(III) in a 3:1 molar ratio. These complexes can easily pass through cell membranes by simple diffusion due to their relatively low molecular weight and non-charged character [74, 75]. Among all hydroxypyridinone derivatives, the bidentate 3-hydroxypyridin-4-ones (Figure 19) have the highest selectivity and affinity for Fe(III) under *in vivo* conditions, showing a high bioavailability [70, 71, 74].

Deferiprone, a 3-hydroxypyridin-4-one, is a bidentate iron chelator that presents great cell membrane permeation due to its lipophilicity. This compound forms non charged octahedral Fe(III) complex, in 3:1 ratio, through α -hydroxyketone groups – Figure 20 [70, 71, 74].

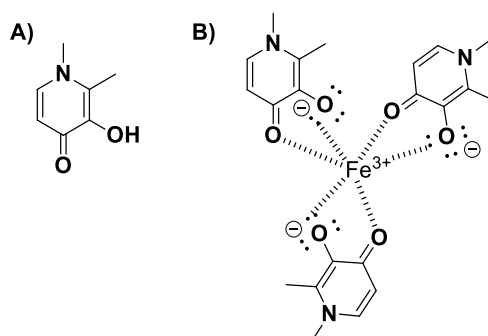


Figure 20 - (A) Chemical structure of deferiprone; (B) Deferiprone-Fe(III) complex

The isosteric compound of 3-hydroxypyridin-4-ones - kojic acid (Figure 21) - is a natural compound used in some skincare products to remove iron from the skin, minimizing the formation of wrinkles. This compound exhibits iron chelating properties, under physiological conditions, forming octahedral complex with Fe(III), in 3:1 molar ratio, through its α -hydroxycarbonyl group (hydroxypyranone group) [76-79].

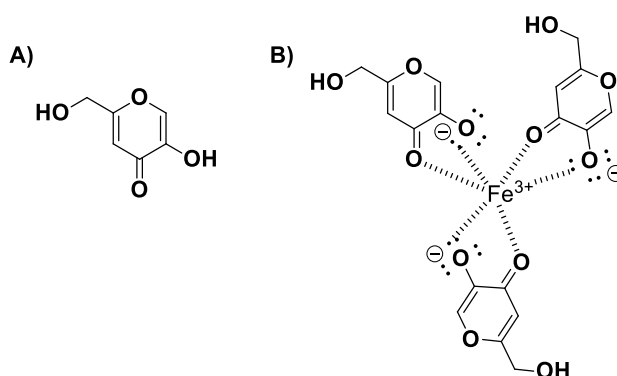


Figure 21 - (a) Chemical structure of kojic acid; (b) Kojic acid-Fe(III) complex

Some studies report that kojic acid and its derivatives, namely chlorokojic acid, present antibacterial effects against both Gram-positive (namely *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Listeria monocytogenes* and *Bacillus subtilis*) and Gram-negative (including *Pseudomonas aeruginosa*, *Escherichia coli*, *Shewanella spp*, *Acinetobacter*, *Salmonella typhimurium*, *Photobacterium*, *Myroides spp.* and *Aeromonas sobria*) bacterial strains [78-81].

Phenolic acids (Figure 22) are secondary natural metabolites that can be divided into two major subgroups: hydroxybenzoic acids (e.g. protocatechuic acid) and hydroxycinnamic acids (e.g. ferulic and caffeic acids) [82, 83]. The iron-chelating properties of caffeic and protocatechuic acids make these structures first-rate siderophore mimetics due to the presence of the catechol moiety (ortho-dihydroxy group) [83-85].

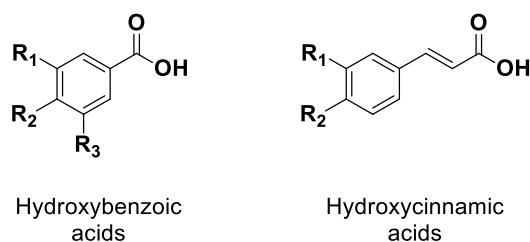


Figure 22 - Chemical structures of phenolic acids: hydroxybenzoic acids (left side) and hydroxycinnamic acids (right side)

The 3,4-dihydroxybenzoic acid, commonly known as protocatechuic acid, during the binding process to iron(III), releases 2 hydrogen ions (H⁺) from catechol moiety – Figure 23 [85-90].

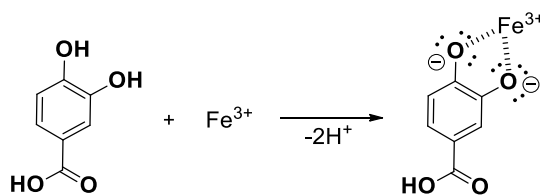


Figure 23 - Iron binding process of protocatechuic acid

This compound shows bactericidal activity against *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Listeria monocytogenes*, *Campylobacter spp*, *Yersinia enterocolitica* and *Staphylococcus aureus* bacterial strains [85-89]. Protocatechuic acid acts by depolarizing bacterial cell membrane which changes membrane permeability and leads to bacterial cell apoptosis due to membrane lysis. When combined with antibiotics, protocatechuic acid enhances, in a synergistic way, the pathogen's vulnerability to the drugs and reduces its intrinsic cytotoxicity [85, 87-89].

Caffeic acid coordinates with iron(III) ions and then form Fe(III)-caffeic acid complex – Figure 24 [91, 92]. This compound presents antibacterial effects and/or increases antimicrobial activity of quinolones antibiotics, against multiple microbes, such as *S. marcescens*, *P. mirabilis*, *E. coli*, *P. aeruginosa*, *B. cereus*, *M. luteus*, *S. aureus*, *S. epidermidis*, *K. pneumoniae*, *L. monocytogenes*, and *C. albicans*. This compound also acts by cell membrane depolarization [93-96].

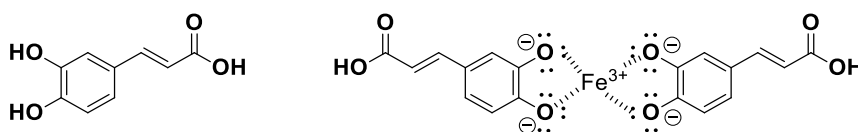


Figure 24 - Chemical structure of caffeic acid (left side) and of caffeic acid-Fe(III) complex (right side)

Ferulic acid is a natural compound that binds to iron(III) ions, in a 2:1 molar ratio, through the carboxylic group – Figure 25 [82].

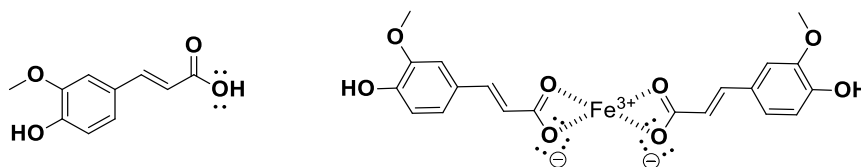


Figure 25 - Chemical structure of ferulic acid (left side) and of ferulic acid-Fe(III) complex (right side)

This compound presents great metal ions chelating, antimicrobial and antiviral properties. It has already been shown that ferulic acid presents antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Staphylococcus aureus*. Its antibacterial activity is based on the perturbation of bacterial membrane potential. In the presence of ferulic acid derivatives it is observed an synergy effect on ciprofloxacin's antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* both *in vitro* and *in vivo* [82, 97-99].

2.5. Fluoroquinolones-siderophore conjugates

In the last years, several siderophore-antibiotic conjugates using fluoroquinolones, namely ciprofloxacin, as an antibacterial agent have been developed and reported in the literature. All these SACs have one common factor: the binding of siderophores or their mimetics to the piperazine group of ciprofloxacin.

Recently, in 2023, a SAC was synthesized linking a 3-hydroxy-pyridin-4(1H)-one (siderophore moiety) to ciprofloxacin, via a non-cleavable linker (succinyl linker) – Figure 26. The resulting conjugate displayed antibiofilm properties and potent antibacterial activity against *P.aeruginosa* bacterial cells (it was observed a MIC of 0.43 μM and 78.3% biofilm reduction at 1/4 MIC) [11, 100].

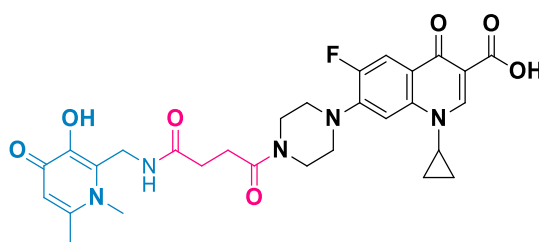


Figure 26 - Dual-acting siderophore-antibiofilm-ciprofloxacin conjugate. Siderophore moiety highlighted in blue, ciprofloxacin in black, linker in pink.

The summary of published ciprofloxacin-siderophore (or mimetic systems) conjugates in the last fifteen years is provided in Table S1.

The vast majority of ciprofloxacin-siderophore conjugates reported in the literature presented a lower or similar antimicrobial activity to that of the parent antibiotic (ciprofloxacin). Nevertheless, the antibacterial activity of these SACs proves that these conjugates are able to reach the cytoplasmic target even in the presence of steric clashes [42, 57, 101]. It is believed that the unsuccess of these type of SACs is due to its inability of being delivered to bacterial cytoplasm and not to piperazine moiety modifications [12, 23]. The introduction of large and bulky siderophores may create difficulties in the incorporation of the conjugates in bacterial cytoplasm via iron-siderophore transporters and/or struggle the binding to the target due to steric hindrance reasons [26, 57-59, 62, 102].

2.6. The Project Rational Design

The main goal of this project is to create new combinations for the ciprofloxacin-siderophore mimetics conjugates (Figure 27). This involves the linkage of the carboxyl group of ciprofloxacin to 3-hydroxypyridin-4-ones, 3-hydroxypyrrone, and catechol moieties using an ester bond, either directly or using linkers. Although it may seem contradictory based on the structure-activity relationship (SAR) of ciprofloxacin, attaching siderophore mimetics to the β -ketoacid group, the ester bond plays a crucial role in creating a pro-drug concept for the designed conjugates, providing the means for intracellular cleavage and release of ciprofloxacin from the SACs, allowing effective targeting of type II topoisomerases [11, 13, 43, 45, 50, 58, 102].

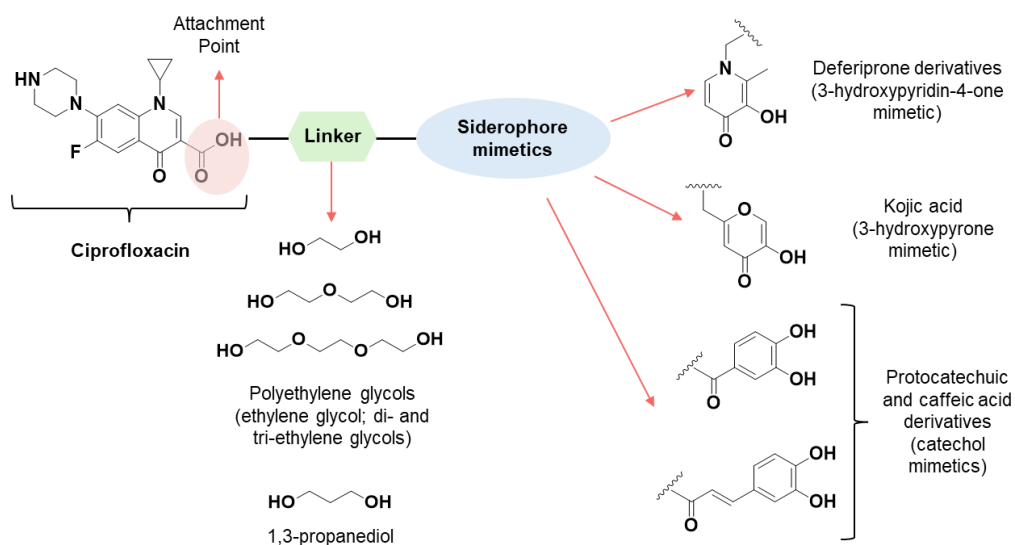


Figure 27 - Rational design of ciprofloxacin-siderophore mimetics conjugates

Moreover, to enhance the likelihood of the conjugate being delivered to bacterial cytoplasm, low molecular weight and not very bulky groups were used as mimetic systems of the natural siderophores – Figure 27 [15].

The ciprofloxacin-siderophore mimetics coupling strategies are outlined in Figure 28. Once these conjugates had been obtained, their iron chelating and antibacterial activities, as well as their toxicological safety, were assessed.

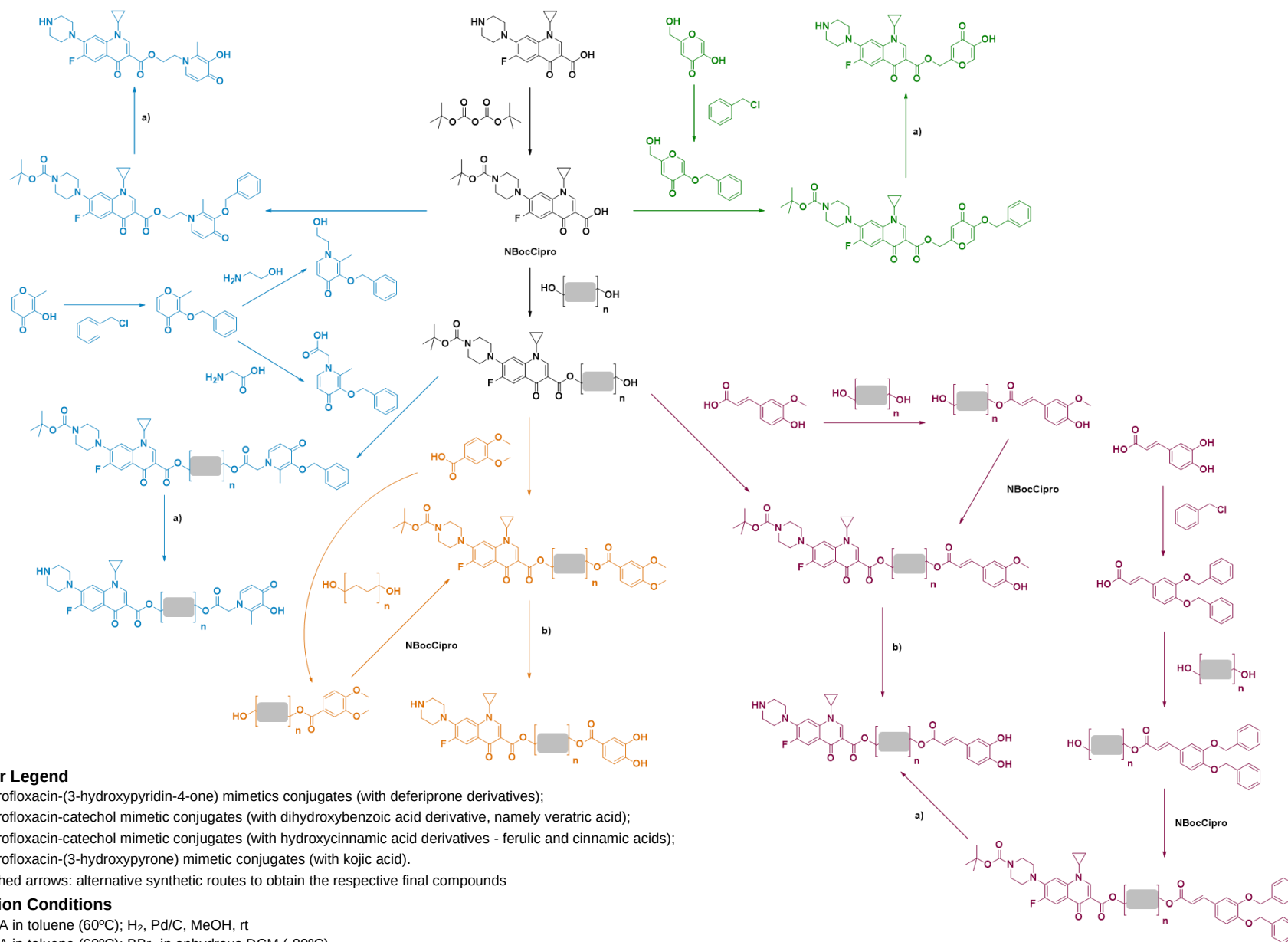


Figure 28 - Synthetic strategies outlined to obtain ciprofloxacin – siderophore mimetics conjugates

Chapter 3 – Experimental Section

3.1. Reactants and solvents

During this project were used reactants and solvents, of pro analysis (p.a) or synthesis quality, obtained from Alfa Aesar, Aldrich-Sigma, Merck, Carlo Erba, Thermoscientific and FluoroChem.

3.2. Materials, instrumentation and methods

All the solid reagents were weighed on Kern ABJ-NB/ABS-N ABS220-4N balance and solvent evaporation was carried out using Büchi Rotavapors R-210 at reduced pressure.

The microwave syntheses were performed in Biotage Initiator EXP Microwave Reactor Synthesis instrument and reactions requiring temperature control were carried out on the Radleys Mya 4 Reaction Station equipment. Catalytic hydrogenation reactions were made in continuous flow H-Cube Mini Plus ThalesNano apparatus.

All reactions were monitored by thin layer chromatography (TLC), using pre-coated silica gel 60 F254 plates (Merck, 0.2 mm of thickness). The TLC elution systems are described in the synthetic procedures for each synthesis, and plates were visualized under ultraviolet light at wavelengths of 254 nm and 365 nm using a Viber Corporation UV chamber.

Flash column chromatography were performed in Biotage Isolera One and Prime systems. The stationary phase used was silica gel 60 (Merck, 0.2 - 0.5mm) and the mobile phases employed are mentioned for each synthesis.

Compounds were dried in MTI Corporation vacuum oven equipment.

All pH measurements were made using an Bante PHS-3BW Benchtop pH meter.

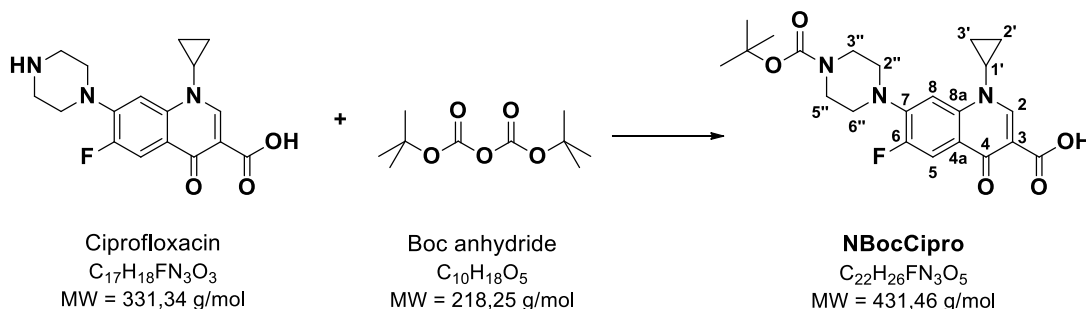
BioTek EPOCH 2 microplate spectrophotometer and Biotek Synergy HT microplate reader, equipped with BioTek Gen5 software, were used to perform UV-Vis absorbance measurements (300-700 nm).

Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker Avance III 400 spectrometer at the Materials Centre of the University of Porto (CEMUP). Proton NMR spectra (^1H NMR) were measured at 400 MHz frequency. Carbon NMR (^{13}C NMR) and Distortionless Enhancement by Polarization Transfer (DEPT) spectra were registered at a frequency of 100 MHz. Chemical Shifts (δ scale, in ppm) were referenced using tetramethylsilane (TMS) as an internal standard. NMR sample preparation used some deuterated solvents, namely dimethylsulfoxide (DMSO-d_6), deuterated methanol (MeOD-d_4) and deuterated chloroform (CDCl_3). "TopSpin" and "MestReNova" software allowed the analysis of the obtained NMR spectra.

3.3. Synthesis of starting compounds

3.3.1. Antibiotic derivative

3.3.1.1. Synthesis of 7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid [NBocCipro]



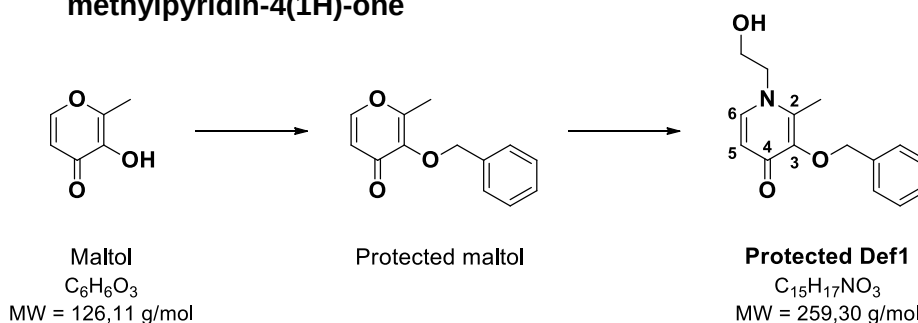
To a ciprofloxacin (4.04g, 12.2 mmol) suspension in dimethylformamide (DMF) (100 mL), di-tert-butyl dicarbonate (Boc anhydride) (3.28g, 15 mmol) and sodium hydrogenocarbonate ($NaHCO_3$) (5.12g, 60.9 mmol) were added. The suspension was stirred at room temperature (rt) for 24h and monitored by TLC using dichloromethane/methanol (DCM/MeOH) (9:1) as eluent. Afterwards, the mixture was vacuum filtered and the resulting solid was rinsed with cold water. The attained white powder was dried in an oven, at 40°C, for 24h and analysed by ^1H NMR, that showed its correspondence to the desired compound.

η =91%; ^1H NMR (400 MHz, $CDCl_3$) δ (in ppm) = 1.21 (2H, m, H-2' or H-3'), 1.40 (2H, m, H-2' or H-3'), 1.50 (9H, s, $-C(CH_3)_3$), 3.30 (4H, t, $J_{H-H} = 5.0$ Hz, H-2'' and H-6''), 3.55 (1H, m, H1'), 3.68 (4H, t, $J_{H-H} = 5.0$ Hz, H-3'' and H-5''), 7.37 (1H, d, $J_{H-F} = 7.1$ Hz, H-8), 8.00 (1H, d, $J_{H-F} = 12.9$ Hz, H-5), 8.74 (1H, s, H-2), 14.92 (1H, s, $-COOH$).

3.3.2. Iron chelators

3.3.2.1. Deferiprone derivatives

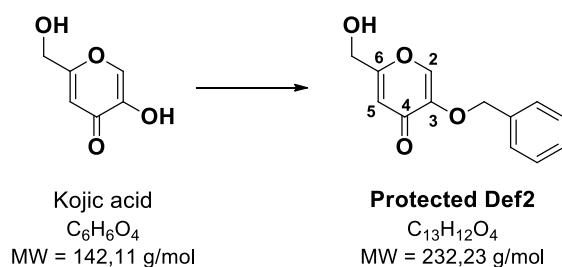
3.3.2.1.1. Synthesis of Protected Def1: 3-(benzyloxy)-1-(2-hydroxyethyl)-2-methylpyridin-4(1H)-one



Maltol (1g, 7.93 mmol) and sodium hydroxide (NaOH) (0.34g, 8.5 mmol) were placed in a microwave (MW) vial and dissolved in a solution of methanol (MeOH, 8mL) and deionized water (H₂O, 4mL). Subsequently, benzyl chloride (BnCl) was added (1mL, 8.7 mmol) and the vial placed into the microwave (MW) equipment. The reaction conditions were as follows: a pre-stirring of 30 seconds followed by heating at 100°C for 30 min in normal absorption mode. A TLC monitoring using an eluent DCM/MeOH (9:1) was performed to check protected maltol formation. After that, 2-aminoethanol (1mL, 16.6 mmol) was added and a new MW cycle was started at 100°C/30min with the 30s of pre-stirring in normal absorption mode. This second reactional cycle was controlled by TLC in DCM/MeOH (9:1). The crude material was placed into a round bottom flask, and MeOH evaporated under reduced pressure. The aqueous phase was extracted with DCM (3x10 mL). The organic phases were joined, dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. The crude product was recrystallised from DCM/n-hexane. The attained solid was dried in an oven at 40°C, overnight, and characterised by ¹H NMR, with an overall yield (Π) of 60%. The ¹H NMR analysis showed that the attained product corresponded to the desired compound.

Π=60% (overall yield of the two-step reaction); ¹H NMR (400 MHz, DMSO) δ (in ppm) = 2.20 (3H, s, -CH₃), 3.59 (2H, m, -CH₂-N), 3.93 (2H, t, J_{H-H}=5.4 Hz, -CH₂-OH), 5.01 (2H, s, -CH₂-Bz), 6.14 (1H, d, J_{H-H}=7.5 Hz, H-5), 7.37 (5H, m, Bz), 7.54 (1H, d, J_{H-H}=7.5 Hz, H-6)

3.3.2.1.2. Synthesis of Protected Def2: 5-(benzyloxy)-2-(hydroxymethyl)-4H-pyran-4-one

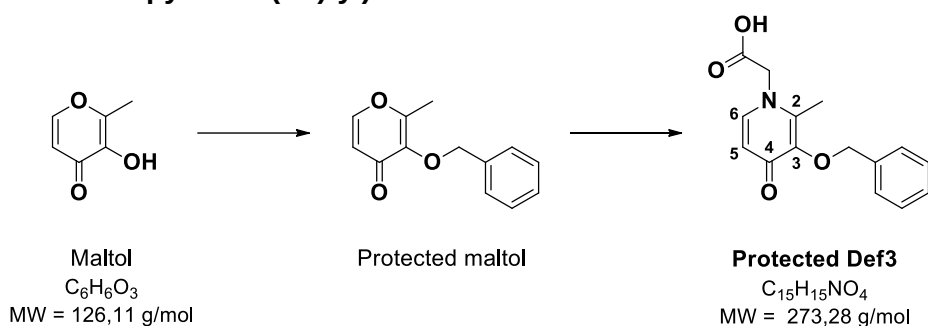


Kojic acid (1g, 7.0 mmol), NaOH (0.34g, 8.5 mmol) and benzyl chloride (1mL, 8.7 mmol) were placed in a MW vial and dissolved in a solution of MeOH (8mL) and deionized H₂O (4mL). Subsequently, the vial was placed into the MW equipment, and the reaction conditions were as follows: a pre-stirring of 30 seconds followed by heating at 100°C for 30 min in normal absorption mode. A TLC monitoring using an eluent DCM/MeOH (9:1) was performed to control the reaction. The crude material was placed into a round bottom flask, and MeOH evaporated under reduced pressure. The MeOH

was then evaporated and the reaction mixture was extracted with DCM (3x10 mL). The organic phases were joined, dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. The crude product was recrystallised from DCM/n-hexane. The attained powder was dried in an oven at 40°C overnight and analysed by ¹H NMR, which showed its correspondence to the desired compound.

η=47%; ¹H NMR (400 MHz, DMSO) δ (in ppm) = 3.60 (1H, m, -OH), 3.93 (2H, m, -CH₂-OH), 5.01 (2H, s, -CH₂-Bz), 6.14 (1H, d, J_{H-H}=7.5 Hz, H-5), 7.37 (5H, m, Bz), 7.54 (1H, d, J_{H-H}=7.5 Hz, H-2)

3.3.2.1.3. Synthesis of Protected Def3: 2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetic acid



The synthesis of protected Def3 following the procedure described in point 3.3.2.1.1 was unsuccessful. The synthesis of the desired compound and yield enhancement was accomplished using glycine instead of 2-aminoethanol and by performing slight modifications to the procedure ascribed in point 3.3.2.1.1, which are summarised in Table 4.

All the reactions were controlled by TLC in DCM/MeOH (9:1) and the pH adjusted to 10 or 12 before each MW cycle with a 5M NaOH aqueous solution – see Table 4. After the 2nd MW Cycle, the MeOH was evaporated, and the reaction mixture was extracted with proper amounts of DCM. The pH of the aqueous phases was adjusted to 2 with a concentrated HCl solution and kept in the fridge overnight, followed by the vacuum filtration of a brownish solid, that was rinsed with cold water. The obtained solid was controlled by TLC in DCM/MeOH (9:1) and recrystallised from DCM/n-hexane. The attained products were dried in an oven at 40°C for 24h and analysed by ¹H NMR.

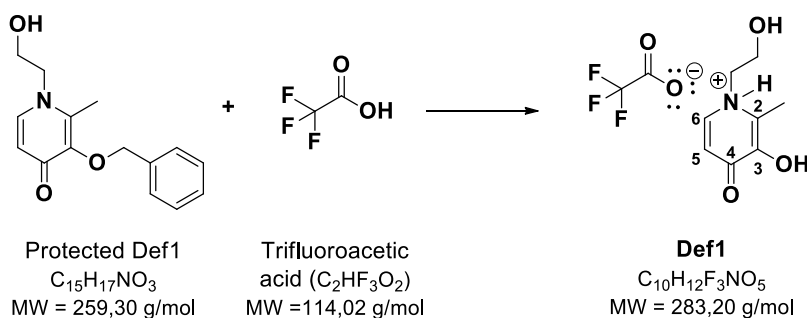
Procedure E (Table 4) showed the best results, affording the desired compound with a yield of η=47%.

¹H NMR (400 MHz, DMSO) δ (in ppm) = 2.06 (3H, s, -CH₃), 4.78 (2H, s, -CH₂-N), 5.02 (2H, s, -CH₂-Bz), 6.15 (1H, d, J_{H-H}=7.5 Hz, H-5), 7.33 (1H, m, Bz), 7.36 (2H, m, Bz), 7.41 (2H, m, Bz), 7.56 (1H, d, J_{H-H}=7.6 Hz, H-6).

Table 4 - Reactional conditions and optimisation procedures to attain compound Protected Def3

Procedure	1 st MW Cycle	2 nd MW Cycle	Reactants (mmol) and Solvents (mL)						pH adjustment	Results
			Maltol	NaOH	BnCl	Glycine	MeOH	H ₂ O		
A	100°C 45 min	120°C 30 min	7.9	8.5	8.7	62	8	4	10	Unsuccessful (protected maltol obtained)
B	100°C 45 min	120°C 30 min	7.9	8.5	8.7	16	8	4	12	Successful (overall η =6%)
C	120°C 60 min	120°C 60 min	7.9	8.5	8.7	24	8	4	12	Successful (overall η =26%)
D	120°C 60 min	120°C 60 min	7.9	8.5	8.7	62	0	10	12	Unsuccessful (protected maltol obtained)
E	120°C 60 min	120°C 60 min	15.9	17.0	8.7	48	8	4	12	Successful (overall η =47%)
F	120°C 60 min	120°C 60 min	7.9	8.5	8.7	48	8	4	12	Successful (overall η =30%)

3.3.2.1.4. Synthesis of Def1: 3-hydroxy-1-(2-hydroxyethyl)-2-methyl-4-oxo-1,4-dihydropyridin-1-ium-2,2,2-trifluoroacetate



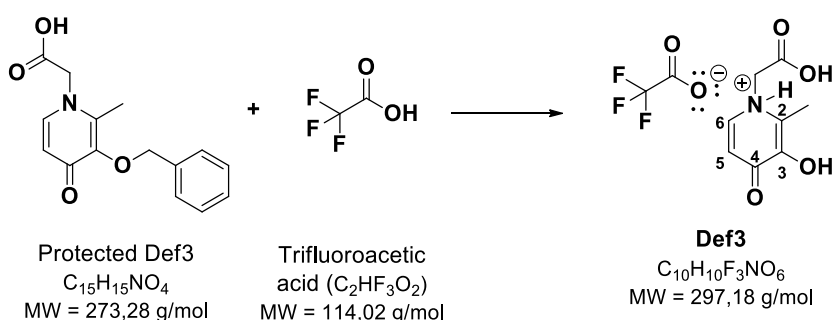
Protected Def1 (1g, 3.86 mmol) was dissolved in trifluoroacetic acid (TFA) (4mL) and toluene (4 mL) and the mixture was kept, with stirring, overnight at 60°C. Then the reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. After evaporating TFA and toluene, under reduced pressure, the crude product was recrystallized from diethyl

ether, yielding a brown powder. This powder was analysed by ^1H NMR and ^{13}C NMR, showing that it corresponded to the desired product.

η =68%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 2.29 (3H, s, $-\text{CH}_3$), 3.63 (2H, t, $J_{\text{H-H}}=5.4$ Hz, $-\text{CH}_2-\text{N}$), 3.98 (2H, t, $J_{\text{H-H}}=5.4$ Hz, $-\text{CH}_2-\text{OH}$), 5.04 (1H, s, $-\text{OH}$), 6.10 (1H, d, $J_{\text{H-H}}=7.3$ Hz, H-5), 7.50 (1H, d, $J_{\text{H-H}}=7.3$ Hz, H-6).

^{13}C NMR (100 MHz, DMSO) δ (in ppm) = 12.1 ($-\text{CH}_3$), 55.2 ($-\text{CH}_2-\text{N}$), 60.7 ($-\text{CH}_2-\text{OH}$), 110.7 (C-5), 129.4 (C-2), 138.7 (C-6), 145.7 (C-3), 169.4 (C-4)

3.3.2.1.5. Synthesis of Def3: 1-(carboxymethyl)-3-hydroxy-2-methyl-4-oxo-1,4-dihydropyridin-1-ium-2,2,2-trifluoroacetate

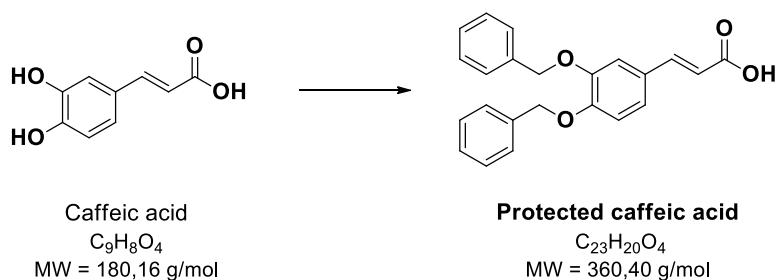


Protected Def3 (0.5g, 1.83 mmol) was dissolved in TFA (2 mL) and toluene (2 mL) and the mixture was kept, with stirring, overnight at 60°C . The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. After evaporation under reduced pressure, the crude product was recrystallized from diethyl ether. The attained product was characterised by ^1H NMR, showing its correspondence to the desired compound.

η =98%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 2.18 (3H, s, $-\text{CH}_3$), 4.87 (2H, s, $-\text{CH}_2-\text{N}$), 6.24 (1H, m, H-5), 7.60 (1H, m, H-6).

3.3.2.2. Cinnamic acid derivative

3.3.2.2.1. Synthesis of Protected caffeic acid: (E)-3-(3,4-bis(benzyloxy)phenyl)acrylic acid



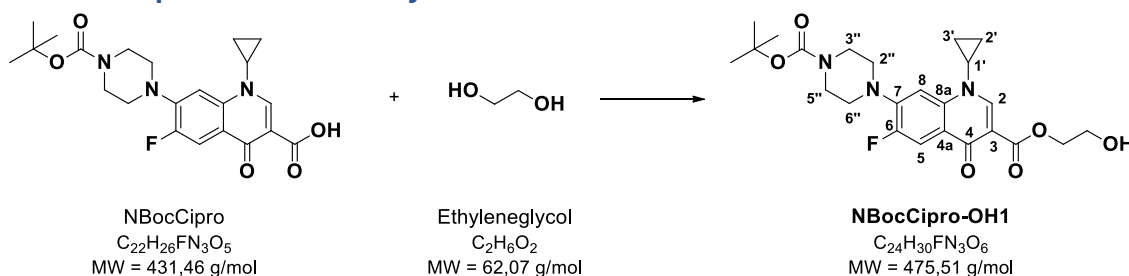
In a MW vial, caffeic acid (0.5g, 2.78 mmol), NaOH (0.106g, 2.65 mmol), benzyl chloride (1.3 mL, 11.2 mmol) were dissolved in a MeOH (2.5 mL) and H₂O (1.3 mL) mixture. The reaction mixture was pre-stirred for 30 seconds and heated, under MW irradiation, at 120°C during 30 min in high absorption mode. The progress of the reaction was followed by TLC in DM/MeOH (9:1) eluent. The reaction mixture was extracted successively with diethyl ether and water. The organic phase was dried with anhydrous sodium sulfate (Na₂SO₄), filtered and the solvent evaporated under reduced pressure. The crude product was purified by automatic flash column chromatography, using an isocratic elution system of DCM/MeOH (9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent and the pure fractions were joined and the solvent evaporated under reduced pressure. The attained product was dried under vacuum and further analysed by ¹H NMR, showing that it didn't correspond to the desired compound.

3.4. Synthesis of OH-linked compounds

3.4.1. NBocCipro-OH compounds

Generic Procedure: NBocCipro (4.6 mmol), hexafluorophosphate benzotriazole tetramethyl uronium (HBTU) (11 mmol), 4-(dimethylamino)pyridine (DMAP) (0.6 mmol) and N,N-diisopropylethylamine (DIPEA) (4mL) were dissolved in DCM (100 mL) and kept, with stirring, at 0°C for 30 min. Afterwards, the proper diol (11.7 mmol) was added, and the mixture was allowed to reach room temperature (rt) and left to stir overnight. The reactions were monitored by TLC using DCM/MeOH (9:1) as eluent. The mixture was extracted with deionized water (3x 25 mL) and a saturated solution of NaHCO₃ (3x 25 mL). The organic phases were dried over anhydrous Na₂SO₄, and the solvent evaporated under reduced pressure. The purification process, yield, and NMR analysis are detailed above for each synthesis.

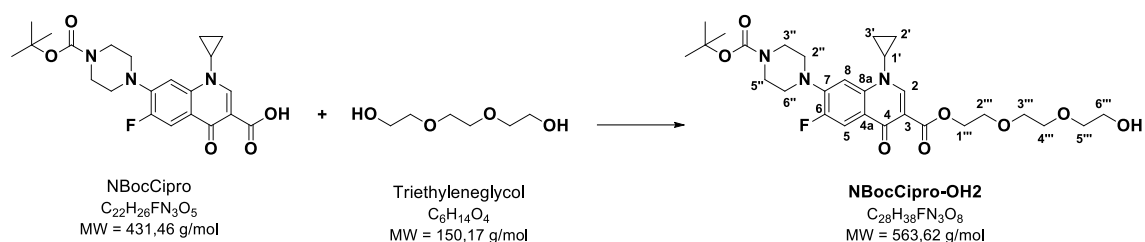
3.4.1.1. Synthesis of NBocCipro-OH1: 2-hydroxyethyl-7-(4-(tert-butoxy-carbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylate



The crude product was purified by flash column chromatography in Biotage equipment using a gradient elution system of DCM/MeOH (9.5:0.5 to 9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected according to the TLC profile, and those presenting the lowest number of impurities were recrystallized from diethyl ether/n-hexane/DCM, yielding a white powder. The attained powder was analysed by ^1H NMR, showing its correspondence to the desired compound.

η =50%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.08 (2H, *Brs*, H-2' or H-3'), 1.27 (2H, m, H-2' or H-3'), 1.42 (9H, s, $-\text{C}(\text{CH}_3)_3$), 3.20 (4H, t, $J_{\text{H-H}} = 5.0$ Hz, H-2'' and H-6''), 3.61-3.66 (6H, m, H-3'', H-5'' and $-\text{O}-\text{CH}_2\text{CH}_2-\text{OH}$), 4.17 (2H, t, $J_{\text{H-H}} = 5.0$ Hz, $-\text{O}-\text{CH}_2\text{CH}_2-\text{OH}$), 7.45 (1H, d, $J_{\text{H-F}} = 7.3$ Hz, H-8), 7.75 (1H, d, $J_{\text{H-F}} = 13.3$ Hz, H-5), 8.48 (1H, s, H-2).

3.4.1.2. Synthesis of NBocCipro-OH2: 2-(2-(2-hydroxyethoxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



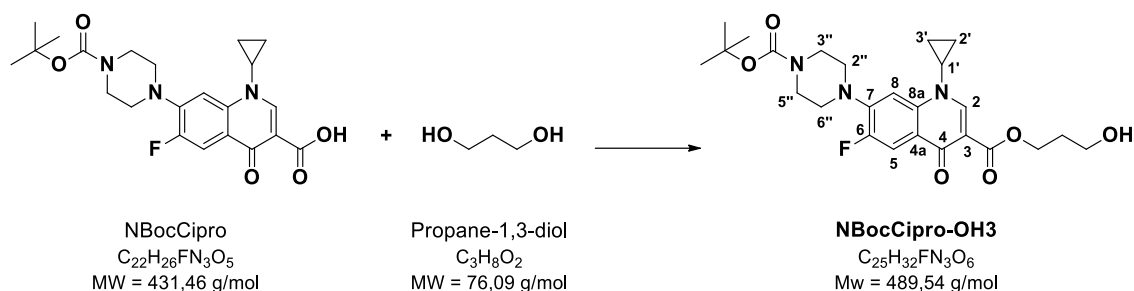
The crude product was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of EtOAc (ethyl acetate)/DCM/MeOH (5:4:1). The purification was tracked by TLC using EtOAc/DCM/MeOH (5:4:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from diethyl ether/DCM/EtOAc/n-hexane, yielding a yellow powder. The attained powder was analysed by ^1H NMR and ^{13}C NMR, showing that it corresponded to the desired compound.

η =29%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.09 (2H, m, H-2' or H-3'), 1.26 (2H, m, H-2' or H-3'), 1.43 (9H, s, $-\text{C}(\text{CH}_3)_3$), 3.14 (1H, m, H-1'), 3.21 (4H, t, $J_{\text{H-H}} = 5.1$ Hz, H-2'' and H-6''), 3.50 (4H, m, H-3'' and H-5''), 3.54 (6H, m, H-3''', H-4''' and H-5'''), 3.64 (2H, m, H-6'''), 3.70 (2H, m, H-2'''), 4.28 (2H, m, H-1'''), 4.60 (1H, m, $-\text{OH}$), 7.47 (1H, d, $J_{\text{H-F}} = 7.3$ Hz, H-8), 7.78 (1H, d, $J_{\text{H-F}} = 13.3$ Hz, H-5), 8.44 (1H, s, H-2).

^{13}C NMR (100 MHz, DMSO) δ (in ppm) = 8.0 (C-2' and C-3'), 28.5 ($-\text{C}(\text{CH}_3)_3$), 35.2 (C-1'), 42.3 (C-2'' and C-6''), 50.0 (C-3'' and C-5''), 60.7 (C-6'''), 68.89 (C-1'''), 70.3

(C-2'''), 70.4 (C-3'''), 70.5 (C-5'''), 72.8 (C-4'''), 76.63 (-C(CH₃)₃), 107.3 (-CH, C-8), 109.3 (C-3), 112.0 (d, J_{C-F} = 22.1 Hz, C-5), 122.2 (C-4a), 139.9 (C-6), 144.2 (C-7), 148.5 (C-2), 151.8 (-COO-N), 155.8 (C8a), 164.5 (-COO-C3), 172.0 (C-4).

3.4.1.3. Synthesis of NBocCipro-OH3: 3-hydroxypropyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate

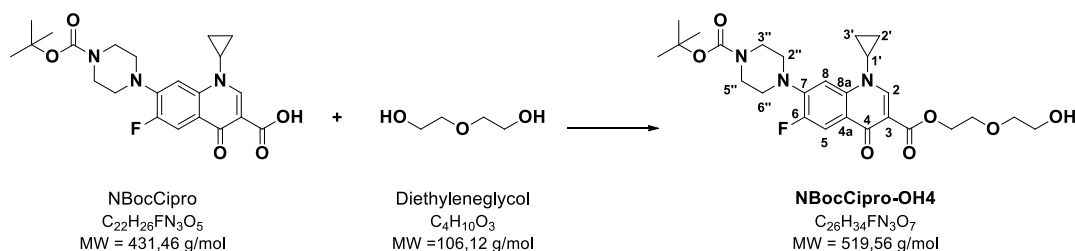


The crude product was purified by recrystallization from n-hexane and EtOAc. The attained product was characterised by ¹H NMR and ¹³C NMR analyses and it corresponded to the desired compound.

η=98%; ¹H NMR (400 MHz, DMSO) δ (in ppm) = 1.10 ppm (2H, m, H-2' or H-3'), 1.26 (2H, m, H-2' or H-3'), 1.44 (9H, s, -C(CH₃)₃), 1.83 (2H, m, -CH₂CH₂-OH), 3.18 (4H, t, J_{H-H} = 5.1 Hz, H-2'' and H-6''), 3.58 (8H, m, H-1', H-3'', H-5'', and -CH₂-OH), 4.23 (2H, t, J_{H-H} = 6.4 Hz, O-CH₂(CH₂)₂OH), 7.43 (1H, d, J_{H-F} = 7.3 Hz, H-8), 7.70 (1H, d, J_{H-F} = 14.0 Hz, H-5), 8.41 (1H, s, H-2).

¹³C NMR (100 MHz, DMSO) δ (in ppm) = 8.0 (C-2' and C-3'), 28.5 (-C(CH₃)₃), 32.1 (-CH₂CH₂-OH), 35.3 (C-1'), 50.0 (C-2'', C-3'', C-5'' and C-6''), 58.3 (-CH₂-OH), 62.2 (-OCH₂(CH₂)₂-OH), 79.6 (-C(CH₃)₃), 107.1 (-d, J_{C-F} = 2.3 Hz, C8), 109.7 (C-3), 112.1 (d, J_{C-F} = 22.5 Hz, C-5), 122.6 (d, J_{C-F} = 6.6 Hz, C4a), 138.5 (C8a), 144.2 (d, J_{C-F} = 10.4 Hz, C-7), 148.7 (C-2), 151.84 (-COO-N), 154.3 (C-F), 165.1 (-COO-C3), 172.1 (C-4).

3.4.1.4. Synthesis of NBocCipro-OH4: 2-(2-hydroxyethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



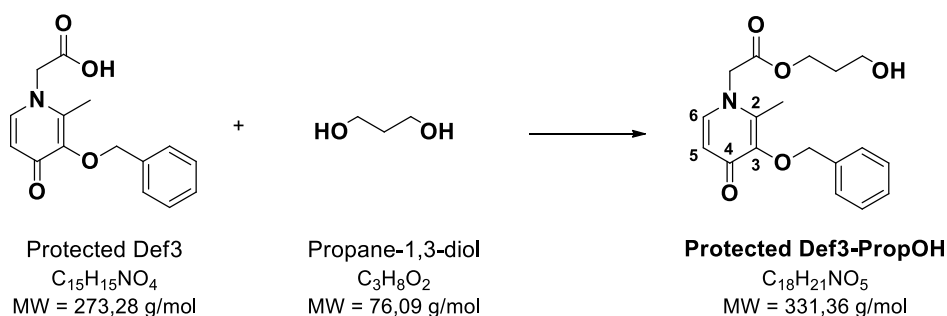
The crude product was purified by recrystallization from diethyl ether yielding a yellow powder and analysed by ^1H NMR and ^{13}C NMR, showing that it corresponded to the desired compound.

η =90%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.09 (2H, m, H-2' or H-3'), 1.25 (2H, m, H-2' or H-3'), 1.43 (9H, s, $-\text{C}(\text{CH}_3)_3$), 3.21 (4H, t, $J_{\text{H-H}} = 5.1$ Hz, H-2'' and H-6''), 3.51 (3H, t, $J_{\text{H-H}} = 4.8$ Hz, $-\text{CH}_2\text{-OH}$), 3.54 (4H, t, $J_{\text{H-H}} = 4.9$ Hz, H-3'' and H-5''), 3.63 (3H, m, $-\text{CH}_2\text{CH}_2\text{-OH}$ and H-1'), 3.70 ppm (2H, m, $-\text{O-CH}_2\text{CH}_2\text{-O-}$), 4.28 (2H, t, $J_{\text{H-H}} = 4.9$ Hz, $-\text{O-CH}_2\text{CH}_2\text{-O-}$), 7.47 (1H, d, $J_{\text{H-F}} = 7.3$ Hz, H-8), 7.78 (1H, d, $J_{\text{H-F}} = 13.2$ Hz, H-5), 8.44 (1H, s, H-2).

^{13}C NMR (100 MHz, DMSO) δ (in ppm) = 8.9 (C-2' or C-3'), 29.4 ($-\text{C}(\text{CH}_3)_3$), 36.2 (C-1'), 43.2 (C-2'' and C-6''), 50.8 (C-3'' and C-5''), 61.6 ($-\text{CH}_2\text{-OH}$), 64.6 ($\text{O-CH}_2\text{CH}_2\text{-O}$), 69.7 ($-\text{O-CH}_2\text{CH}_2\text{-O-}$), 73.7 ($-\text{CH}_2\text{CH}_2\text{-OH}$), 80.5 ($-\text{C}(\text{CH}_3)_3$), 108.0 (C-8), 110.4 (C-3), 112.9 (d, $J_{\text{C-F}} = 22.1$ Hz, C-5), 123.4 (C-4a), 139.4 (C-8a), 145.1 (C-7), 149.5 (C-2), 152.7 ($-\text{COO-N}$), 155.1 (C-6), 165.6 ($-\text{COO-C3}$), 173.0 (C-4)

3.4.2. Iron chelators-OH compounds

3.4.2.1. Synthesis of Protected Def3-PropOH: 3-hydroxypropyl-2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetate

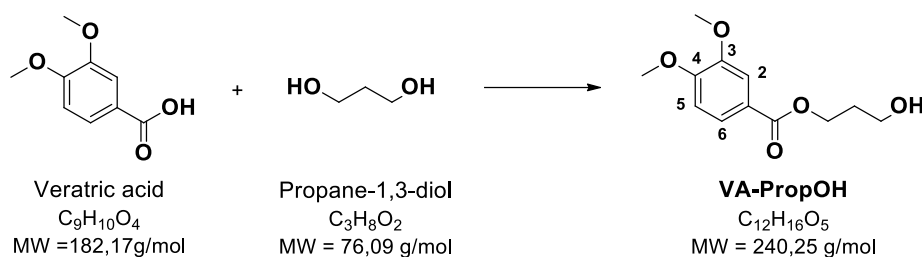


In a 250mL round-bottom flask, protected Def3 (0.5g, 1.8 mmol), HBTU (1.6g, 4.2 mmol), DMAP (29.2mg, 0.2 mmol) and DIPEA (1.6 mL) were dissolved in DCM (80 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then, propane-1,3-diol (333 μL , 4.61 mmol) was added and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was extracted with deionized water (3x 20mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of DCM/MeOH

(9:1). The fractions were collected regarding the TLC profile, the pure ones were joined and the solvents were evaporated under reduced pressure. The attained product was analysed by ^1H NMR, showing that it corresponded to the desired compound.

$\eta=14\%$; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.72 (2H, m, $-\text{O}-\text{CH}_2\text{CH}_2-\text{OH}$), 2.07 (3H, s, $-\text{CH}_3$), 3.44 (2H, t, $J_{\text{H-H}} = 6.2$ Hz, $-\text{CH}_2-\text{OH}$), 4.19 (2H, t, $J_{\text{H-H}} = 6.5$ Hz, $-\text{O}-\text{CH}_2(\text{CH}_2)_2\text{OH}$), 4.55 (1H, s, $-\text{OH}$), 4.90 (2H, s, $\text{N}-\text{CH}_2$), 5.02 (2H, s, $-\text{CH}_2-\text{Bz}$), 6.19 (1H, d, $J_{\text{H-H}} = 7.5$ Hz, H-5), 7.31-7.42 (5H, m, Bz), 7.58 (1H, d, $J_{\text{H-H}} = 7.6$ Hz, H-6)

3.4.2.2. Synthesis of VA-PropOH: 3-hydroxypropyl-3,4-dimethoxybenzoate



Procedure A

In a 100mL round-bottom flask, veratric acid (0.5g, 2.8 mmol), HBTU (2.4g, 6.3 mmol), DMAP (43.7 mg, 0.4 mmol), DIPEA (2.4 mL) were dissolved in DCM (50 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then propane-1,3-diol (510 μL , 7.0 mmol) was added and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was extracted with deionized water (3x15 mL), a 1M HCl aqueous solution (3x15mL) and a saturated solution of NaHCO_3 (3x15mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using a gradient elution system of DCM/MeOH (9.5:0.5 to 9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and the less impure fractions were recrystallized from EtOAc/diethyl ether/n-hexane. The resulting powder was analysed by ^1H NMR, showing that it corresponded to the desired product.

$\eta=7\%$;

Procedure B

Veratric acid (0.5g, 2.8 mmol), propane-1,3-diol (198 μL , 2.8 mmol), H_2SO_4 (96% in catalytic amounts) were dissolved in 10 mL of tetrahydrofuran (THF). The mixture was kept, with stirring, at the reflux temperature for 24h. The reaction was monitored by TLC

using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was extracted (3x) with deionized water (10 mL) and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The resulting oil was dried in an oven, at 40°C, overnight and then analysed by ¹H NMR, showing that it corresponded to veratric acid.

Procedure C

Veratric acid (1g, 5.5 mmol), propane-1,3-diol (2.2mL, 30 mmol) and H₂SO₄ (96% in catalytic amounts) were placed in a MW vial. The MW reactional conditions were as follows: a pre-stirring of 30 seconds and heating at 100°C for 45 min in high absorption mode. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, DCM (20 mL) was added and the mixture was extracted (3x) with deionized water (10 mL) and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and evaporated under reduced pressure. The crude product was recrystallised from DCM/diethyl ether, dried in an oven, at 40°C, overnight and further characterised by ¹H NMR, showing that it corresponded to the starting material (veratric acid).

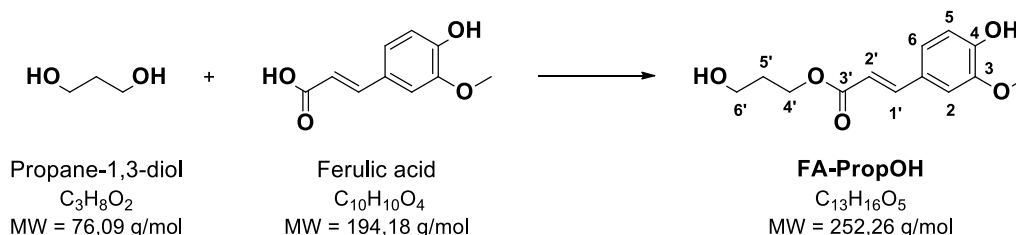
Procedure D

In a two-neck round-bottom flask, veratric acid (1g, 5.5 mmol), propane-1,3-diol (4mL, 54.9 mmol) and p-toluenesulfonic acid monohydrate (209mg, 1.1 mmol) were dissolved in 1,4-dioxane (25 mL). The mixture was kept, with stirring, at reflux temperature overnight. The reaction was monitored by TLC using EtOAc/DCM (7:3) as eluent. Afterwards, the mixture was washed (3x) with deionized water (10 mL), a 1M HCl aqueous solution (10mL) and a saturated solution of NaHCO₃ (10mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude product was purified by recrystallization from n-hexane/DCM and the resulting product was dried in an oven, at 40°C for 24h, and analysed by ¹H NMR. The ¹H NMR analysis showed that the attained product corresponded to the desired compound.

$$\eta = 44\%$$

¹H NMR (400 MHz, CDCl₃) δ (in ppm) = 2.01 (2H, m, -CH₂CH₂-OH), 2.84 (1H, m, -OH), 3.77 (2H, t, J_{H-H} = 6.1 Hz, -CH₂-OH), 3.92 (3H, s, -OCH₃), 3.93 (3H, s, -OCH₃), 4.46 (2H, t, J_{H-H} = 6.2 Hz, -CH₂(CH₂)₂-OH), 6.88 (1H, d, J_{H-H} = 8.4 Hz, H-5), 7.53 (1H, d, J_{H-H} = 2.0 Hz, H-2), 7.67 (1H, dd, J_{H-H} = 8.4 and 2.0 Hz, H-6).

3.4.2.3. Synthesis of FA-PropOH: (*E*)-3-hydroxypropyl-3-(4-hydroxy-3-methoxyphenyl)acrylate



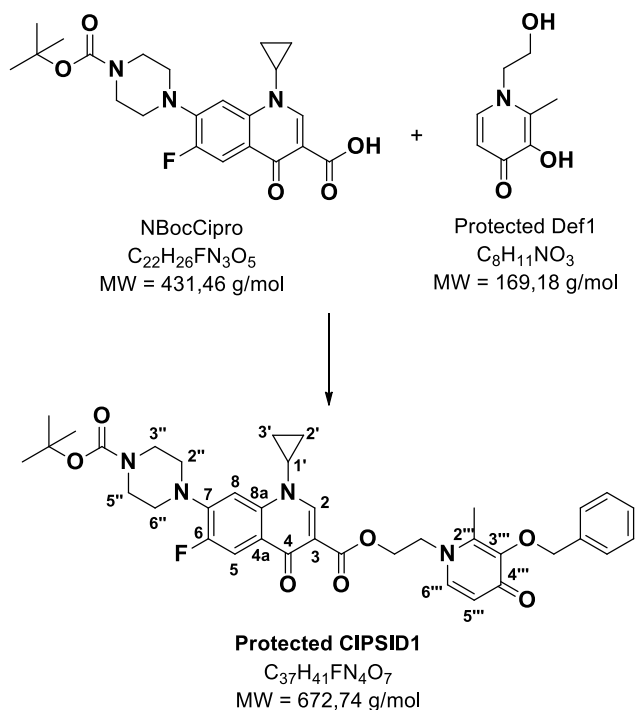
In a two-neck round-bottom flask, ferulic acid (0.5g, 2.6 mmol), propane-1,3-diol (1.9mL, 25.8mmol) and p-toluenesulfonic acid monohydrate (98 mg, 0.5 mmol) were dissolved in 1,4-dioxane (15 mL). The mixture was kept, with stirring, at reflux temperature overnight. The reaction was monitored by TLC using EtOAc/DCM (7:3) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL), a 1M HCl aqueous solution (10mL) and a saturated solution of NaHCO₃ (10mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude product was purified by recrystallization from DCM/n-hexane/ diethyl ether and the attained product was dried in a oven, at 40°C for 24h. The product was analysed by ¹H NMR, showing its correspondence to the desired compound.

η=55%; ¹H NMR (400 MHz, CDCl₃) δ (in ppm) = 2.07 (1H, m, -OH), 1.95 (2H, m, H-5'), 3.74 (2H, t, J_{H-H} = 6.0 Hz, H-6'), 3.93 (3H, s, -OCH₃), 4.37 (2H, t, J_{H-H} = 6.1 Hz, H4'), 5.96 (*Brs*, -OH), 6.29 (1H, d, J_{H-H} = 15.8 Hz, H2'), 6.92 (1H, d, J_{H-H} = 8.2 Hz, H-5), 7.03 (1H, d, J_{H-H} = 2.0 Hz, H-2), 7.07 (1H, dd, J_{H-H} = 8.2 and 2.0 Hz, H-6), 7.63 (1H, d, J_{H-H} = 15.9 Hz, H-1').

3.5. Synthesis of protected CIPSID1 and protected CIPSID2

Generic Procedure: In a 250 mL round-bottom flask, NBocCipro (1g, 2.3 mmol), HBTU (2g, 5.3 mmol), DMAP (0.040g, 0.3 mmol) and DIPEA (2mL, 11.4 mmol) were suspended in DCM (100 mL) and kept, with stirring, at 0°C for 30 min. Then, the corresponding protected iron chelator (5.8 mmol) was added and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (25 mL) and a saturated solution of NaHCO₃ (25 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered, and the solvent evaporated under reduced pressure. The purification process, yield, and NMR analysis for each synthesis are detailed below.

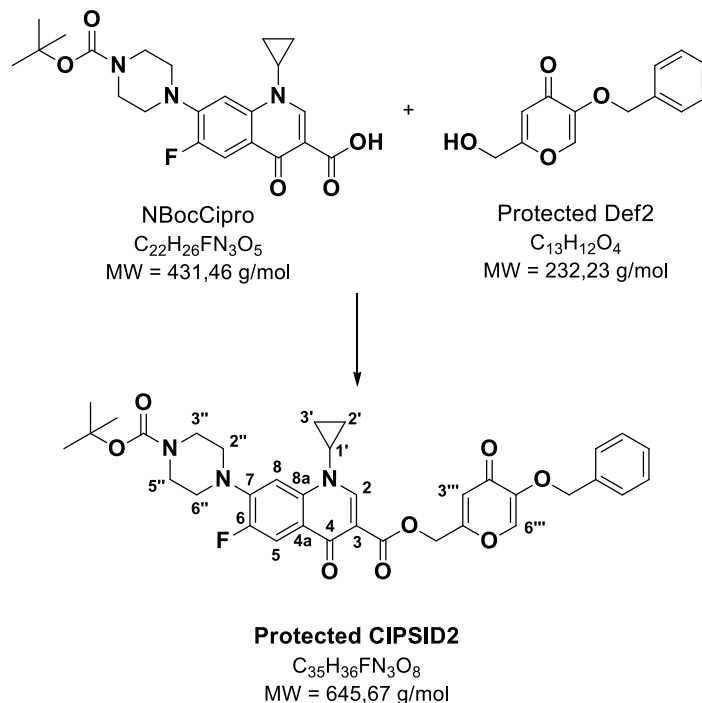
3.5.1. Synthesis of Protected CIPSID1: 2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)ethyl 7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



The crude product was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of DCM/MeOH (9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from DCM/n-hexane. The attained powder was analysed by ^1H NMR, showing that it corresponded to the desired compound.

$\eta = 19\%$; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.04 (2H, m, H-2' or H-3'), 1.26 (2H, m, H-2' or H-3'), 1.44 (9H, s, $-C(CH_3)_3$), 2.34 (3H, s, CH_3-C2''), 3.20 (4H, t, $J_{H-H} = 5.1$ Hz, H-2'' and H-6''), 3.54 (4H, t, $J_{H-H} = 5.0$ Hz, H-3'' and H-5''), 3.62 (1H, m, H-1'), 4.35 (2H, t, $J_{H-H} = 4.9$ Hz, $-CH_2-N-$), 4.41 (2H, t, $J_{H-H} = 4.9$ Hz, $-COO-CH_2-$), 5.01 (2H, s, $-CH_2-Bz$), 6.35 (1H, d, $J_{H-H} = 7.5$ Hz, H-5'''), 7.37 (6H, m, Bz and H-8), 7.70 (1H, d, $J_{H-F} = 13.2$ Hz, H-5), 7.91 (1H, d, $J_{H-H} = 7.5$ Hz, H-6'''), 8.37 (1H, s, H-2).

3.5.2. Synthesis of Protected CIPSID2: (5-(benzyloxy)-4-oxo-4H-pyran-2-yl)methyl 7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



The crude product was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of DCM/MeOH (9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from DCM/n-hexane. The attained powder was analysed by ^1H NMR, showing its correspondence to the desired compound.

η =20%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.12 (4H, m, H-2' and H-3'), 1.26 (9H, s, $-C(CH_3)_3$), 3.15 (1H, m, H-1'), 3.61 (4H, m, H-2'' and H-6''), 3.71 (4H, m, (H-3'' and H-5''), 4.95 (2H, s, $-\underline{CH}_2-Bz$), 5.13 (2H, s, $-\underline{COO}-\underline{CH}_2$), 6.65 (1H, *Brs*, H-3'''), 7.43 (5H, m, Bz), 7.49 (1H, d, J_{H-F} = 7.4 Hz, H-8), 7.83 (1H, J_{H-F} = 13.4 Hz, H-5), 8.10 (1H, s, H-6'''), 8.37 (1H, s, H-2).

3.6. Synthesis of protected CIPSID3 to protected CIPSID6

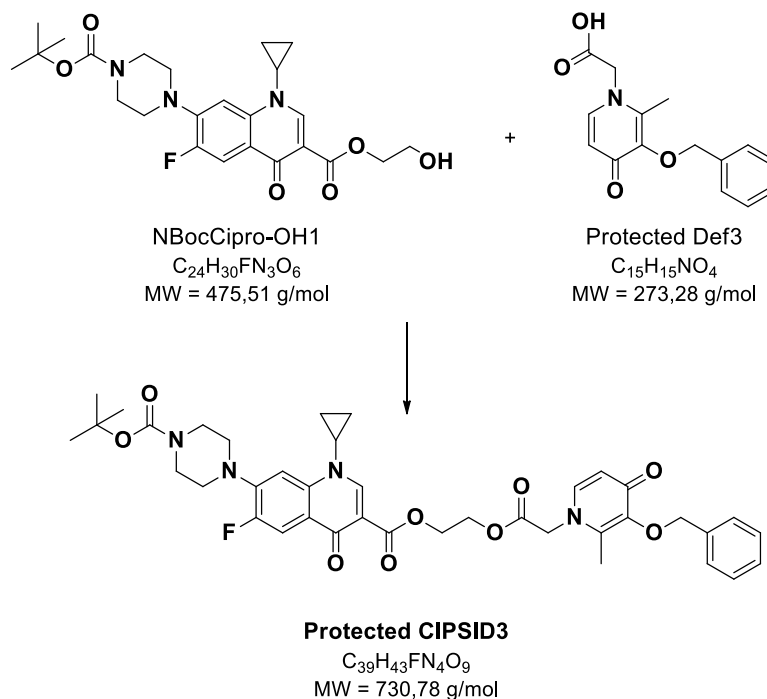
Two Generic Procedures were performed to attain the desired compounds:

Procedure A — To a solution of Protected Def3 (0.19g, 0.7 mmol) in DCM (30 mL), DIPEA (610 μ L, 3.5 mmol), HBTU (0.6 g, 1.6 mmol), and DMAP (0.01 g, 0.09 mmol) was added, and the mixture was kept, with stirring, at 0°C for 30 min. Then, the corresponding NBocCipro-OH (1.8 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL) and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered, and the solvent evaporated under reduced pressure.

Procedure B — To a solution of Protected Def3 (0.7g, 2.7 mmol) in DCM (50 mL), triethylamine (TEA) (1.9 mL, 13.9 mmol), HBTU (2.5g, 6.6 mmol) and DMAP (0.13g, 1.1 mmol) were added, and the mixture was kept, with stirring, at 0°C for 30 min. Then, the corresponding NBocCipro-OH (1.8 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (8:2) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (15 mL) and a saturated solution of NaHCO₃ (15 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered, and the solvent evaporated under reduced pressure.

Below are the results of each synthesis for both procedures along the purification process, yield, and NMR analysis.

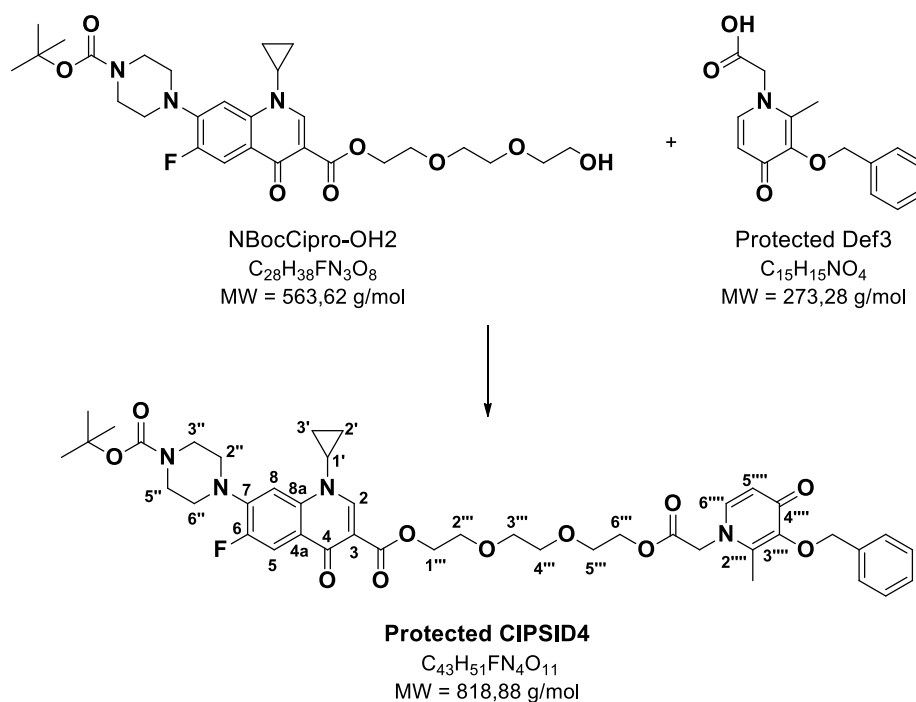
3.6.1. Synthesis of Protected CIPSID3: 2-(2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetoxylethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



Procedure A

From this procedure, the crude product was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of DCM/MeOH (9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest amount of impurities were recrystallized from DCM/n-hexane. The resulting powder proceeded to deprotection without any further ^1H NMR analysis.

3.6.2. Synthesis of Protected CIPSID4: 2-(2-(2-(2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetoxylethoxy)ethoxy)ethyl-7-(4-(tertbutoxy carbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



Procedure A

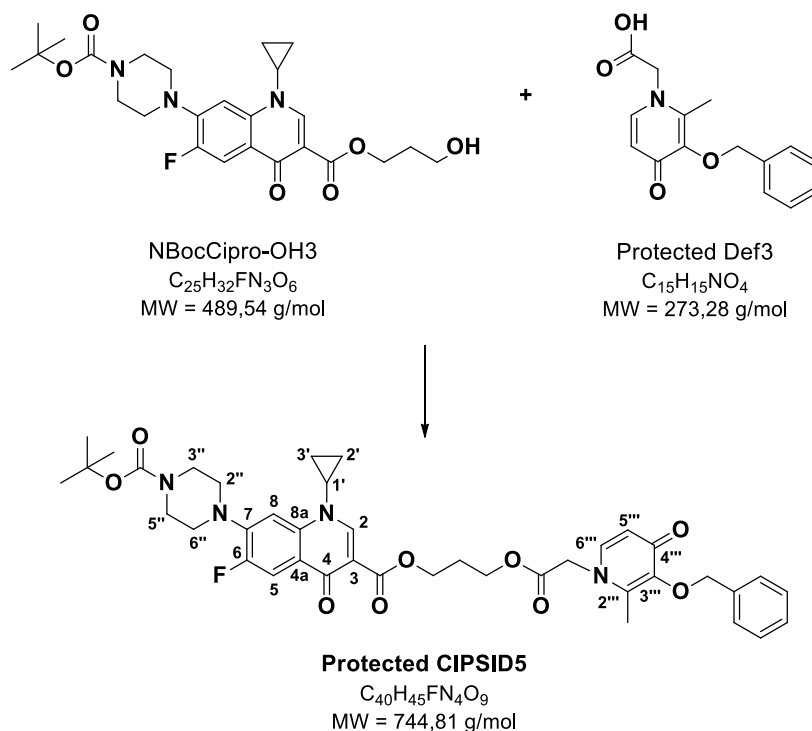
The oil obtained from this procedure was recrystallized from diethyl ether, yielding a yellow powder. The ^1H NMR analysis showed that it didn't correspond to the desired product but to the starting alcohol (NBocCipro-OH2).

Procedure B

The oil obtained from this procedure that was recrystallized from diethyl ether, yielding a yellow powder. The ^1H NMR analysis showed it corresponded to the desired compound.

η =22%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.25 (4H, m, H-2' and H-3'), 1.43 (9H, s, $-C(CH_3)_3$), 2.05 (3H, s, $-CH_3-C2''''$), 3.21 (4H, m, H-2'' and H-6''), 3.62 ppm (13H, m, H-1', H-3'', H-5'', H-2''', H-3''', H-4''' and H-5'''), 4.26 (4H, m, H-1''' and H-6'''), 4.90 (2H, s, $-CH_2-N$), 4.99 (2H, s, CH_2-Bz), 6.13 ppm (1H, d, $J_{H-H} = 7.6$ Hz, H-5'''), 7.36 (5H, m, Bz), 7.45 (1H, d, $J_{H-F} = 7.4$ Hz, H-8), 7.54 (1H, d, $J_{H-H} = 7.5$ Hz, H-6'''), 7.76 (1H, d, $J_{H-F} = 13.2$ Hz, H-5), 8.43 (1H, s, H-2).

3.6.3. Synthesis of Protected CIPSID5: 3-(2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetoxypentyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



Procedure A

The crude product, attained from this procedure, was purified by flash column chromatography in Biotage equipment, using a gradient elution system of DCM/MeOH (98:2 to 80:20). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from diethyl ether/n-hexane. The resulting powder was analysed by ^1H NMR, however it corresponded to NBocCipro-OH3.

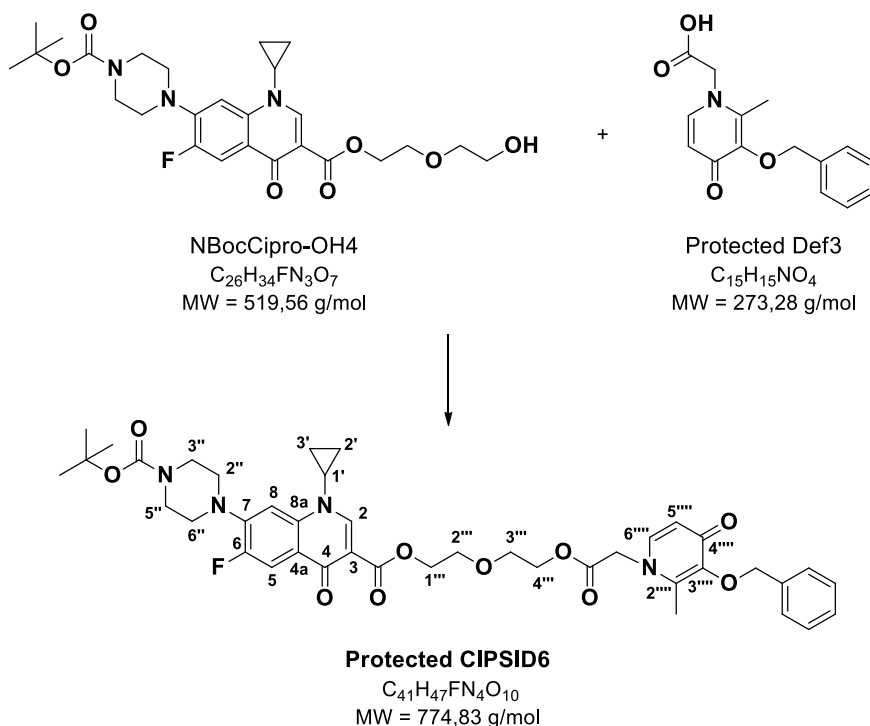
Procedure B

The oil attained from this procedure was recrystallized from diethyl ether/n-hexane, yielding a brown powder. The ^1H NMR analysis showed its correspondence to the desired compound.

η =16%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.07 (2H, m, H-2' or H-3'), 1.26 (2H, m, H-2' or H-3'), 1.43 (9H, s, $-C(CH_3)_3$), 2.00 (2H, m, H-2'''), 2.07 (3H, s, $-CH_3-C2''$), 3.21 (4H, m, H-2'' and H-6''), 3.54 (4H, m, H-3'' and H-5''), 3.63 (1H, m, H-1'), 4.23 (2H, t, $J_{H-H} = 6.2$ Hz, $-OCH_2CH_2CH_2-O-$), 4.32 (2H, t, $J_{H-H} = 6.2$ Hz, $-OCH_2CH_2CH_2-O-$), 4.93

(2H, s, $-\text{CH}_2\text{-N}$), 5.00 (2H, s, $-\text{CH}_2\text{-Bz}$), 6.16 (1H, d, $J_{\text{H-H}} = 7.5$ Hz, H-5'''), 7.35 (5H, m, Bz), 7.45 (1H, d, $J_{\text{H-F}} = 7.3$ Hz, H-8), 7.58 (1H, d, $J_{\text{H-H}} = 7.6$ Hz, H-6'''), 7.77 (1H, d, $J_{\text{H-F}} = 13.2$ Hz, H-5), 8.43 (1H, s, H-2).

3.6.4. Synthesis of Protected CIPSID6: 2-(2-(2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetoxylethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



Procedure A

The crude product, attained from this procedure, was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of EtOAc/DCM/MeOH (5:4:1). The purification was tracked by TLC using EtOAc/DCM/MeOH (5:4:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from diethyl ether/DCM/EtOAc/n-hexane. The resulting product was analysed by ^1H NMR, showing that it corresponded to the starting alcohol (NBocCipro-OH4).

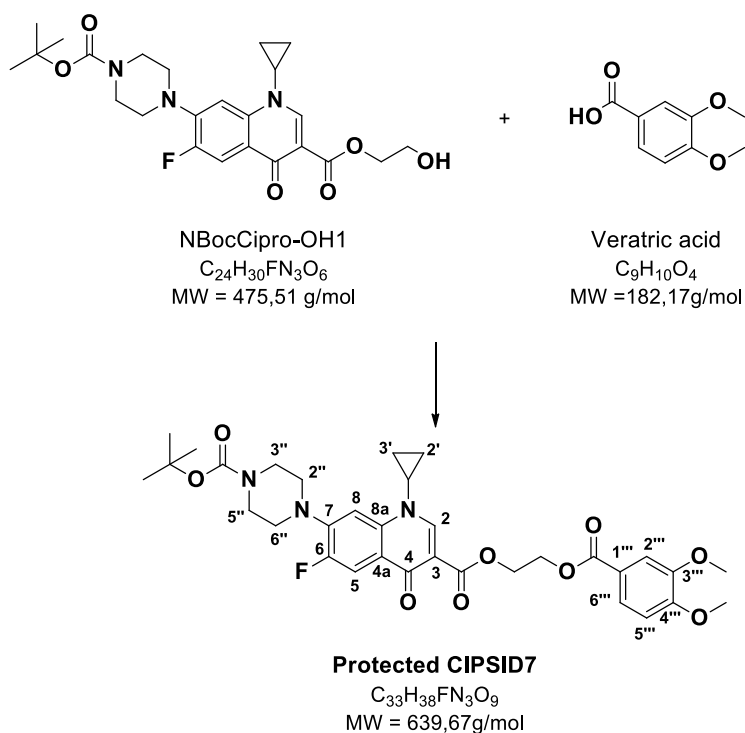
Procedure B

The oil obtained from this procedure was recrystallized from DCM/n-hexane/EtOAc/diethyl ether, yielding a yellow powder. The attained product was analysed by ^1H NMR, showing its correspondence to the desired compound.

η =49%; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.07 (2H, m, H-2' or H-3'), 1.25 (2H, m, H-2' or H-3'), 1.43 (9H, s, $-\text{C}(\text{CH}_3)_3$), 2.24 (3H, s, $-\text{CH}_3-\text{C}2''''$), 3.21 ppm (6H, t, $J_{\text{H-H}} = 5.2$ Hz, H-2'', H-6'' and H-2'''), 3.64 (2H, m, H-3'''), 3.72 (5H, m, H-3'', H-5'' and H-1'), 4.29 (4H, m, H-1''' and H-4'''), 5.05 (2H, s, $-\text{CH}_2-\text{N}$), 5.25 (2H, s, $-\text{CH}_2-\text{Bz}$) 6.79 (1H, m, H-5'''), 7.33-7.44 (5H, m, Bz), 7.47 (1H, d, $J_{\text{H-F}} = 7.4$ Hz, H-8), 7.78 (1H, d, $J_{\text{H-F}} = 13.3$ Hz, H-5), 8.06 (1H, d, $J_{\text{H-H}} = 7.4$ Hz, H-6'''), 8.45 (1H, s, H-2).

3.7. Synthesis of protected CIPSID7 to protected CIPSID11

3.7.1. Synthesis of Protected CIPSID7: 2-((3,4-dimethoxybenzoyl)oxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate

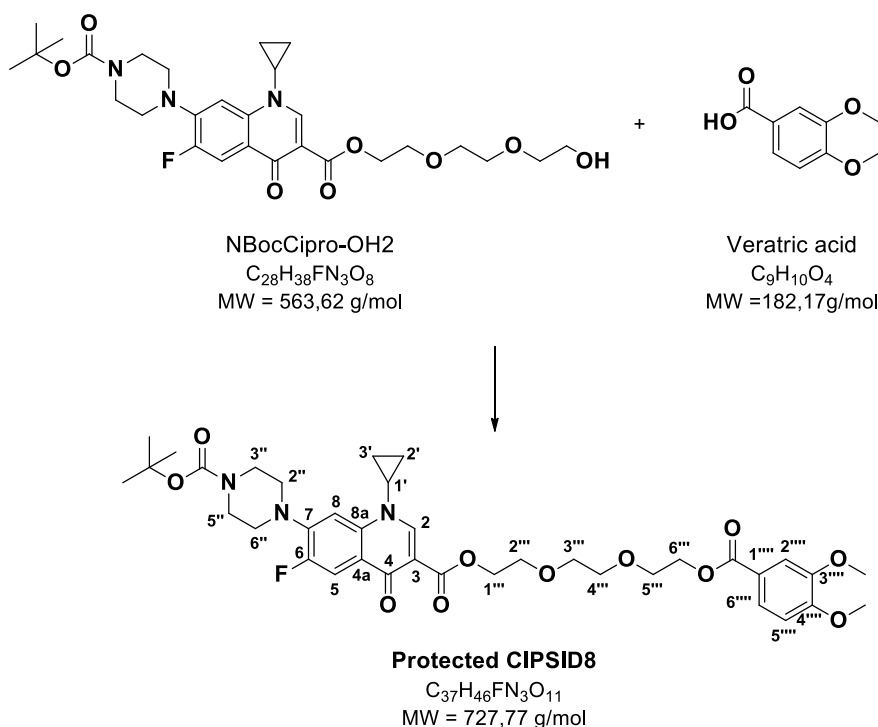


In a 100 mL round-bottom flask, veratric acid (0.29 g, 1.6 mmol), HBTU (1.5 g, 3.9 mmol), DMAP (77.1 mg, 0.6 mmol), TEA (1.1 mL, 7.9 mmol) were dissolved in DCM (40 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then, NBocCipro-OH1 (0.5g, 1 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL) and a saturated solution of NaHCO_3 (10 mL). The organic phase was dried over anhydrous

Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using a gradient elution system of DCM/MeOH (9.5:0.5 to 9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were joined, and the solvent evaporated under reduced pressure. A second flash column chromatography was performed on the attained product, using an isocratic elution system of DCM/MeOH (9:1). The purest fractions were recrystallised from diethyl ether/n-hexane/MeOH, yielding a white powder. The attained powder was characterised by ¹H NMR, showing its correspondence to the desired product.

η=12%; ¹H NMR (400 MHz, CDCl₃) δ (in ppm) = 1.12 (2H, m, H-2' or H-3'), 1.31 (2H, m, H-2' or H-3'), 1.68 (9H, s, -C(CH₃)₃), 3.24 (4H, t, J_{H-H} = 4.9 Hz, H-2'' and H-6''), 3.45 (1H, m, H-1'), 3.66 (4H, t, J_{H-H} = 4.8 Hz, H-3'' and H-5''), 3.93 (6H, s, -OCH₃), 3.97-4.05 (1H, m, O-CH₂CH₂-O), 6.89 (1H, d, J_{H-H} = 8.4 Hz, H-8), 7.30 (1H, d, J_{H-H} = 6.8 Hz, H-2'''), 7.46 (1H, dd, J_{H-H} = 8.8 Hz, H-5'''), 7.73 (1H, dd, J_{H-H} = 8.8 Hz, H-6'''), 8.05 (1H, d, J_{H-F} = 13.3 Hz, H-5), 8.53 (1H, s, H-2).

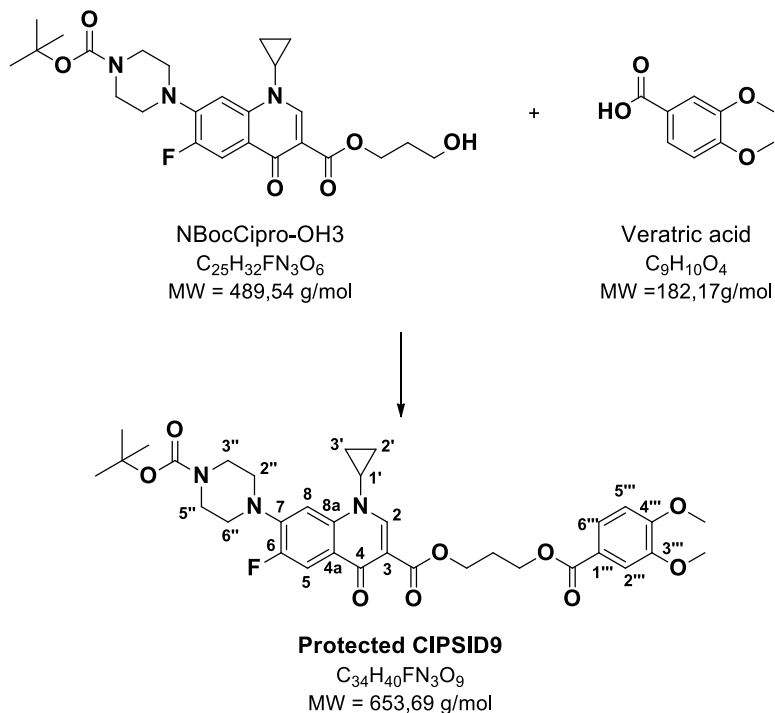
3.7.2. Synthesis of Protected CIPSID8: 2-(2-(2-((3,4-dimethoxybenzoyl)oxy)ethoxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



In a 100 mL round-bottom flask, veratric acid (0.24 g, 1.3 mmol), HBTU (1.25 g, 3.3 mmol), DMAP (65mg, 0.5 mmol), TEA (930 μ L, 6.7 mmol) were suspended in DCM (40 mL), and the suspension was kept, with stirring, at 0°C for 30 min. Then, NBocCipro-OH2 (0.5g, 0.9 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL) and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using a gradient elution system of DCM/MeOH (9.5:0.5 to 9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were joined, and the solvent evaporated under reduced pressure. A second flash column chromatography was performed on the attained product, using an isocratic elution system of DCM/MeOH (9:1). The pure fractions were joined, and the solvent evaporated under reduced pressure. The attained product was analysed by ¹H NMR, showing that it corresponded to the desired compound.

η =6%; ¹H NMR (400 MHz, CDCl₃) δ (in ppm) = 1.13 (2H, m, H-2' or H-3'), 1.33 (2H, m, H-2' or H-3'), 1.50 (9H, s, -C(CH₃)₃), 3.24 (4H, t, J_{H-H} = 5.0 Hz, H-2'' and H-6''), 3.45 (1H, m, H-1'), 3.66 (4H, t, J_{H-H} = 5.8 Hz, H-3'' and H-5''), 3.74 (4H, m, H-3''' and H-4'''), 3.85 (4H, m, H-2''' and H-5'''), 3.91 (3H, s, -OCH₃), 3.92 (3H, s, -OCH₃), 4.46 (4H, m, H-1''' and H-6'''), 6.86 (1H, d, J_{H-H} = 8.5 Hz, H-5'''), 7.31 (1H, d, J_{H-H} = 7.0 Hz, =H-8), 7.52 (1H, d, J_{H-H} = 2.0 Hz, H-2'''), 7.67 (1H, dd, J_{H-H} = 8.5 and 4.1 Hz, H-6'''), 8.05 (1H, d, J_{H-F} = 13.1 Hz, H-5), 8.56 (1H, s, H-2).

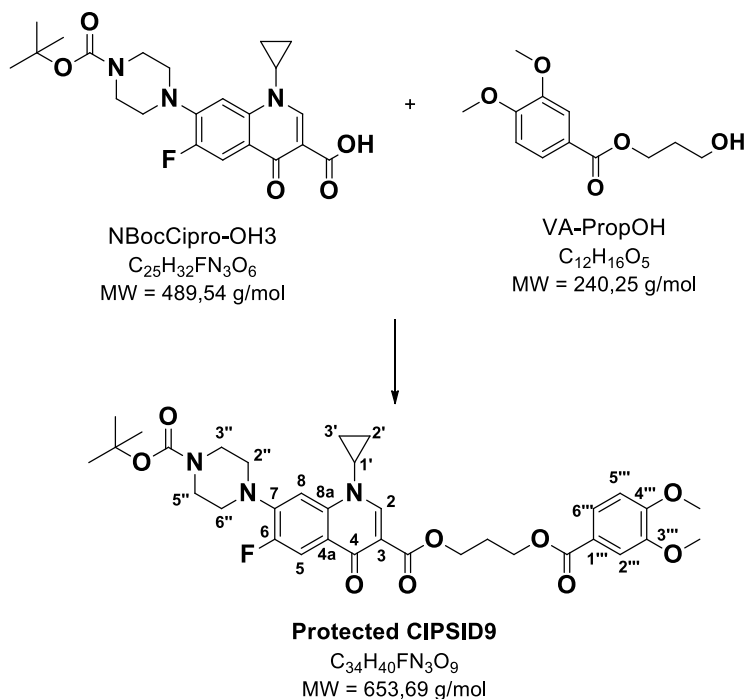
3.7.3. Synthesis of Protected CIPSID9: 3-((3,4-dimethoxybenzoyl)oxy)propyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



Procedure A

In a 100mL round-bottom flask, veratric acid (0.28 g, 1.5 mmol), HBTU (1.43 g, 3.8 mmol), DMAP (75 mg, 0.6 mmol) and TEA (1.1 mL, 7.5 mmol) were dissolved in DCM (40 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then, NBocCipro-OH3 (0.5 g, 1.0 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL) and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using a gradient elution system of DCM/MeOH (9.5:0.5 to 9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and the less impure fraction was recrystallized from diethyl ether, yielding a white powder. The attained powder was analysed by ¹H and ¹³C NMR, showing its correspondence to the desired compound with a yield of 29%.

Procedure B



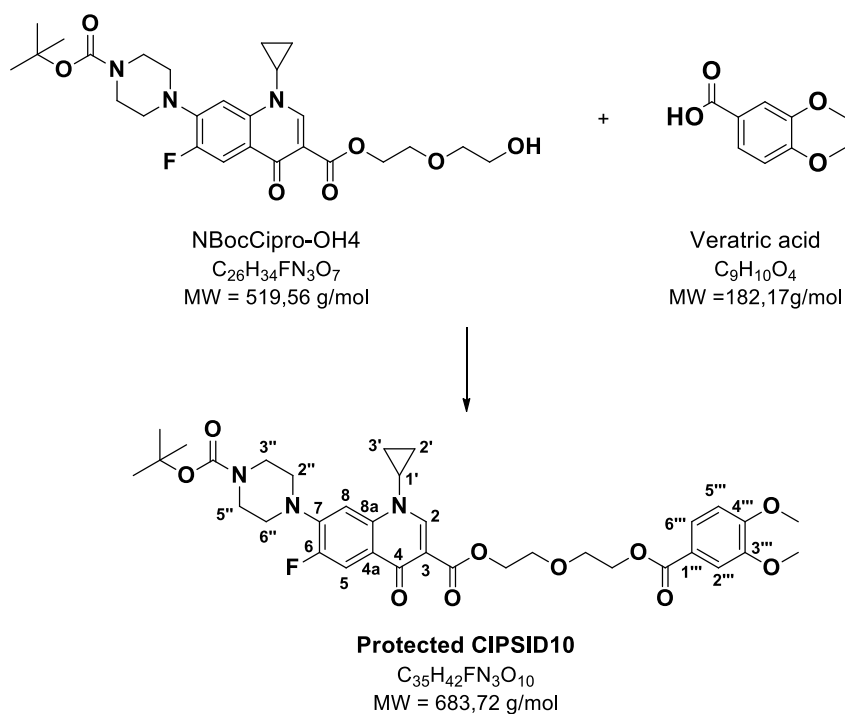
NBocCipro (0.24 g, 0.56 mmol), HBTU (0.49 g, 1.3 mmol), DMAP (9.0 mg, 0.08 mmol) and DIPEA (490 μ L, 2.8 mmol) were dissolved in DCM (30 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then, VA-PropOH (0.34 g, 1.4 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using EtOAc/DCM (7:3) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL), a 1M HCl aqueous solution (10 mL), and a saturated solution of $NaHCO_3$ (10 mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of EtOAc/DCM (7:3). The purification was tracked by TLC using EtOAc/DCM (7:3) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from diethyl ether/n-hexane, yielding a white powder. The attained powder was analysed by ^1H and ^{13}C NMR, showing its correspondence to the desired compound, with a yield of 38%.

^1H NMR (400 MHz, $CDCl_3$) δ (in ppm) = 1.12 (2H, m, H-2' or H-3'), 1.32 (2H, m, H-2' or H-3'), 1.50 (9H, s, $-C(CH_3)_3$), 2.26 (2H, m, $-O-CH_2CH_2CH_2-O-$), 3.23 (4H, t, $J_{H-H} = 5.0$ Hz, H-2'' and H-6''), 3.41 (1H, m, H-1'), 3.65 (4H, t, $J_{H-H} = 5.0$ Hz, H-3'' and H-5''), 3.90 (3H, s, $-OCH_3$), 3.91 (3H, s, $-OCH_3$), 4.50 (4H, t, $J_{H-H} = 6.3$ Hz, $-O-CH_2CH_2CH_2-O-$),

6.82 (1H, d, $J_{H-H} = 8.4$ Hz, H-5'''), 7.27 (1H, d, $J_{H-H} = 7.4$ Hz, H-8), 7.53 (1H, d, $J_{H-H} = 1.9$ Hz, H-2'''), 7.67 (1H, dd, $J_{H-H} = 8.4$ and 1.9 Hz, H-6'''), 8.04 (1H, d, $J_{H-F} = 13.1$ Hz, H-5), 8.50 (1H, s, H-2).

^{13}C NMR (100 MHz, CDCl_3) δ (in ppm) = 8.2 (C-2' and C-3'), 28.4 (-O-CH₂-CH₂-CH₂-O-), 28.4 (-C(CH₃)₃), 34.7 (C-1'), 50.0 (C-2'', C-3'', C-5'' and C-6''), 56.0 (-OCH₃), 61.8 (-O-CH₂-CH₂-CH₂-O-), 80.25 (-C(CH₃)₃), 105.1 (C-8), 110.0 (C-3), 110.2 (C-5'''), 112.0 (C-2'''), 113.4 (C-5), 122.8 (C-1'''), 123.1 (C-4a), 123.7 (C-6'''), 138.1 (C-8a), 144.6 (d, $J_{C-F} = 10$ Hz, C-7), 148.2 (C-2), 148.6 (C-3'''), 153.0 (-COO-N and C-4'''), 153.4 (d, $J_{C-F} = 242$ Hz, C-6), 165.7 (-COO-C3), 166.3 (-COO-C1'''), 173.0 (C-4).

3.7.4. Synthesis of Protected CIPSID10: 2-(2-((3,4-dimethoxybenzoyl)oxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate



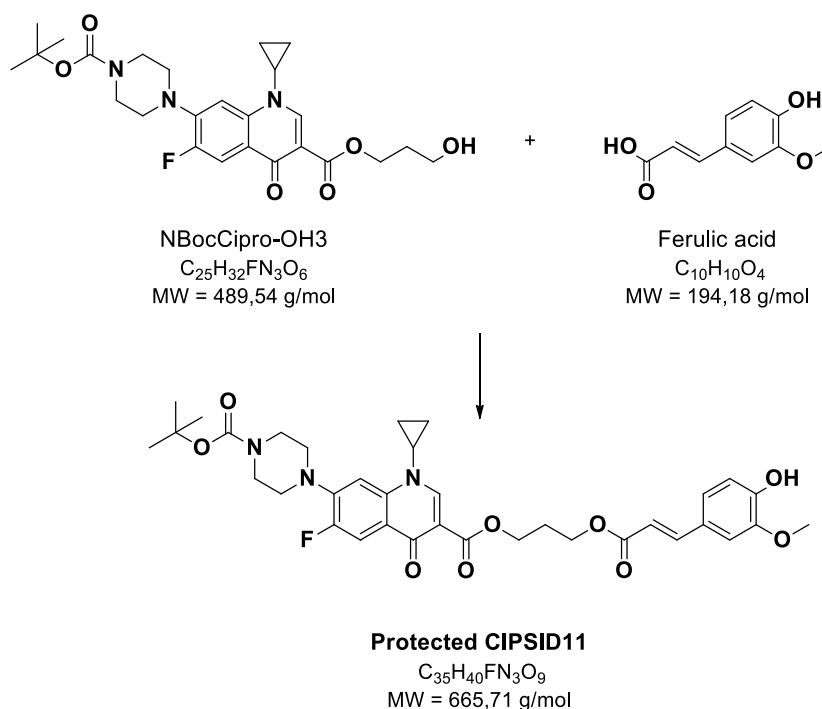
In a 100mL round-bottom flask, veratric acid (0.26 g, 1.4 mmol), HBTU (1.36 g, 3.6 mmol), DMAP (70.5 mg, 0.6 mmol) and TEA (1 mL, 7.2 mmol) were dissolved in DCM (40 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then, NBocCipro-OH4 (0.5 g, 1.0 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL) and a

saturated solution of NaHCO_3 (10 mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using a gradient elution system of DCM/MeOH (9.5:0.5 to 9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from diethyl ether, yielding a pale yellow powder. The attained powder was characterised by ^1H NMR, showing that it corresponded to the desired compound.

$\eta = 37\%$; ^1H NMR (400 MHz, CDCl_3) δ (in ppm) = 1.09 (2H, m, H-2' or H-3'), 1.27 (2H, m, H-2' or H-3'), 1.50 (9H, s, $-\text{C}(\text{CH}_3)_3$), 3.22 (4H, t, $J_{\text{H-H}} = 5.1$ Hz, H-2'' and H-6''), 3.65 (4H, t, $J_{\text{H-H}} = 5.0$ Hz, H-3'' and H-5''), 3.70 (1H, m, H-1'), 3.89 (6H, s, $-\text{OCH}_3$) 3.91 (4H, m, $-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-$), 4.48 (4H, m, $-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-$), 6.81 (1H, d, $J_{\text{H-H}} = 8.4$ Hz, H-5'''), 7.25 (1H, d, $J_{\text{H-H}} = 7.1$ Hz, H-8), 7.51 (1H, d, $J_{\text{H-H}} = 2.0$ Hz, H-2'''), 7.66 (1H, dd, $J_{\text{H-H}} = 8.4$ and 2.0 Hz, H-6'''), 8.02 (1H, d, $J_{\text{H-F}} = 13.2$ Hz, H-5), 8.49 (1H, s, H-2).

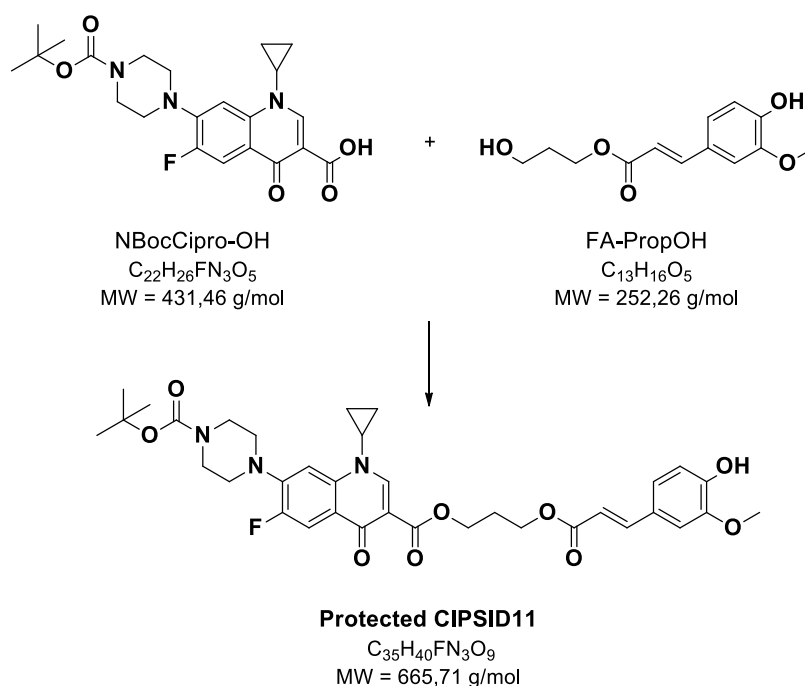
3.7.5. Synthesis of Protected CIPSID11: (*E*)-3-((3-(4-hydroxy-3-methoxyphenyl)acryloyl)oxy)propyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate

Procedure A



In a 100mL round-bottom flask, ferulic acid (0.3 g, 1.5 mmol), HBTU (1.43 g, 3.8 mmol), DMAP (74.9mg, 0.6 mmol) and TEA (1.1 mL, 7.5 mmol) were dissolved in DCM (40 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then, NBocCipro-OH3 (0.5g, 1.0 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL) and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using a gradient elution system of DCM/MeOH starting with DCM to DCM/MeOH (8:2). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from diethyl ether and DCM. The attained product was analysed by ¹H NMR, indicating that the reaction didn't occur and it corresponded to a mixture of ferulic acid and NBocCipro-OH3.

Procedure B



NBocCipro (0.25 g, 0.6 mmol), HBTU (0.5g, 1.3 mmol), DMAP (9.1 mg, 0.075 mmol) and DIPEA (498μL, 2.8 mmol) were dissolved in DCM (35 mL) and the mixture was kept, with stirring, at 0°C for 30 min. Then, FA-PropOH (0.36g, 1.4 mmol) was added, and the mixture was allowed to reach rt and left, with stirring, overnight. The reaction was monitored by TLC using DCM/MeOH (9:1) as eluent. Afterwards, the reaction

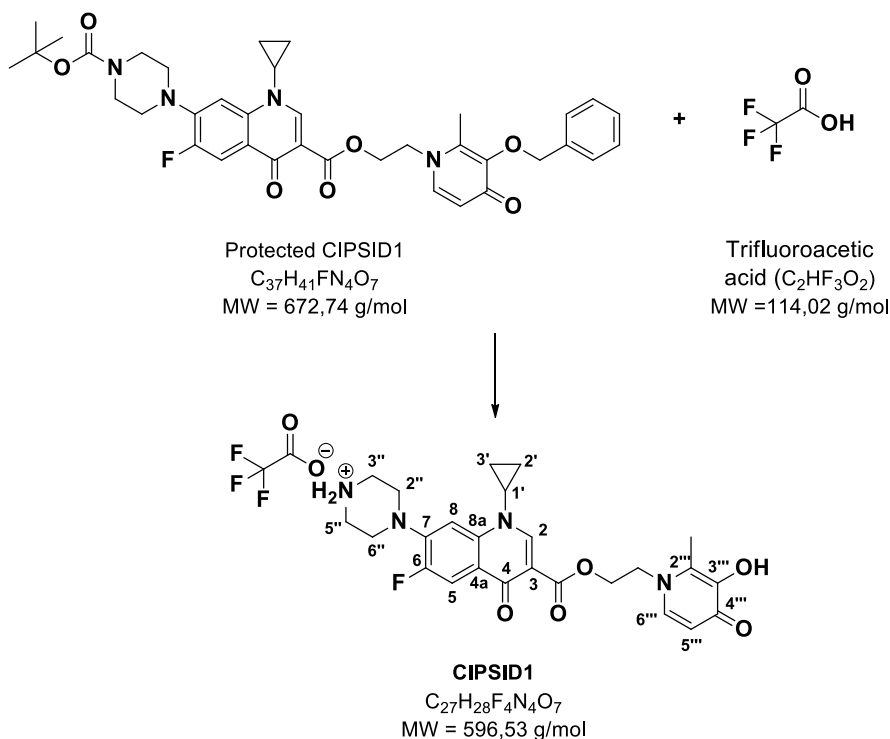
mixture was washed (3x) with deionized water (10 mL), a 1M HCl aqueous solution (10 mL), and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography in Biotage equipment, using an isocratic elution system of DCM/MeOH (9:1). The purification was tracked by TLC using DCM/MeOH (9:1) as eluent. The fractions were collected regarding the TLC profile, and those presenting the lowest number of impurities were recrystallized from MeOH/n-hexane. The attained product was analysed by ¹H NMR, indicating that the reaction didn't occur and it corresponded to a mixture of NBocCipro and FA-PropOH.

3.8. Synthesis of Final Compounds: Deprotection Reactions

3.8.1. Deprotection Reactions of the protected CIPSID1 to protected CIPSID6

Generic Procedure: The corresponding Protected CIPSID (0.74 mmol) was dissolved in TFA (2mL) and toluene (2mL), and the mixture was kept, with stirring, overnight at 60°C. After evaporating TFA and toluene under reduced pressure, the crude product was dissolved in a proper solvent and a catalytic hydrogenation was carried out, for ~2h, in the presence of a Pd/C (palladium on carbon) catalyst. The purification process, yield, and NMR analysis for each synthesis are detailed below.

3.8.1.1. Synthesis of CIPSID1: 4-(1-cyclopropyl-6-fluoro-3-((2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)-piperazin-1-ium-2,2,2-trifluoroacetate

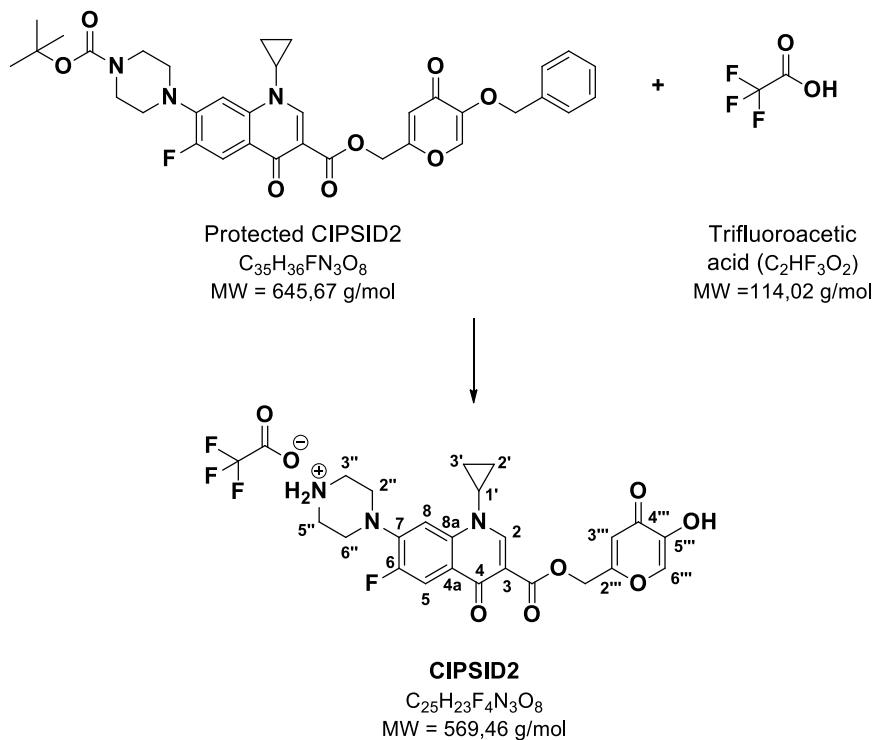


The crude product was dissolved in MeOH, and after the catalytic hydrogenation, the resulting oil was recrystallized from diethyl ether/MeOH. The attained powder was characterized by ^1H NMR and ^{13}C NMR, showing its correspondence to the desired compound.

$\eta=12\%$; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.06 (2H, m, H-2' or H-3'), 1.28 (2H, m, H-2' or H-3'), 2.58 (3H, s, $-CH_3$), 3.34 (4H, m, H-2'' and H-6''), 3.47 (4H, m, H-3'' and H-5''), 3.68 (1H, m, H-1'), 4.53 (2H, t, $J_{H-H} = 4.7$ Hz, $-OCH_2CH_2-N-$), 4.65 (2H, m, $-OCH_2CH_2-N-$), 6.97 (1H, d, $J_{H-H} = 6.8$ Hz, H-5'''), 7.48 (1H, d, $J_{H-H} = 7.2$ Hz, H-8), 7.81 (1H, d, $J_{H-F} = 13.1$ Hz, H-5), 8.26 (1H, d, $J_{H-H} = 6.9$ Hz, H-6'''), 8.36 (1H, s, H-2), 9.21 (2H, *Brs*, $-NH$ and OH).

^{13}C NMR (100 MHz, DMSO) δ (in ppm) = 8.0 (C-2' and C-3'), 12.9 ($-CH_3$), 35.4 (C-1'), 43.2 (C-2'' and C-6''), 47.0 (C-3'' and C-5''), 54.2 ($-O-CH_2CH_2-N-$), 62.7 ($-O-CH_2CH_2-N-$), 107.3 (C-8), 108.6 (C3), 110.9 (C-5'''), 112.3 (d, $J_{C-F} = 23.0$ Hz, C-5), 123.0 (d, $J_{C-F} = 7$ Hz, C-4a and C-2'''), 138.4 (C-8a), 139.5 (C-6'''), 143.3 (d, $J_{C-F} = 10.0$ Hz, C-7), 143.97 (C-3'''), 149.2 (C-2), 153.0 (d, $J_{C-F} = 245$ Hz, C-6), 162.0 ($-COO-C3$), 164.2 (C-4'''), 173.0 (C-4).

3.8.1.2. Synthesis of CIPSID2: 4-(1-cyclopropyl-6-fluoro-3-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate

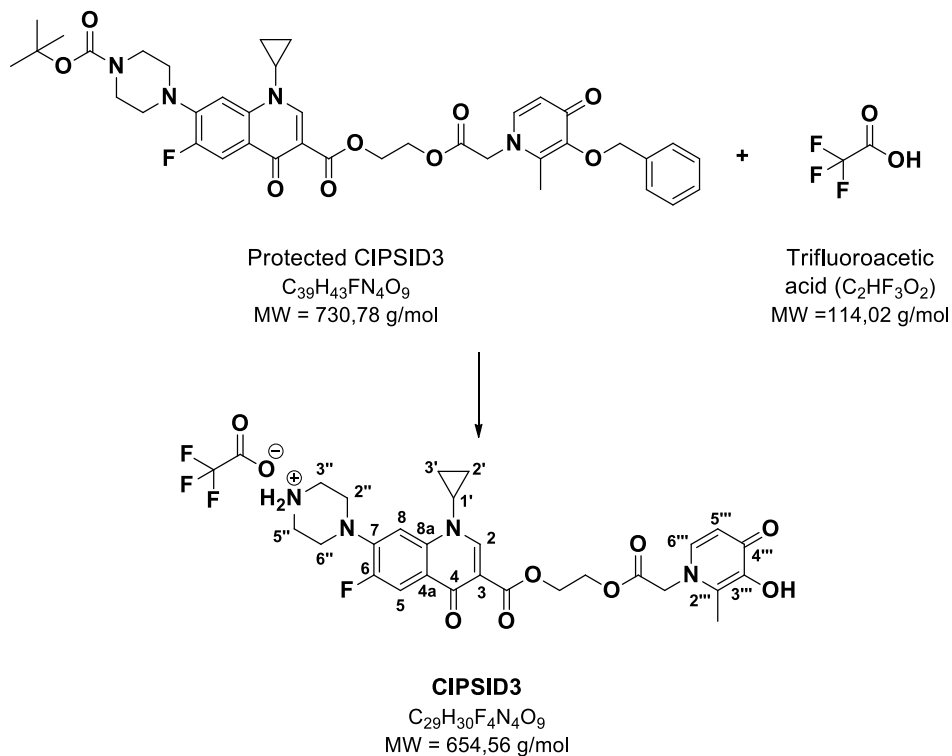


The crude product was dissolved in MeOH, and after the catalytic hydrogenation, the resulting oil was recrystallized from diethyl ether/MeOH. The product was analysed by ^1H NMR and ^{13}C NMR, showing its correspondence to the desired compound.

$\eta = 11\%$; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.11 (2H, m, H-2' or H-3'), 1.26 (2H, m, H-2' or H-3'), 3.20 (1H, m, H-1'), 3.29 (4H, m, H-2'' and H-6''), 3.73 (4H, m, H-3'' and H-5''), 4.95 (1H, s, -OH), 5.12 (2H, s, -O-CH₂-), 6.63 (1H, s, H-3'''), 7.47 (1H, d, $J_{H-H} = 7.2$ Hz, H-8), 7.82 (1H, d, $J_{H-F} = 13.0$ Hz, H-5), 8.09 (1H, s, C-6'''), 8.51 (1H, s, H-2).

^{13}C NMR (100 MHz, DMSO) δ (in ppm) = 8.1 (C-2' and C-3'), 35.5 (C-1'), 43.2 (C-2'' and C-6''), 47.1 (C-3''' and C-5'''), 61.4 (-CH₂, -O-CH₂-), 107.4 (C-8), 108.5 (C-3), 112.4 (d, $J_{C-F} = 14$ Hz, C-5), 123.1 (d, $J_{C-F} = 6$ Hz, C-4a), 128.6 (C-3'''), 138.5 (C-8a), 140.2 (C-6'''), 143.3 (d, $J_{C-F} = 11.0$ Hz, C-7), 146.5 (C-5'''), 149.5 (C-2), 153.0 (d, $J_{C-F} = 246$ Hz, C-6), 162.8 (C-2'''), 164.3 (C3-COO), 172.0 (C-4'''), 174.2 (C-4).

3.8.1.3. Synthesis of CIPSID3: 4-(1-cyclopropyl-6-fluoro-3-((2-(2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)acetoxyl)ethoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate

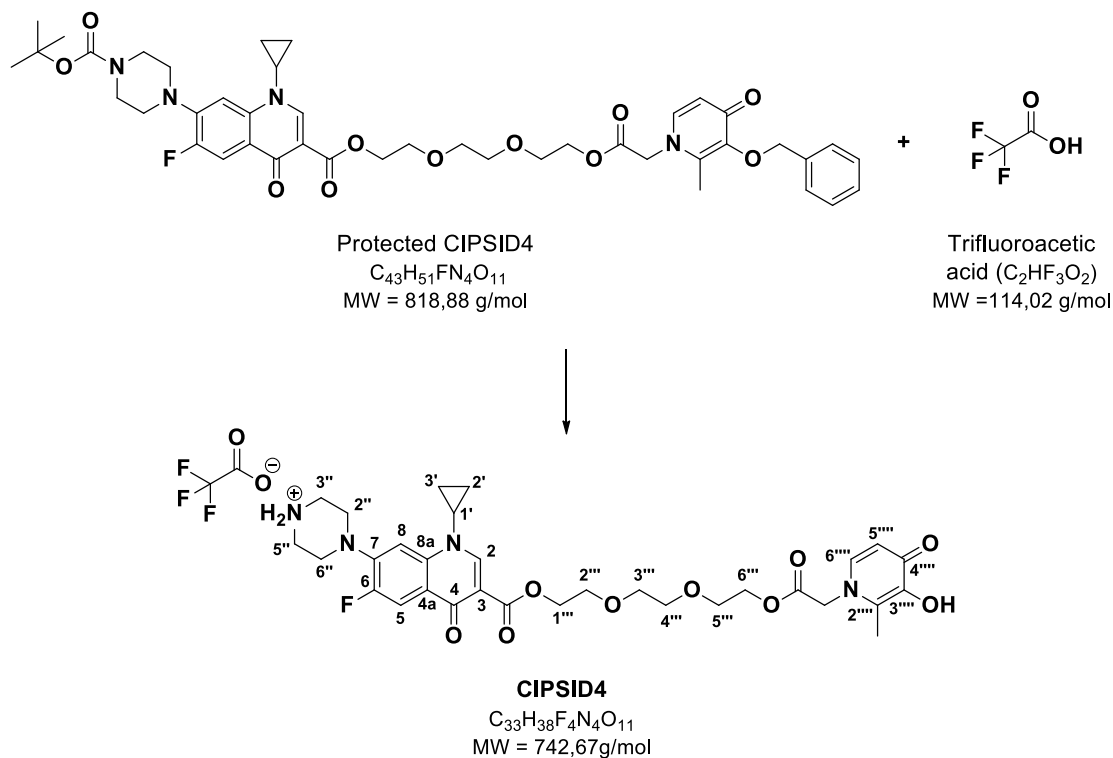


The crude product was dissolved in MeOH, and after the catalytic hydrogenation, the resulting oil was recrystallized from diethyl ether/MeOH. The attained product was analysed by ^1H NMR and ^{13}C NMR, showing its correspondence to the desired compound.

$\eta = 16\%$; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.08 (2H, m, H-2' or H-3'), 1.24 (2H, m, H-2' or H-3'), 2.29 (3H, s, $-CH_3$), 3.47 (5H, m, H-1', H-2'' and H-6''), 3.70 (4H, m, H-3'' and H-5''), 4.41 (2H, t, $J_{H-H} = 4.7$ Hz, $-OCH_2CH_2-O$), 4.51 (2H, m, $-OCH_2CH_2-O$), 5.24 (2H, s, $-COO-CH_2-N$), 6.69 (1H, d, $J_{H-H} = 7.1$ Hz, H-5'''), 7.49 (1H, d, $J_{H-H} = 7.3$ Hz, H-8), 7.83 (1H, d, $J_{H-F} = 13.1$ Hz, H-5), 7.92 (1H, d, $J_{H-H} = 7.3$ Hz, H-6'''), 8.44 (1H, s, H-2), 9.03 (2H, *Brs*, $-NH$ and OH).

^{13}C NMR (100 MHz, DMSO) δ (in ppm) = 8.0 (C-2' and C-3'), 12.4 ($-CH_3$), 35.2 (C-1'), 43.2 (C-2'' and C-6''), 47.0 (C-3'' and C-5''), 55.4 ($-COO-CH_2-N$), 59.6 ($-O-CH_2CH_2-O$), 64.0 ($-O-CH_2CH_2-O$), 107.3 (C-8), 109.2 (C-3), 111.2 (C-5'''), 112.3 (d, $J_{C-F} = 22.0$ Hz, C-5), 118.7 (d, $J_{C-F} = 7$ Hz, $-C(CH_3)$), 123.0 (C-4a), 138.5 (C-8a), 139.7 (C-6'''), 143.3 (d, $J_{C-F} = 10.0$ Hz, C-7), 144.0 (C-3'''), 148.9 (C-2), 153.0 (d, $J_{C-F} = 244$ Hz, C-6), 158.7 (d, $J_{C-N} = 31$ Hz, $-COO-CH_2-N$), 164.4 (C3- COO), 168.1 (C-4'''), 172.1 (C-4).

3.8.1.4. Synthesis of CIPSID4: 4-(1-cyclopropyl-6-fluoro-3-(13-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)-12-oxo-2,5,8,11-tetraoxatridecan-1-oyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate



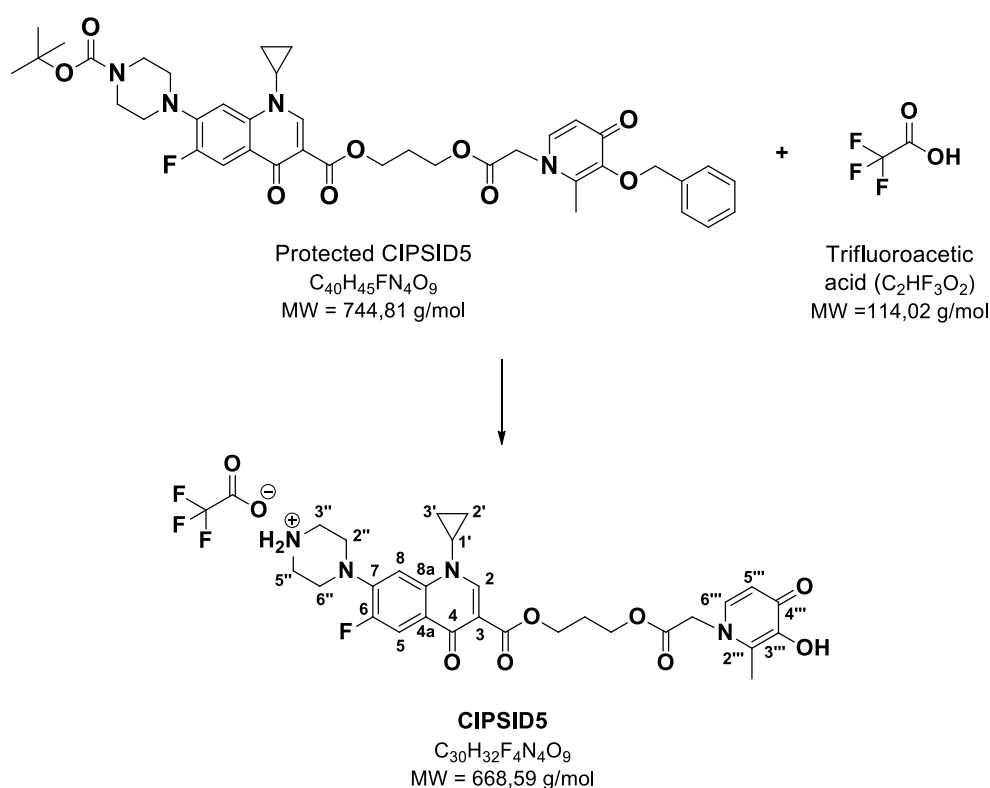
The crude product's dissolution in MeOH for catalytic hydrogenation, followed by recrystallization from diethyl ether/EtOAc/MeOH, didn't yield the desired product. The ^1H NMR analysis of the attained powder showed that only a partial deprotection occurred, with the removal of the NBoc group and maintenance of the benzyl moiety. Thus, after evaporating TFA and toluene under reduced pressure, the attained oil was dissolved in a mixture of DMF/MeOH followed by a catalytic hydrogenation, carried out for ~2h, in the presence of a Pd/C catalyst. The resulting oil was recrystallized from diethyl ether/MeOH. The attained product was analysed by ^1H NMR and ^{13}C NMR, showing its correspondence to the desired compound.

$\eta=8\%$; ^1H NMR (400 MHz, MeOD) δ (in ppm) = 1.17 (2H, m, H-2' or H-3'), 1.37 (2H, m, H-2' or H-3'), 2.38 (3H, s, $-CH_3$), 3.49 (4H, m, H-2'' and H-6''), 3.57 (4H, m, H-3'' and H-5''), 3.64 (3H, m, H-1' and H-3'''), 3.70 (5H, m, H-4''', H-5''' and $-OH$), 3.83 (2H, m, H-2'''), 4.33 (2H, m, H-6'''), 4.44 (2H, m, H-1'''), 5.14 (2H, s, $-COO-CH_2-N$), 6.79 (1H, d, $J_{H-H} = 7.1$ Hz, H-5'''), 7.55 (1H, d, $J_{H-H} = 7.2$ Hz, H-8), 7.86 (1H, d, $J_{H-H} = 7.3$ Hz, H-6'''), 7.87 (1H, d, $J_{H-F} = 13.1$ Hz, H-5), 8.70 (1H, s, H-2).

^{13}C NMR (100 MHz, MeOD) δ (in ppm) = 8.7 (C-2' and C-3'), 12.6 ($-CH_3$), 36.4 (C-1'), 44.8 (C-2'' and C-6''), 48.2 (C-3'' and C-5''), 56.9 ($-COO-CH_2-N$), 64.9 (C-1'''),

66.4 (C-6'''), 69.7 (C-2'''), 70.3 (C-5'''), 71.6 (C-3'''), 73.7 (C-4'''), 108.0 (C-8), 110.2 (C-3), 112.1 (C-5'''), 113.4 (d, $J_{C-F} = 24.0$ Hz, C-5), 119.6 (C-2''') 124.3 (C-4a), 139.8 (C-8a), 140.5 (C-6'''), 145.1 (d, $J_{C-F} = 11.0$ Hz, C-7), 145.7 (C-3'''), 150.4 (C-2), 154.8 (d, $J_{C-F} = 246$ Hz, C-6), 162.9 (d, $J_{C-N} = 36$ Hz, -C(=O)-CH₂-N), 166.0 (C3-C(=O)), 168.4 (C-4'''), 175.4 (C-4)

3.8.1.5. Synthesis of CIPSID5: 4-(1-cyclopropyl-6-fluoro-3-((3-(2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)acetoxyl)propoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate

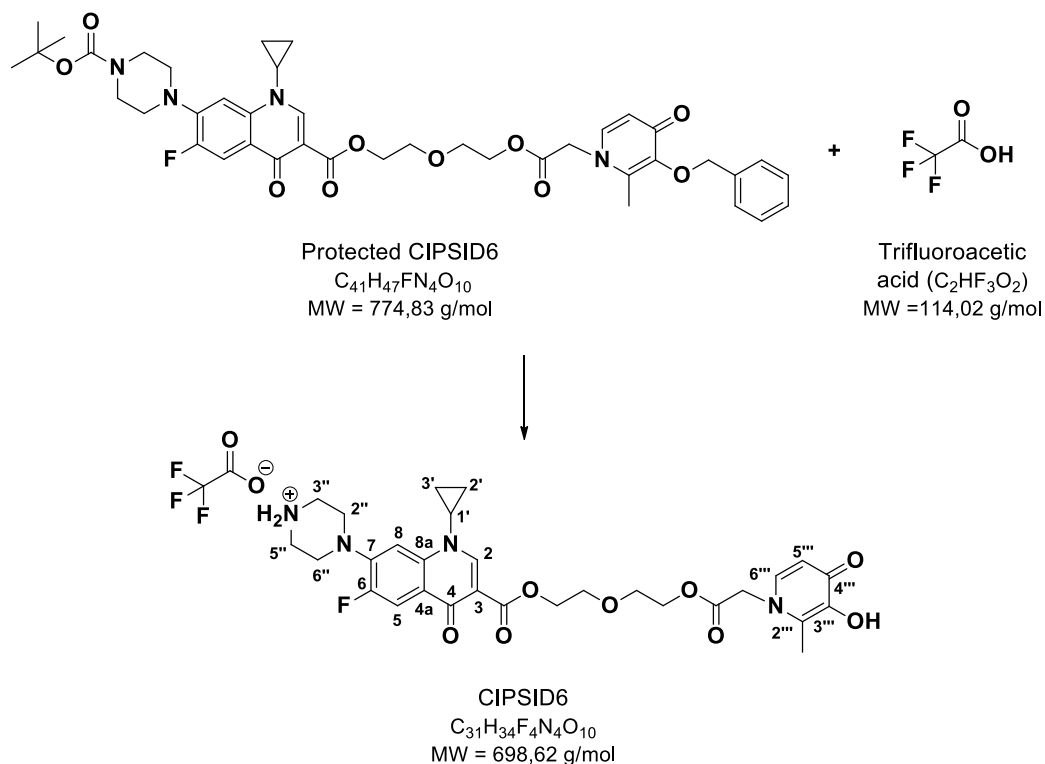


The crude product's dissolution in MeOH for catalytic hydrogenation, followed by recrystallization from MeOH/diethyl ether, didn't yield the desired product. The ¹H NMR analysis of the attained powder showed that only a partial deprotection occurred, with the removal of the NBoc group and maintenance of the benzyl moiety. Thus, after evaporating TFA and toluene under reduced pressure, the attained oil was dissolved in a mixture of DMF/MeOH followed by a catalytic hydrogenation, carried out for ~2h in the presence of a Pd/C catalyst. The resulting oil was recrystallised from diethyl EtOAc/diethyl ether/MeOH. The attained product was analysed by ¹H NMR and ¹³C NMR, showing its correspondence to the desired compound.

$\eta=9\%$; ^1H NMR (400 MHz, DMSO) δ (in ppm) = 1.08 (2H, m, H-2' or H-3'), 1.90 (2H, m, H-2' or H-3'), 2.17 (3H, s, $-\text{CH}_3$), 3.42 (4H, t, $J_{\text{H-H}} = 4.9$ Hz, H-2'' and H-6''), 3.53 (4H, t, $J_{\text{H-H}} = 4.8$ Hz, H-3'' and H-5''), 3.59 (3H, m, H-1' and $-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{O}-$), 4.28 (4H, t, $J_{\text{H-H}} = 4.8$ Hz, $-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{O}-$), 4.77 (2H, s, $-\text{COO}-\text{CH}_2-\text{N}$), 4.93 (1H, *Brs*, $-\text{OH}$), 6.19 (1H, d, $J_{\text{H-H}} = 7.1$ Hz, H-5'''), 7.47 (1H, m, H-6'''), 7.55 (1H, d, $J_{\text{H-H}} = 7.3$ Hz, H-8), 7.79 (1H, d, $J_{\text{H-F}} = 13.2$ Hz, H-5), 8.47 (1H, s, H-2).

^{13}C NMR (100 MHz, DMSO) δ (in ppm) = 8.0 (C-2' and C-3'), 11.9 ($-\text{CH}_3$), 36.3 (C-1'), 43.2 (C-2'' and C-6''), 47.0 (C-3'' and C-5''), 55.1 ($-\text{COO}-\text{CH}_2-\text{N}$), 68.8 ($-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{O}-$), 70.3 ($-\text{O}-(\text{CH}_2)_2\text{CH}_2-\text{O}-$), 72.7 ($-\text{O}-\text{CH}_2(\text{CH}_2)_2-\text{O}-$), 107.2 (C-8), 109.5 (C-3), 111.1 (C-5'''), 112.2 (d, $J_{\text{C-F}} = 22.0$ Hz, C-5), 122.9 (C-4a), 130.8 (C-2'''), 138.5 (C-8a), 139.2 (C-6'''), 143.3 (d, $J_{\text{C-F}} = 9.0$ Hz, C-7), 145.3 (C-3'''), 148.8 (C-2), 153.0 (d, $J_{\text{C-F}} = 245$ Hz, C-6), 164.7 ($-\text{COO}-\text{CH}_2-\text{N}$), 169.5 (C3- COO), 170.2 (C-4'''), 172.3 (C-4).

3.8.1.6. Synthesis of CIPSID6: 4-(1-cyclopropyl-6-fluoro-3-((2-(2-(2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)acetoxyl)ethoxyl)ethoxyl)ethoxyl)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate



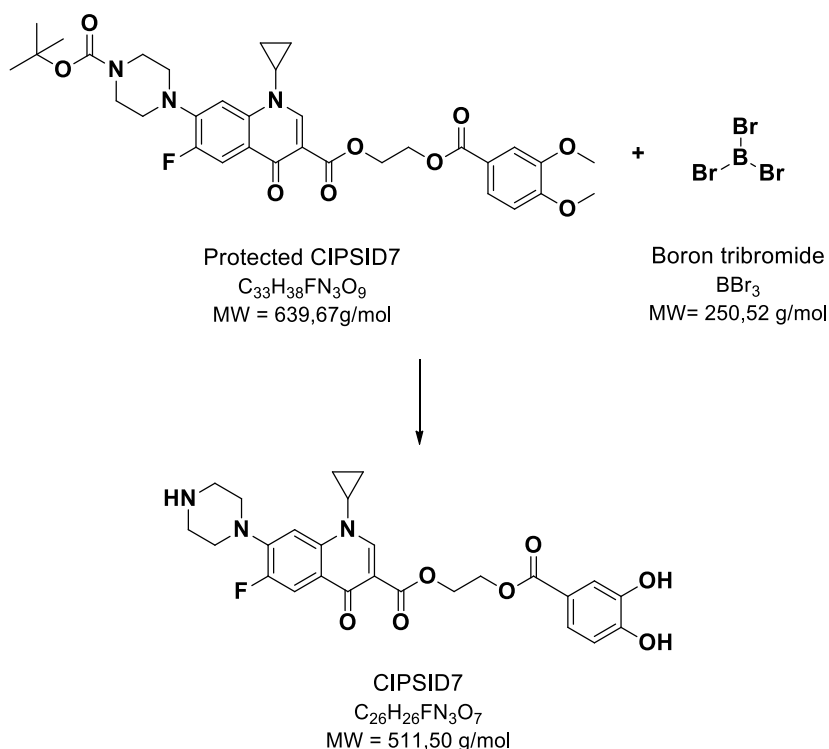
The crude product's dissolution in MeOH for catalytic hydrogenation, followed by recrystallization from MeOH/diethyl ether, didn't yield the desired product. The ^1H NMR analysis of the attained powder showed that only a partial deprotection occurred, with

the removal of the NBoc group and maintenance of the benzyl moiety. Thus, after evaporating TFA and toluene under reduced pressure, the attained oil was dissolved in a mixture of DMF/MeOH followed by a catalytic hydrogenation, carried out for ~2h in the presence of a Pd/C catalyst. The resulting oil was recrystallized from diethyl ether/MeOH. The attained product was analysed by ^1H NMR, showing its correspondence to the desired compound.

$\eta=9\%$; ^1H NMR (400 MHz, MeOD) δ (in ppm) = 1.17 (2H, m, H-2' or H-3'), 1.36 (2H, m, H-2' or H-3'), 2.45 (3H, s, $-\text{CH}_3$), 3.47 (4H, m, H-2'' and H-6''), 3.57 (4H, m, H-3'' and H-5''), 3.81 (5H, m, $-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-$ and $-\text{OH}$), 4.42 (4H, m, $-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-$), 5.28 (2H, s, $-\text{COO}-\text{CH}_2-\text{N}$), 6.92 (1H, d, $J_{\text{H-H}} = 7.1$ Hz, H-5'''), 7.59 (1H, d, $J_{\text{H-H}} = 7.2$ Hz, H-8), 7.98 (1H, d, $J_{\text{H-F}} = 13.1$ Hz, H-5), 8.05 (1H, d, $J_{\text{H-H}} = 7.1$ Hz, H-6'''), 8.73 (1H, s, H-2).

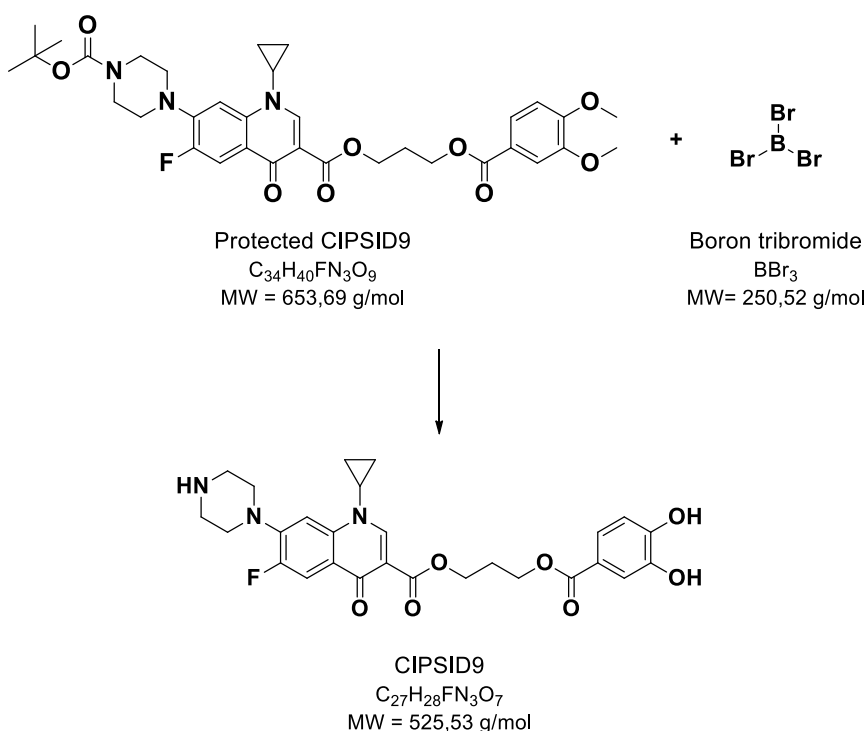
3.8.2. Deprotection Reactions of the protected CIPSID7 and protected CIPSID9

3.8.2.1. Synthesis of CIPSID7: 2-((3,4-dihydroxybenzoyl)oxy)ethyl-1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylate



In a three-neck round-bottom flask, protected CIPSID7 (0.5g, 0.8 mmol) was suspended in anhydrous DCM (26 mL), and the suspension was cooled to -80°C under an argon atmosphere. Then, boron tribromide BBr₃ (6.3 mL, 6.25 mmol) (1 M BBr₃ in anhydrous DCM) was added, and the mixture was kept, with stirring, at -80°C for 4h, in an argon atmosphere. The reaction was monitored by TLC using DCM/MeOH (8:2) as eluent. The BBr₃ was destroyed by adding ice/cold water to the reaction mixture. Afterwards, the reaction mixture was washed (3x) with deionized water (10 mL) and a saturated solution of NaHCO₃ (10 mL). The organic phase was dried over anhydrous Na₂SO₄, filtered, and the solvent evaporated under reduced pressure. The attained oil was dried in an oven at 40°C overnight and analysed by ¹H NMR. The ¹H NMR analysis showed that it didn't correspond to the desired compound but to a mixture of NBocCipro and protocatechuic acid linked to ethylene glycol.

3.8.2.2. Synthesis of CIPSID9: 3-((3,4-dihydroxybenzoyl)oxy)propyl-1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylate



In a three-neck round-bottom flask, protected CIPSID9 (200 mg, 0.3 mmol), was suspended in anhydrous DCM (10 mL) and the suspension was cooled to -80°C, under an argon atmosphere. Then, boron tribromide BBr₃ (2.5 mL, 2.5 mmol) (1 M BBr₃ in anhydrous DCM) was added, and the mixture was kept, with stirring, at -80°C for 4h, in

an argon atmosphere. The reaction was monitored by TLC using DCM/MeOH (8:2) as eluent. The BBr_3 was destroyed by adding ice/cold water to the reaction mixture. Afterwards, the reaction mixture was washed (3x) with deionized water (5 mL) and a saturated solution of NaHCO_3 (5 mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered, and the solvent evaporated under reduced pressure. The resulting oil was recrystallized from diethyl ether/MeOH, yielding a brown powder that was dried in an oven at 40°C overnight. The ^1H NMR analysis showed that it didn't correspond to the desired compound but to a mixture of NBocCipro and VA-PropOH.

3.9. Evaluation of the antimicrobial activity

The antimicrobial activity of synthesized compounds was evaluated in collaboration with CO-ADD (Community for Open Antimicrobial Drug Discovery) and with the group coordinated by Prof. Manuel Simões from LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy).

Briefly, CO-ADD screened the conjugates CIPSID1 to CIPSID6 against a panel of some of the most serious microbial threats to human health, namely *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, as well as the fungal pathogen *Candida albicans* through a biased antimicrobial high-throughput screening (HTS) [103]. The primary screening was performed by measuring absorbance at 600 nm (OD600) using a monochromator plate reader. The conjugates were tested at a single concentration (32 $\mu\text{g}/\text{ml}$) and the percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. The MIC (minimum inhibitory concentration) assessment was performed against microorganisms that showed susceptibility to the compounds tested in the Primary Screen. These assays were performed by whole cell growth inhibition assays, conducted as an 8-point dose response in duplicate ($n=2$). The MIC was determined as the lowest concentration at which the growth was fully inhibited, defined by an inhibition $\geq 80\%$.

The encouraging results from CO-ADD prompted the study of iron's effect on the conjugate's antibacterial activity. Hence, the antibacterial activity of CIPSID1, CIPSID2, and SID-CIP 3 was determined in iron-repleted (MHB) and iron-depleted media (ID-MHB). The iron-depleted media (ID-MHB) was prepared following the methodology of Hackel *et.al* [104]. These studies were performed with three bacteria species:

Pseudomonas aeruginosa PAO1, *Escherichia coli* ATCC 19138, and *Staphylococcus aureus* CECT 976. The bacteria were cultured in plate count agar (PCA) plates and allowed to grow for 24 h at 37 °C. The inoculum needed for each assay was prepared by growing bacteria overnight in Mueller-Hinton Broth (MHB), iron-free MHB, and MHB supplemented with iron (0.25 mg/L, 0.50 mg/L, and 0.75 mg/L) at 37°C with agitation (150 rpm). The bacteria culture was then adjusted to an optical density (OD) of 0.132 (around 10^8 CFU/mL). Ciprofloxacin and the deferiprone derivatives, Def2 and Def3, were also included in the assays. The MICs of each molecule were determined by the microdilution method according to the guidelines of the Clinical & Laboratory Standards Institute (CLSI) in sterile 96-well flat-bottomed polystyrene tissue culture microtiter plates (Avantor, VWR, Radnor, PA, USA). The concentrations tested were between 0.015625 mg/L and 32 mg/L. Briefly, 10 μ L of each dilution was incubated and 190 μ L of inoculum was added per well. The absorbance at 600 nm was measured at 0 h and 24 h using a microtiter plate reader. The concentration from which the values at 24 h were equal to or lower than those recorded at 0 h was recorded as the MIC.

Minimum bactericidal concentrations (MBCs) were determined by the drop plate method according to the CLSI guidelines. After the microtiter plates used for MIC determination were incubated, 10 μ L were taken from three different wells of each column, gently scraping the bottom of each well with the tip of the micropipette to loosen the adhered biofilm. Then, the plates were plated in Petri dishes with PCA and incubated at 37°C for 24 h. The MBC was then determined visually by observing the lowest concentration from which colonies did not grow.

3.10. Assessment of the toxicological profile

The toxicological profile of synthesized compounds was assessed, taking into account two parameters, cytotoxicity in human embryonic kidney cells and hemolysis of human red blood cells, to profile some adverse effects of the conjugates. The cytotoxicity in HEK293 cells was counted manually in a Neubauer hemocytometer and then plated in the 384-well plates containing the compounds to give a density of 5000 cells/well in a final volume of 50 μ L. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was used as growth media, and the cells were incubated with the compounds for 20 h at 37 °C in 5% CO₂. The cell viability was measured by fluorescence, ex: 560/10 nm, em: 590/10 nm (F560/590), after addition of 5 μ L of 25 μ g/mL resazurin (2.3 μ g/mL final concentration) and after incubation for further 3 h at 37 °C in 5% CO₂.

The hemolysis assay used human whole blood that was washed three times with 3 volumes of 0.9% NaCl and then resuspended in the same to a concentration of 0.5×10^8 cells/mL, as determined by manual cell count in a Neubauer hemocytometer. The washed cells were then added to the 384-well compound-containing plates for a final volume of 50 μ L. After a 10-minute shake on a plate shaker, the plates were then incubated for 1h at 37 °C. After incubation, the plates were centrifuged at 1000g for 10 min to pellet cells and debris, 25 μ L of the supernatant was then transferred to a polystyrene 384-well assay plate. The hemolysis was determined by measuring the supernatant absorbance at 405 nm (OD405). The values of cytotoxicity in HEK293 cells and hemolysis of human red blood cells were calculated by curve fitting the inhibition values vs. log(concentration) using a sigmoidal dose-response function with variable fitting values for top, bottom and slope. The curve fitting was implemented using Pipeline Pilot's dose-response component, resulting in similar values to curve fitting tools such as GraphPad's Prism and IDBS's XIFit

3.11. Evaluation of the Iron Chelating Activity

The iron chelating activity was assessed using a colorimetric ferrozine assay. This method is based on forming a purple-coloured complex between Fe^{2+} ions and ferrozine, whose absorbance (Abs) can be measured at a wavelength of 562 nm. Therefore, the compound's Fe chelating potential can be evaluated by the decrease in absorbance at 562 nm [105-107].

For this assay, standard solutions were prepared:

- ✓ 2.6 M aqueous ammonium acetate ($\text{CH}_3\text{COOHNH}_4$) buffer solution (pH of 6.5);
- ✓ 0.12 M aqueous hydrochloric acid (HCl);
- ✓ 0.6 mM ammonium iron(II) sulfate hexahydrate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) in 0.12 M aqueous HCl solution;
- ✓ 10 mM aqueous hydroxylamine hydrochloride ($\text{NH}_2\text{OH} \cdot \text{HCl}$) solution;
- ✓ 2.5 mM aqueous ferrozine solution;
- ✓ 100 mM EDTA (ethylenediaminetetraacetic acid) in dimethyl sulfoxide (DMSO) solution;
- ✓ 10 mM compound's solutions in DMSO: ciprofloxacin, CIPSID1, CIPSID2, CIPSID3, kojic acid, Def1 and Def3.

Preliminary to the ferrozine assay, the absorbance spectra of all the tested compounds, at a concentration of 10 mM, were recorded within the wavelength range of 350-700 nm to ensure that none of them exhibited absorbance at 562 nm.

The ferrozine assay was performed in a 96-well plate reader, using ammonium acetate buffer (pH=6.5) and a 10 mM aqueous solution of hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$) as a reducing agent.

Briefly, 20 μL of a range of sample/ EDTA standard dilutions (1, 2, 3, 4, and 5 mM) were placed in triplicate in a 96-well plate, and 100 μL of a 2.6 M aqueous ammonium acetate ($\text{CH}_3\text{COOH}\cdot\text{NH}_4$) buffer solution (pH of 6.5) and 20 μL of DMSO were added to each well. The negative control was performed by using 20 μL DMSO instead of compound/standard solution.

Subsequently, 20 μL of a 10 mM aqueous hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$) solution and 20 μL of a 0.6 mM ammonium iron(II) sulfate hexahydrate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$) in 0.12 M aqueous HCl solution. The plate was allowed to a 10-minute incubation period, at 25°C, and its absorbance was read at 562 nm – read 1.

Finally, 20 μL of a 2.5 mM aqueous ferrozine solution was placed in each well of the plates, and after a 10-minute incubation period, the plate's absorbance was read again at 562 nm – read 2.

The results were calculated according to the formula:

$$\text{Iron chelating activity (\%)} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100$$

Where $\text{Abs}_{\text{blank}}$ (without compound's sample) = $\text{Abs}_{\text{read2}} - \text{Abs}_{\text{read1}}$

$\text{Abs}_{\text{sample}}$ (with compound's sample) = $\text{Abs}_{\text{read2}} - \text{Abs}_{\text{read1}}$

Chapter 4 – Results and Discussion

4.1. Synthesis

4.1.1. Ciprofloxacin protection

Ciprofloxacin was used as the starting point for synthesising the conjugates outlined in Figures 27 and 28 of Chapter 2. One of the main purposes of this work was the structural modification of ciprofloxacin by introducing siderophore mimetics into the carboxylic acid in position 3 (Figure 29). Therefore, to diminish parallel reactions and avoid the formation of unwanted products, the amine group of the piperazine ring (at position 7, Figure 29) was protected by introducing a tert-butyloxycarbonyl protecting group (Boc).

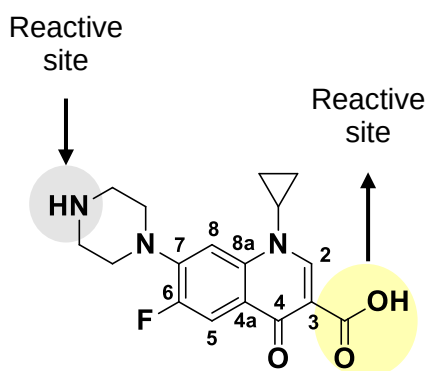


Figure 29 - Representation of the 2 main reactive points of ciprofloxacin

This protection was accomplished by the reaction of ciprofloxacin with di-tert-butyl dicarbonate (Boc anhydride), whose mechanism is outlined in Figure 30. Briefly, this mechanism encompasses a base-mediated (NaHCO_3) acyl nucleophilic substitution starting by the attack of the amine to the carbonyl group of Boc anhydride, with the formation of reactive tetrahedral intermediate, followed by the elimination of the carbonate moiety, that spontaneously decarboxylates into carbon dioxide (CO_2) and tert-butoxide anion. The protonated carbamate formed within this reaction is then neutralized by the t-butoxide anion originating the desired NBocCipro and tert-butanol as a by-side product [108, 109]. In this synthetic step, it was possible to attain the desired product, henceforth denominated as NBocCipro, with a quantitative yield.

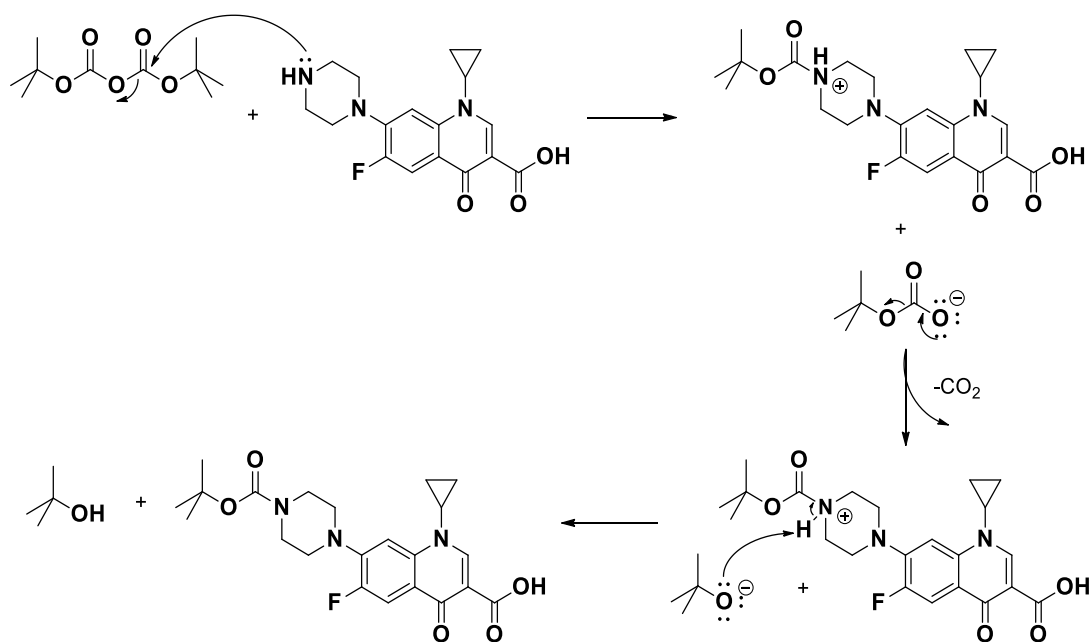


Figure 30 - Boc (di-tert-butyl dicarbonate) Protection Mechanism

4.1.1.1. NBocCipro ^1H NMR analysis

The ^1H NMR spectrum of NBocCipro (attachment 1) indicates that the desired product was obtained and is summarized in Table 5 [110-114].

Table 5 - ^1H NMR data of NBocCipro in CDCl_3

Hydrogens	Chemical shift (ppm, CDCl_3)	Integration	Multiplicity and coupling constant (J in Hz)
H2	8.74	1H	s
H5	8.00	1H	d, $J_{\text{H-F}} = 12.9$ Hz
H8	7.37	1H	d, $J_{\text{H-F}} = 7.1$ Hz
H1'	3.55	1H	m
H2' and H3'	1.21 and 1.40	4H	m
H2'' and H6''	3.30	4H	t, $J_{\text{H-H}} = 5.0$ Hz
H3'' and H5''	3.68	4H	t, $J_{\text{H-H}} = 5.0$ Hz
-C(CH ₃) ₃	1.50	9H	s
-COOH	14.92	1H	s

4.1.2. Synthesis of deferiprone derivatives – Iron chelators moieties

As ascribed in the rational design of this project (Chapter 2, point 2.6), siderophore mimetics were preconized to be attached, directly or through a linker, to NBocCipro. The chosen siderophore mimetics in this work, namely deferiprone and kojic acid derivatives (Def1, Def2, and Def3), are depicted in Figure 31.

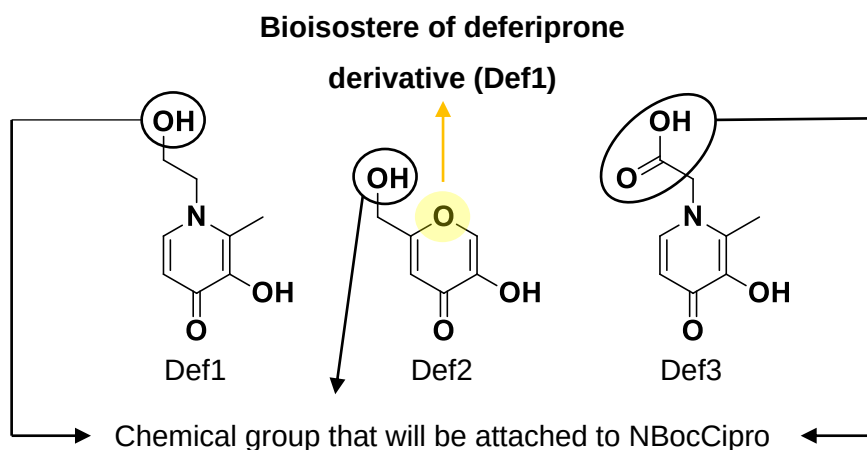


Figure 31 - Chemical structures of Def1, Def2, Def3

The linkage of these siderophore mimetics to NBocCipro via aliphatic hydroxyl or carboxyl side chains present in their structures required the synthesis of these non-commercial compounds. Thus, starting with maltol or kojic acid and postponing the reactivity of the hydroxyl group at position 3 of the pyrone ring, the 3-OH group was protected with a benzyl group (step a, Figure 32) to avoid secondary and unwanted by-side products. This step yielded protected maltol and Protected Def2.

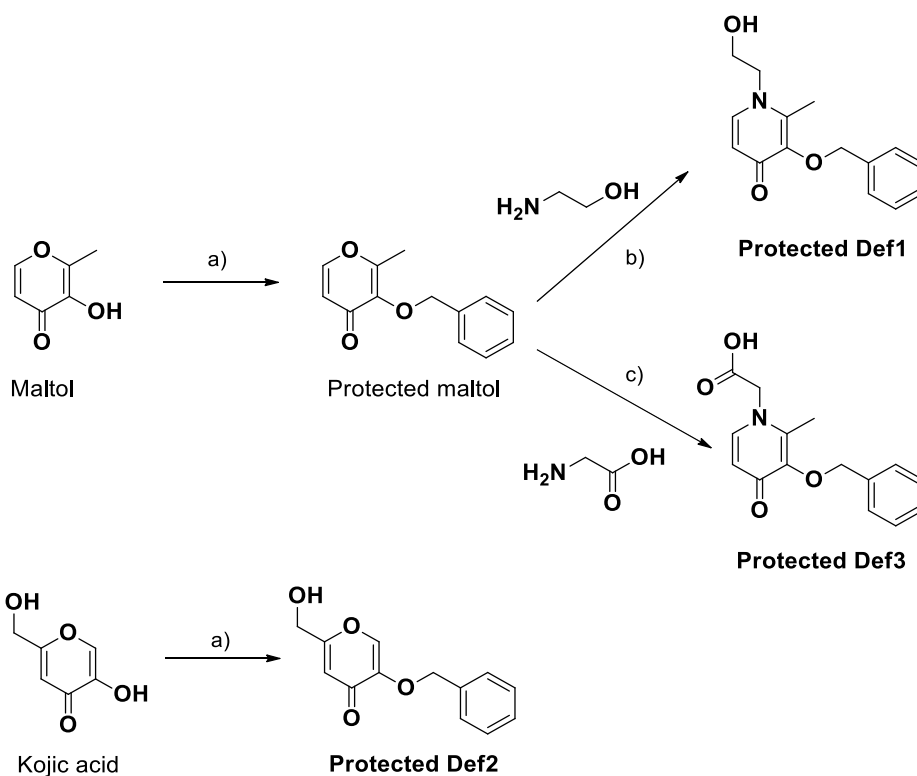


Figure 32 - Synthetic strategies used to attain the O-benzylated deferiprone and kojic acid derivatives

The O-benzylated derivatives (protected maltol and protected Def2) were accomplished by microwave-assisted organic synthesis (MW) between maltol or kojic acid with benzyl chloride under basic conditions. The reactional mechanism is shown in Figure 33 [115]. The protected Def2 was attained with a yield of 47%, and the protected maltol was used without any purification for the following reactions (steps b) and c) of Figure 32).

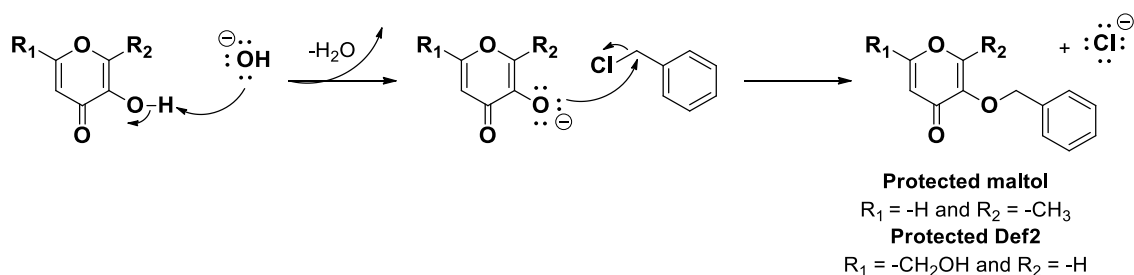


Figure 33 - O-Benzylation of maltol and kojic acid mechanism

A second MW cycle was performed with protected maltol for the coupling with a proper amine, namely 2-aminoethanol and glycine, to attain the protected deferiprone derivatives, protected Def1 and protected Def3, respectively. The mechanism of this

reaction is outlined in Figure 34. The conversion of an oxygen heterocycle to a nitrogen one occurs through the nucleophilic addition of a primary amine at the β -carbon of α,β -unsaturated carbonyl moiety. This nucleophilic attack causes the ring opening followed by a condensation reaction (with the loss of a water molecule) and ring closure [116].

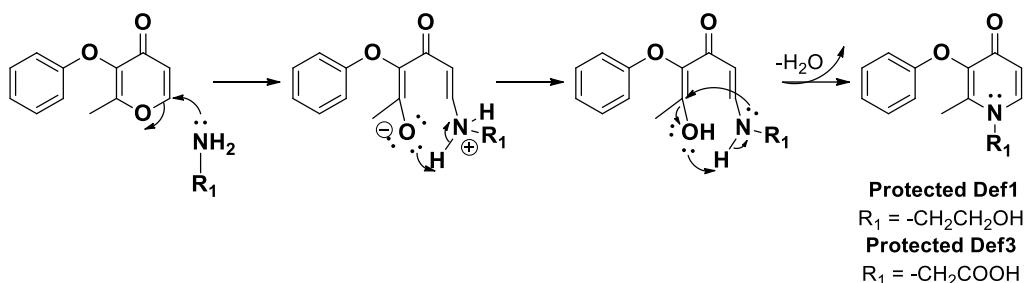


Figure 34 - Synthesis of 3-hydroxypyran-4-ones from the reaction of protected maltol with primary amines (2-aminoethanol and glycine) under basic conditions

Protected Def1 and protected Def3 were obtained with an overall yield of 60 and 47%, respectively.

4.1.2.1. Synthetic optimization methodologies to attain protected Def3

It is worth mentioning that the synthesis of protected Def3 had some drawbacks. Initial attempts using the same synthetic procedure to obtain protected Def1 were unsuccessful. The generic procedure to attain the deferiprone derivatives involves two sequential MW cycles, the first one for the formation of the protected maltol, followed by a second one to attain the desired compound. Moreover, previous work carried out by the research group has shown that the pH significantly impacts the outcomes of the second MW cycle. Thus, a pH assessment is conducted between cycles to ensure the proper pH conditions to yield the desired compound. During the first attempt to achieve protected Def3 (Chapter 3 - experimental section, synthesis of protected Def 3, procedure A, point 3.3.2.1.3), it was observed that only protected maltol was obtained, suggesting that the pH adjustment to 10 was not sufficient to provide the necessary conditions for N-heterocycle formation.

In fact, the pH adjustment will allow the primary amine to be in its free form (unprotonated), thereby promoting the nucleophilic attack on the β -carbon (C-2) of the pyrone ring.

The amine used to obtain protected Def3 is glycine, an amino acid with two pK_a values ($pK_a = 2.4$ and 9.8) – Figure 35 - and the adjustment of pH of reaction to 10 may

not be sufficient to ensure that the amine group of glycine is not protonated [117, 118]. So, the first procedure modification involved changes in the pH adjustment between the MW cycles. It has been found that a pH adjustment to 12 instead of 10 was crucial for protected Def3 synthesis. However, this modification only yielded 6% of the desired product.

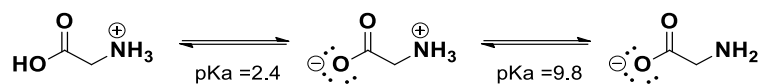


Figure 35 - pKa values of glycine

To enhance the yield, several other modifications regarding the reactional temperature and stoichiometric proportions of reagents and solvents were also performed. Increasing the reaction temperature from 100 to 120°C and the reaction time from 45 min (1st MW cycle) and 30 min (2nd MW cycle) to 1h in both MW cycles led to an increase in yield from 6% to 26%. It was also noticed that stoichiometry of the maltol and glycine reactants influenced the yield of the synthesis. It was observed a decrease in the yield (η) values according to the following ratios of maltol: glycine, 1:3 (η = 47%) > 1:4 (η = 30%) > 1:2 (η = 6 %) [119, 120].

It was also found that it is necessary a solvent mixture of MeOH/water (2:1) for the success of the reaction. A reactional attempt using water as the only solvent was unsuccessful (Chapter 3 - experimental section, synthesis of protected Def 3, procedure D, point 3.3.2.1.3).

Restoring the solvent to a MeOH/water (2:1) mixture and halving its amount led to an increase in yield from 26% to a 47% (experimental section, synthesis of protected Def 3, procedure E, point 3.3.2.1.3).

4.1.2.2. Synthetic optimization methodologies to attain protected Def3

To better understand the antibacterial and iron chelating properties of the conjugates it is also important to evaluate, in the same conditions, the siderophore mimetics *per se*. Thus, the benzyl group was removed from the protected Def1 and protected Def3 in order to have Def1 and Def3 compounds. This deprotection was achieved by the reaction of protected Def1 and protected Def3 with TFA, in toluene at 60°C, whose mechanism is depicted in Figure 36 [121, 122]. It was possible to attain Def1 and Def3 with yields of 68% and 98%, respectively. The deprotection reaction of the protected Def2 wasn't performed, since the resulting compound would correspond to kojic acid, a commercially available compound.

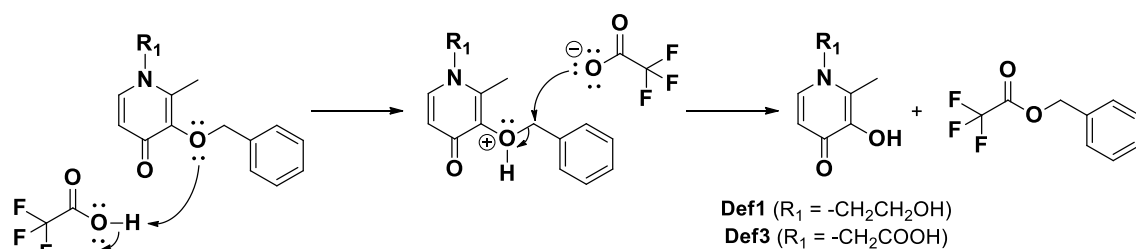


Figure 36 - O-Debenzylation of protected Def1 and protected Def3 mechanism

4.1.2.3. NMR analysis of deferiprone derivatives – Iron chelator moieties

The structural elucidation of protected Def 1, protected Def2 and protected Def3 by ^1H NMR spectra (attachments 2-5) are summarized in Table 6. The full structural elucidation of Def 1 by 1D and 2D NMR techniques, namely ^1H NMR, COSY (^1H - ^1H Homonuclear Correlated Spectroscopy), ^{13}C , DEPT, HSQC (^1H - ^{13}C Heteronuclear Simple Quantum Correlation) and HMBC (^1H - ^{13}C Heteronuclear Multiple Bond Correlation) - attachments 6 to 11 - and the ^1H NMR analysis of Def3 (attachment 12) are depicted in Table 7 [123-126].

Table 6 - ¹H NMR data of protected deferiprone derivatives (protected Def1, protected Def2 and protected Def3) in DMSO-d₆

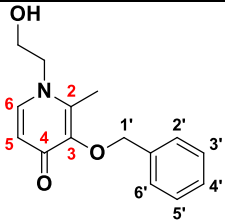
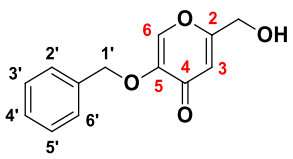
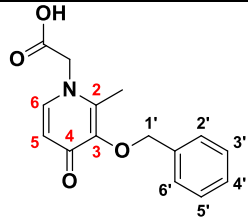
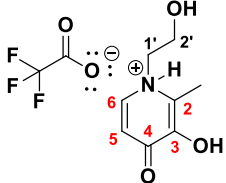
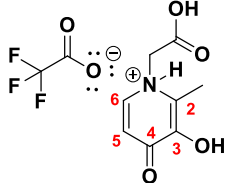
Hydrogens									
	Chemical shift (ppm, DMSO-d ₆)	Integration	Multiplicity and coupling constant (J in Hz)	Chemical shift (ppm, DMSO-d ₆)	Integration	Multiplicity and coupling constant (J in Hz)	Chemical shift (ppm, DMSO-d ₆)	Integration	Multiplicity and coupling constant (J in Hz)
H3	-	-	-	6.14	1H	d, J _{H-H} = 7.5 Hz	-	-	-
H5	6.14	1H	d, J _{H-H} = 7.5 Hz	-	-	-	6.15	1H	d, J _{H-H} = 7.5 Hz
H6	7.54	1H	d, J _{H-H} = 7.5 Hz	7.54	1H	d, J _{H-H} = 7.5 Hz	7.56	1H	d, J _{H-H} = 7.6 Hz
H1'	5.01	2H	s	5.01	2H	s	5.02	2H	s
H2' to H6'	7.33 to 7.43	5H	m	7.33 to 7.43	5H	m	7.33-7.41	5H	m
-CH ₃	2.20	3H	s	-	-	-	2.06	1H	m
-N-CH ₂ -	3.59	2H	m	-	-	-	4.78	2H	s
-CH ₂ OH	3.93	2H	t, J _{H-H} = 5.4 Hz	3.93	2H	t, J _{H-H} = 5.4 Hz	-	-	-
-OH	-	-	-	3.60	2H	m	-	-	-

Table 7 - NMR data of O-debenzylation of protected deferiprone derivatives (Def1 and Def3) in DMSO-d₆

Position								
	Chemical shift (ppm, DMSO-d ₆)			COSY correlations	HMBC correlations	Chemical shift (ppm, DMSO-d ₆)	Integration	Multiplicity and coupling constant (J in Hz)
	δ ¹ H (mult, J, Hz)	Integration	δ ¹³ C					
2	-	-	129.4	-	-	-	-	-
-CH₃	2.29 (s)	3H	<u>12.1</u>	-	129.4 (C2), 145.7 (C3), <u>55.2</u> (C2')	2.18	3H	s
3	-	-	145.7	-	-	-	-	-
4	-	-	169.4	-	-	-	-	-
5	6.10 (d, J _{H-H} = 7.3 Hz)	1H	<u>110.7</u>	7.50 (H6)	<u>138.7</u> (C6), 145.7 (C3), 169.4 (C4)	6.24	1H	m
6	7.50 (d, J _{H-H} = 7.3 Hz)	1H	<u>138.7</u>	6.10 (H5)	<u>12.1</u> (CH ₃), <u>55.2</u> (C2'), <u>110.7</u> (C5), 129.4 (C2), 145.7 (C3), 169.4 (C4)	7.60	1H	m
1'	3.63 (t, J _{H-H} = 5.4 Hz)	2H	<u>60.7</u>	3.98 (H2')	<u>55.2</u> (C2')	-	-	-
2'	3.98 (t, J _{H-H} = 5.4 Hz)	2H	<u>55.2</u>	3.63 (H1')	<u>60.7</u> (C1'), 129.4 (C2), <u>138.7</u> (C6)	-	-	-
-OH	5.04 (s)	1H	-	-	-	-	-	-
-CH ₂ COOH	-	-	-	-	-	4.87	2H	s

4.1.3. Catecholate-type siderophore mimetics

In addition to deferiprone derivatives (3-hydroxy-pyran-4-one systems), other compounds that are catecholate-type mimetic systems, namely hydroxybenzoic (protocatechuic acid derivatives) and hydroxycinnamic acids (caffeic acid derivatives), were explored. The aim was to link, via an ester bond, these catecholate mimetics to NBocCipro.

Veratric acid, a commercially available compound corresponding to 3,4-dimethoxybenzoic acid, was used as protected catechuic acid – Figure 37A. An attempt at synthesising O-benzylated caffeic acid was performed by the reaction between caffeic acid (Figure 37B) and benzyl chloride using the same procedure of the O-benylation of maltol and kojic acid (Figure 33). However, the protection reaction didn't occur. Since this reaction wasn't successful, ferulic acid, a commercially available compound, was used as the caffeic acid derivative, to be attached to NBocCipro – Figure 37C. The phenolic group of ferulic acid barely presents reactivity due to the electron-withdrawing effect of acrylic acid group [127-129].

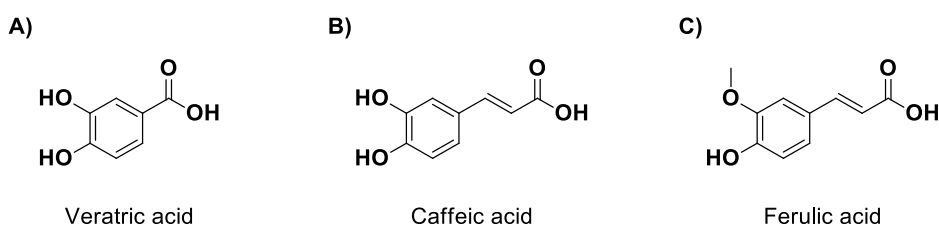


Figure 37 - Chemical structures of A) veratric acid, B) caffeic acid and C) ferulic acid

4.1.4. Synthesis of OH-linked compounds

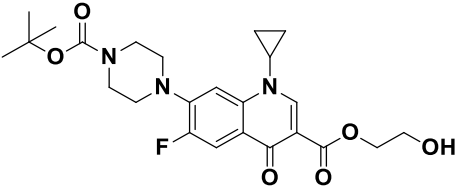
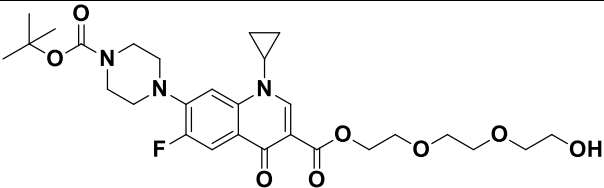
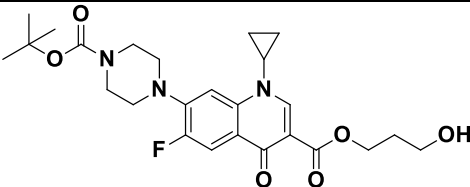
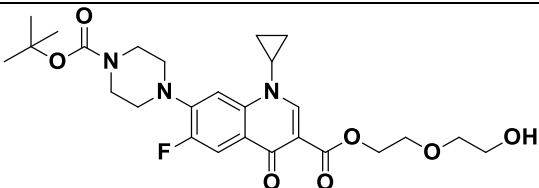
4.1.4.1. Synthesis of NBocCipro-OH compounds

To attain the conjugates with different linkers between ciprofloxacin and the siderophore mimetics (CIPSID3 to CIPSID11), the synthetic methodology encompassed an intermediary step that involved the attachment of NBocCipro with polyethylene glycols (namely ethylene glycol, triethylene glycol and diethylene glycol) and diols, to synthesize the precursors named as NBocCipro-OH.

The NBocCipro-OH derivatives were synthesized following an esterification involving the use of HBTU as a coupling agent, DMAP as a catalyst and DIPEA as a

tertiary amine. Four NBocCipro-OH derivatives were obtained, and their yields are depicted in Table 8.

Table 8 - Reaction yields of NBocCipro-OH compounds synthesis

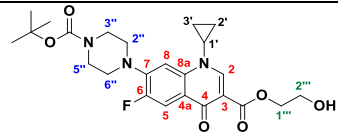
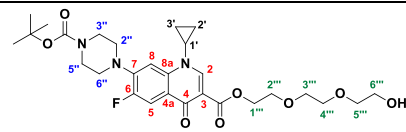
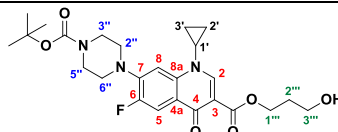
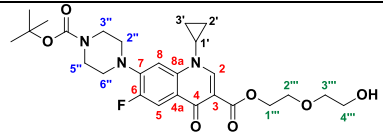
Compound	Chemical structure	Yield
NBocCipro-OH1		50%
NBocCipro-OH2		29%
NBocCipro-OH3		98%
NBocCipro-OH4		90%

NBocCipro-OH1 and NBocCipro-OH2 were synthesized with lower yields than NBocCipro-OH3 and NBocCipro-OH4. The low yield values may be due to the need of purification by column chromatography, since for NBocCipro-OH1 and NBocCipro-OH2 compounds, recrystallization wasn't enough to attain the desired pure products. These observations suggested that there was a loss of compound during column chromatography probably caused by the adsorption of hydroxyl groups of these compounds on the silica gel surface [130, 131].

4.1.4.2. NMR analysis of NBocCipro-OH compounds

The structural elucidation of NBocCipro-OH compounds (attachments 13-22) by ^1H NMR, ^{13}C NMR and DEPT techniques are summarized in Table 9 [110-114].

Table 9 - ^1H , ^{13}C and DEPT NMR data of NBocCipro-OH compounds in DMSO- d_6

Number								
	δ ^1H (mult, J, Hz)	δ ^{13}C (mult, J, Hz) *	δ ^1H (mult, J, Hz)	δ ^{13}C (mult, J, Hz)	δ ^1H (mult, J, Hz)	δ ^{13}C (mult, J, Hz)	δ ^1H (mult, J, Hz)	δ ^{13}C (mult, J, Hz)
2	8.48 (s)	-	8.44 (s)	<u>148.5</u>	8.41 (s)	<u>148.7</u>	8.44 (<i>Brs</i>)	<u>149.5</u>
3	-	-	-	109.3	-	109.7	-	110.4
4	-	-	-	172.0	-	172.1	-	173.0
4a	-	-	-	122.2	-	122.6 (d, J _{C-F} = 6.6 Hz)	-	123.4
5	7.75 (d, J _{H-F} = 13.3 Hz)	-	7.78 (d, J _{H-F} = 13.3 Hz)	<u>112.0</u> (d, J _{C-F} = 22.1 Hz)	7.70 (d, J _{H-F} = 14.0 Hz)	<u>112.1</u> (d, J _{C-F} = 22.5 Hz)	7.78 (d, J _{H-F} = 13.2 Hz)	<u>112.9</u> (d, J _{C-F} = 22.1 Hz)
6	-	-	-	139.9	-	154.3	-	155.1
7	-	-	-	144.2	-	144.2 (J _{C-F} = 10.4 Hz)	-	145.1
8	7.45 (d, J _{H-F} = 7.3 Hz)	-	7.41 (d, J _{H-F} = 7.3 Hz)	<u>107.3</u>	7.43 (d, J _{H-F} = 7.3 Hz)	<u>107.1</u> (d, J _{C-F} = 2.3 Hz)	7.47 (d, J _{H-F} = 7.3 Hz)	<u>108.0</u>
8a	-	-	-	155.8	-	138.5	-	139.4
1'	3.64 (m)	-	3.14 (m)	<u>35.3</u>	3.58 (m)	<u>35.3</u>	3.63 (m)	<u>36.1</u>
2' and 3'	1.08 and 1.27 (m)	-	1.09 and 1.26 (m)	<u>8.0</u>	1.10 and 1.26 (m)	<u>8.0</u>	1.09 and 1.25 (m)	<u>8.9</u>
2'' and 6''	3.20 (t, J _{H-H} = 5.0 Hz)	-	3.21 (t, J _{H-H} = 5.1 Hz)	<u>42.3</u>	3.18 (t, J _{H-H} = 5.1 Hz)	<u>50.0</u>	3.21 (t, J _{H-H} = 5.1 Hz)	<u>43.2</u>
3'' and 5''	3.61 to 3.66	-	3.50 (m)	<u>50.0</u>	3.58 (m)	<u>50.0</u>	3.54 (t, J _{H-H} = 4.9 Hz)	<u>50.8</u>
1'''	4.17 (t, J _{H-H} = 5.0 Hz)	-	4.28 (m)	<u>68.9</u>	4.23 (t, J _{H-H} = 6.4 Hz)	<u>62.2</u>	4.28 (t, J _{H-H} = 4.9 Hz)	<u>64.6</u>
2'''	3.61 to 3.66	-	3.70 (m)	<u>70.3</u>	1.83 (m)	<u>32.1</u>	3.70 (m)	<u>69.7</u>
3'''	-	-	3.54 (6 H, m)	<u>70.4</u>	3.58 (m)	<u>58.3</u>	3.63 (m)	<u>73.7</u>
4'''	-	-		<u>72.8</u>	-	-	3.51 (t, J _{H-H} = 4.8 Hz)	<u>61.6</u>
5'''	-	-		<u>70.5</u>	-	-	-	-
6'''	-	-		<u>60.7</u>	-	-	-	-
-C(CH ₃) ₃	1.42 (s)	-	1.43 (s)	<u>28.5</u>	1.44 (s)	<u>28.5</u>	1.43 (s)	<u>29.4</u>
- <u>C</u> (CH ₃) ₃	-	-	-	76.6	-	<u>79.6</u>	-	80.5
-OH	-	-	4.60 (m)	-	3.58 (m)	-	3.51(t, J _{H-H} = 4.8 Hz)	-
- <u>COO</u> -N	-	-	-	151.8	-	<u>151.8</u>	-	152.7
- <u>COO</u> -linker	-	-	-	164.5	-	<u>165.1</u>	-	165.6

*Not determined

4.1.4.3. Synthesis of Iron chelators-OH compounds

With the purpose of improving yields of protected CIPSID compounds other synthetic routes were thought for their synthesis, involving the attainment of Iron chelators-OH by coupling the same alcohols (above described) to Protected Def3, veratric acid and ferulic acid, followed by the linkage of the resulting products to NBocCipro (Figure 38).

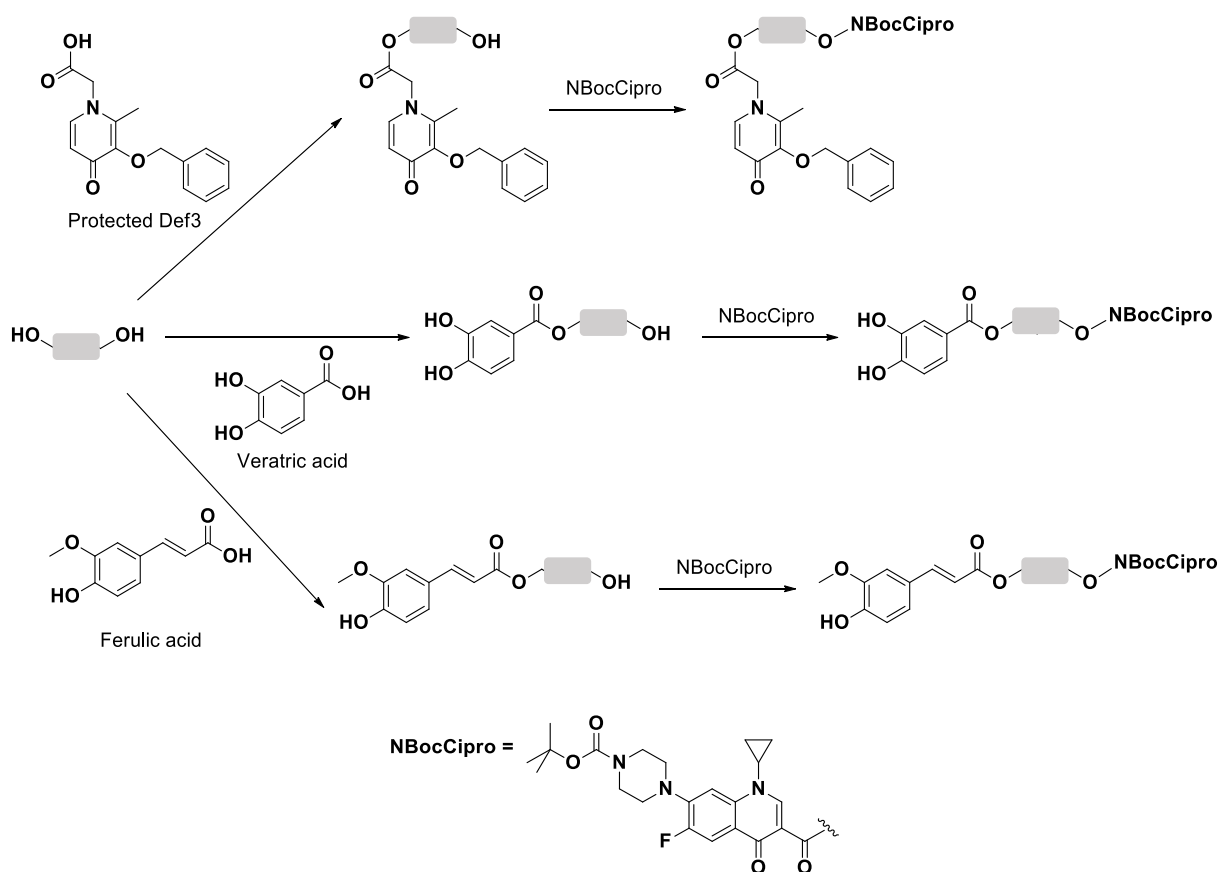


Figure 38 - Alternative synthetic route for the synthesis of protected CIPSID compounds

This synthetic approach was first performed by linking propane-1,3-diol to Protected Def3, veratric acid and ferulic acid. The synthesis of Protected Def3-OH derivative was made by an esterification reaction (using HBTU as coupling agent and DMAP as a catalyst) and the desired product was attained with a yield of 14%. Nevertheless, this synthetic procedure presented lower yield than the esterification reaction of NBocCipro-OH3 so it was decided to abandon this pathway.

Several attempts to attain veratric acid-propane-1,3-diol derivative (VA-PropOH) were performed. The esterification reaction using HBTU as a coupling agent occurred, however, in a very low the yield (7%). The Fischer esterification, using H_2SO_4 as catalyst, was ineffective but the replacement of this catalyst by p-toluenesulfonic acid (PTSA) in catalytic amounts, allowed the synthesis of VA-PropOH with a yield of 44%. The mechanism of esterification using PTSA as a catalyst is presented in Figure 39 [132-134]. PTSA proved to be a better catalyst than H_2SO_4 , due to its higher hydrophobicity, which facilitates the attack of the hydrophobic veratric acid molecule [135, 136].

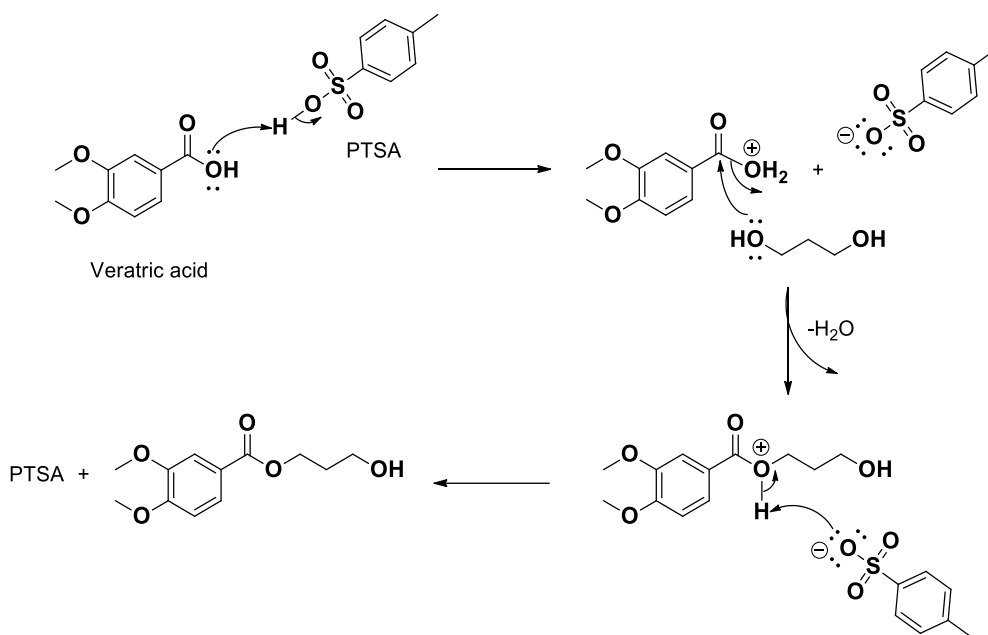


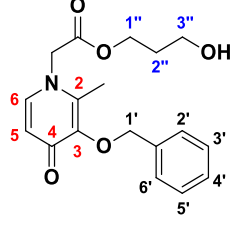
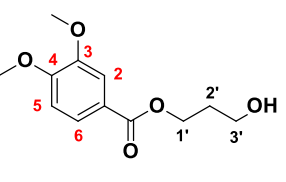
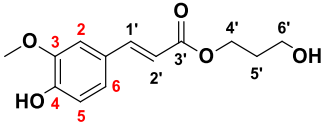
Figure 39 - PTSA catalysed esterification of veratric acid with propane-1,3-diol

Following the same line of thought, ferulic acid was coupled to propane-1,3-diol using PTSA in catalytic amounts, with a yield of 55%. The esterification reaction using HBTU as a coupling agent didn't occur.

4.1.4.4. NMR analysis of Iron chelators-OH compounds

The structural elucidation of Iron chelators-OH compounds (attachments 23-25) by ^1H NMR are summarized in Table 10 [123-126].

Table 10 - ¹H NMR data of Iron chelators-OH compounds (* in DMSO-d₆ and ** in CDCl₃)

Hydrogens			
	δ ¹ H (mult, J, Hz)*	δ ¹ H (mult, J, Hz)**	δ ¹ H (mult, J, Hz)**
H2	-	7.53 (d, J _{H-H} = 2.0 Hz)	7.03 (d, J _{H-H} = 2.0 Hz)
H5	6.19 (d, J _{H-H} = 7.5 Hz)	6.88 (d, J _{H-H} = 8.4 Hz)	6.92 (d, J _{H-H} = 8.2 Hz)
H6	7.58 (d, J _{H-H} = 7.6 Hz)	7.67 (dd, J _{H-H} = 8.4 and 2.0 Hz)	7.07 (dd, J _{H-H} = 8.2 and 2.0 Hz)
-CH ₃	2.07 (s)	-	
H1'	5.02 (s)	4.46 (t, J _{H-H} = 6.2 Hz)	7.63 (t, J _{H-H} = 15.9 Hz)
H2'	7.31-7.42 (m)	2.01 (m)	6.29 (t, J _{H-H} = 15.8 Hz)
H3'		3.77 (t, J _{H-H} = 6.1 Hz)	-
H4'		-	4.37 (t, J _{H-H} = 6.1 Hz)
H5'			1.95 (m)
H6'			3.74 (t, J _{H-H} = 6.0 Hz)
H1''	4.19 (t, J _{H-H} = 6.5 Hz)	-	-
H2''	1.72 (m)		
H3''	3.44 (t, J _{H-H} = 6.2 Hz)		
-OCH ₃	-	3.92 (s)	3.93 (s)
-OCH ₃	-	3.93 (s)	
-OH	4.55 (Brs)	2.84 (m)	5.96 (Brs)
-CH ₂ -N	4.90 (s)	-	-

4.1.5. Synthesis of conjugates protected CIPSID1 and protected CIPSID2

Through an esterification reaction, coupling between NBocCipro and protected Def1 and protected Def2 was carried out to obtain the precursors named protected CIPSID1 and protected CIPSID2 with a yield of ~20%. The ester bond formation was based on the activation of carboxylic group using the coupling reagent HBTU, and its generic mechanism is depicted in Figure 40 [137, 138].

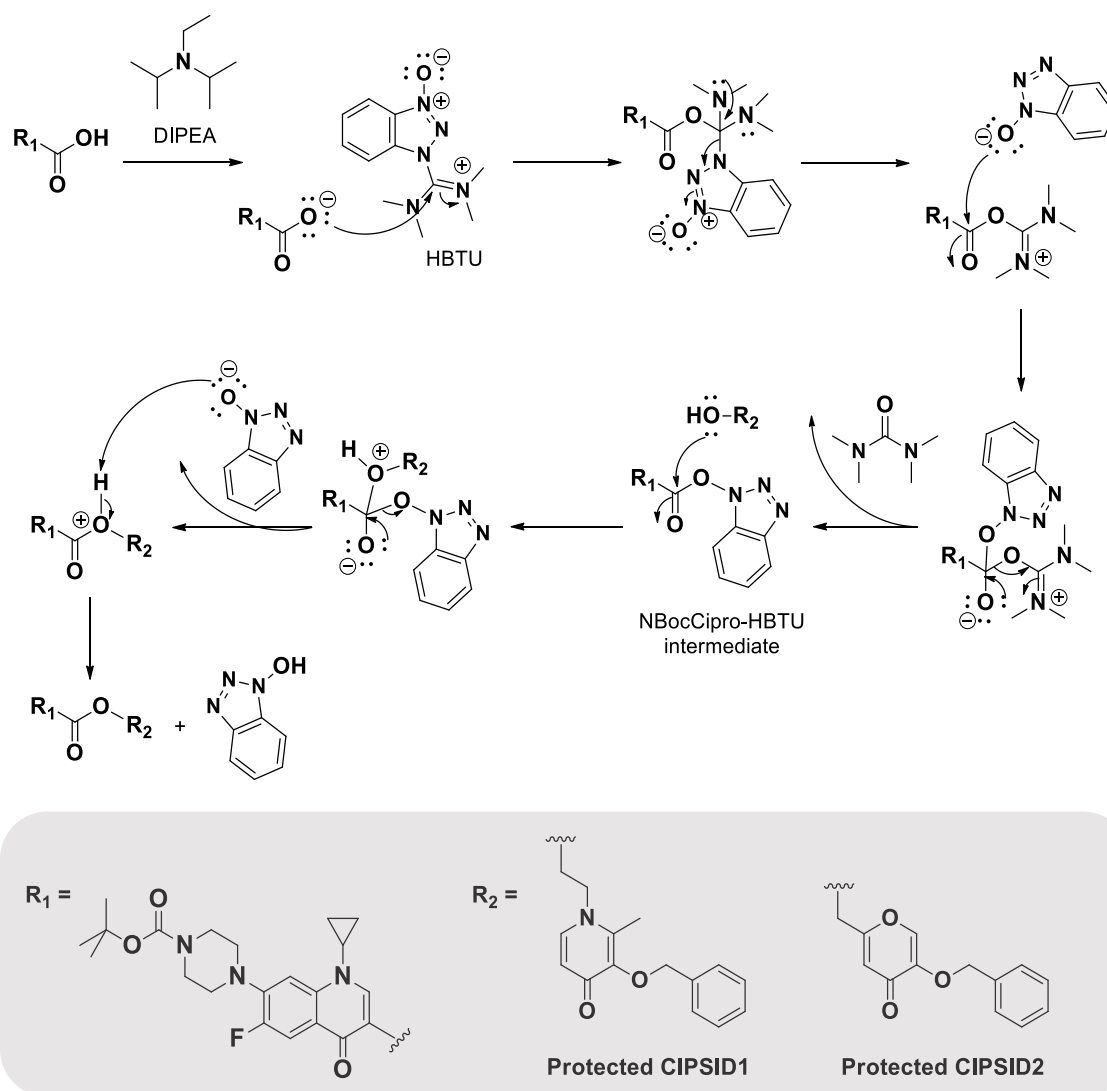


Figure 40 - Mechanism of ester bond formation using HBTU

The synthetic procedure is based on Steglich esterification with some modifications. DMAP, commonly known as an activating agent of carboxylic groups, was used as nucleophilic base catalyst in these reactions acting as a “accelerator” of the esterification reaction. As a stronger nucleophile than the respective alcohol, DMAP reacts with NBocCipro-HBTU intermediate (generated during HBTU activation reaction) which leads to the formation of a reactive amide – Figure 41. Then, this reactive amide, rapidly reacts with alcohols, giving the ester product. DMAP catalyst mechanism is based on the fact that alcohols interact more quickly with the reactive amide than with the NBocCipro-HBTU intermediate, due to its higher reactivity and chemical instability [139-141].

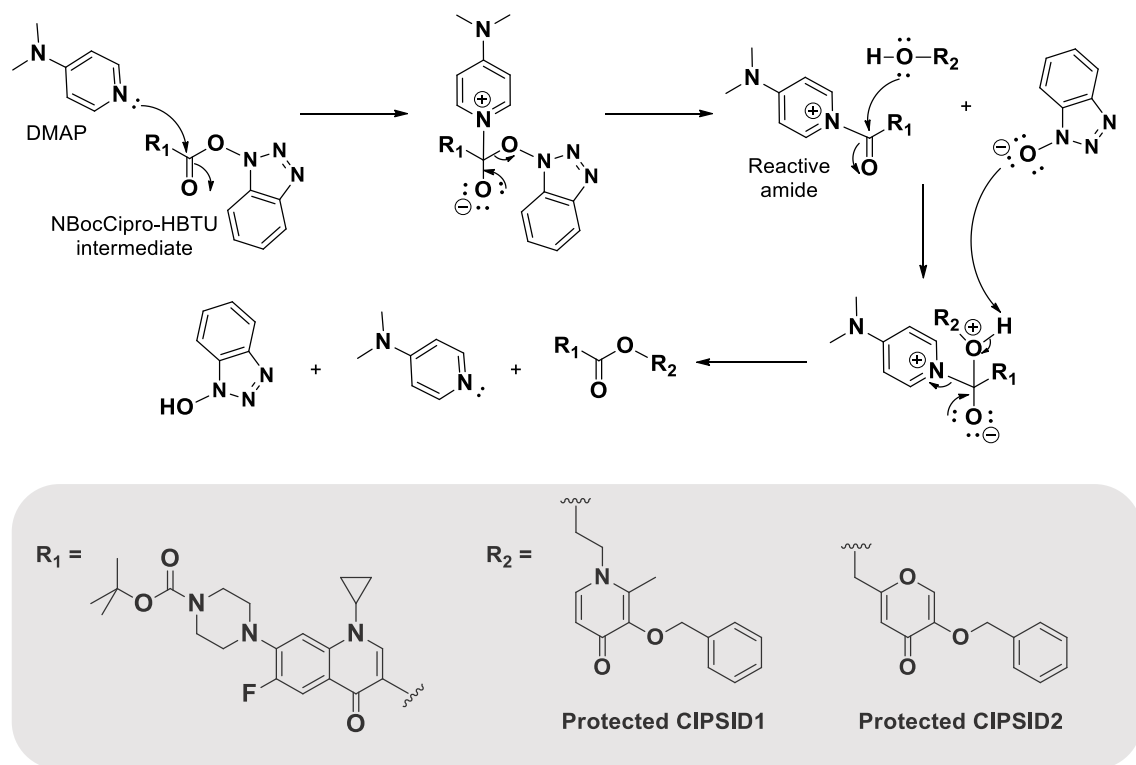


Figure 41 - DMAP catalyst mechanism

The low yield values observed for the coupling between NBocCipro and protected Def1 and protected Def2 may be due to the formation of a bulky NBocCipro-HBTU intermediate. The steric hindrance of this bulky compound makes its interaction with the nucleophilic catalyst (DMAP) difficult, which in turn causes a decrease in its activation and diminishes the formation of the intermediate corresponding to the reactive amide. The lower reactivity of NBocCipro-HBTU intermediate, when compared to the reactive amide, leads to a decrease in the reaction rate and yield values [142, 143].

4.1.5.1. NMR analysis of conjugates protected CIPSID1 and protected CIPSID2

The structural elucidation of conjugates protected CIPSID1 and protected CIPSID2 (attachments 26 and 27) by ¹H NMR are summarized in Table 11 [110-114].

Table 11 - ¹H NMR data of conjugates protected CIPSID1 and protected CIPSID2 in DMSO-d₆

Hydrogens		
	δ ¹ H (mult, J, Hz)	δ ¹ H (mult, J, Hz)
H2	8.37 (s)	8.37 (s)
H5	7.70 (d, J _{H-F} = 13.2 Hz)	7.83 (d, J _{H-F} = 13.4 Hz)
H8	7.37 (m)	7.49 (d, J _{H-F} = 7.4 Hz)
H1'	3.62 (m)	3.15 (m)
H2' and H3'	1.04 and 1.26 (m)	1.12 (m)
H2'' and H6''	3.20 (t, J _{H-H} = 5.1 Hz)	3.61 (m)
H3'' and H5''	3.54 (t, J _{H-H} = 5.0 Hz)	3.71 (m)
H1'''	4.41 (t, J _{H-H} = 4.9 Hz)	5.13 (s)
H2'''	4.35 (t, J _{H-H} = 4.9 Hz)	-
H3'''	-	6.65 (Brs)
H5'''	6.35 (d, J _{H-H} = 7.5 Hz)	-
H6'''	7.91 (d, J _{H-H} = 7.5 Hz)	8.10 (s)
-C2'''-CH ₃	2.34 (s)	-
H2''''', H3''''', H4''''', H5''''', and H6'''''	7.37 (m)	7.43 (m)
-O-CH ₂ -Bz	5.01 (s)	4.95 (s)
-C(CH ₃) ₃	1.44 (s)	1.26 (s)

4.1.6. Synthesis of conjugates protected CIPSID3 to protected CIPSID6

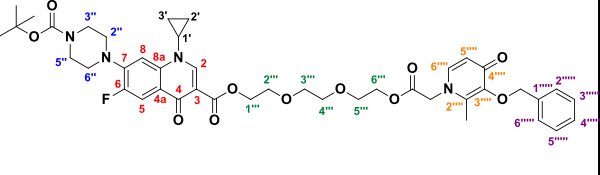
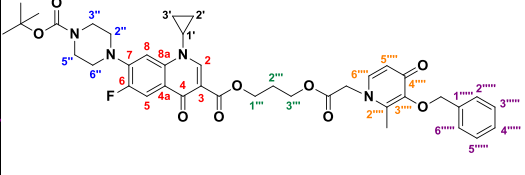
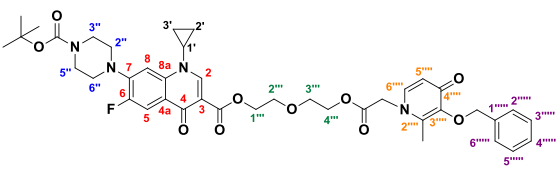
The NBocCipro-OH derivatives were attached to protected Def3 using the same esterification procedure previously mentioned. Unfortunately, these reactional conditions didn't allow the attainment of protected CIPSID3 to protected CIPSID6. The successful synthesis of these compounds was achieved with slightly modifications on the esterification procedure namely, changing the stoichiometric proportions of acid and alcohol (from 1.2:5 to 1.5:1) and by replacing DIPEA with TEA. The Protected CIPSID4 to protected CIPSID6 were synthesized, with yields ranging from 22% to 49%. These

relative low yields may be due to the low nucleophilicity of NBocCipro-OH intermediates [143, 144]. The results also suggest that the tertiary amine (DIPEA or TEA) plays a pivotal role in this reaction. Attending that both present a similar basicity ($pK_a = 8.5$ and 9.0 for DIPEA and TEA, respectively), it can be hypothesized that their chemical structure may dictate the success of this reaction due to steric hindrance reasons [145, 146].

4.1.6.1. NMR analysis of conjugates protected CIPSID3 to protected CIPSID6

The structural elucidation of conjugates protected CIPSID4 to protected CIPSID6 (attachments 28-30) by ^1H NMR, ^{13}C and DEPT techniques are summarized in Table 12. The conjugate protected CIPSID3 proceeded to deprotection without any further ^1H NMR analysis [110-114].

Table 12 - ¹H NMR data of conjugates protected CIPSID4 to protected CIPSID6 in DMSO-d₆

Hydrogens						
	δ ¹ H (mult, J, Hz)	Integration	δ ¹ H (mult, J, Hz)	Integration	δ ¹ H (mult, J, Hz)	Integration
H2	8.43 (s)	1H	8.43 (s)	1H	8.45 (s)	1H
H5	7.76 (d, J _{H-F} = 13.2 Hz)	1H	7.77 (d, J _{H-F} = 13.2 Hz)	1H	7.78 (d, J _{H-F} = 13.3 Hz)	1H
H8	7.45 (d, J _{H-F} = 7.4 Hz)	1H	7.45 (d, J _{H-F} = 7.3 Hz)	1H	7.47 (d, J _{H-F} = 7.4 Hz)	1H
H1'	3.62 (m)	1H	3.63 (Brs)	1H	3.72 (m)	1H
H2' and H3'	1.25 (m)	4H	1.07 and 1.26 (Brs)	4H	1.07 and 1.25 (m)	4H
H2'' and H6''	3.21 (m)	4H	3.21 (Brs)	4H	3.21 (t, J _{H-H} = 5.2 Hz)	4H
H3'' and H5''	3.62 (m)	4H	3.54 (Brs)	4H	3.72 (m)	4H
H1'''	4.26 (m)	2H	4.32 (t, J _{H-H} = 6.2 Hz)	2H	4.29 (m)	2H
H2'''	3.62 (m)	8H	2.00 (m)	2H	3.21 (t, J _{H-H} = 5.2 Hz)	2H
H3'''			4.23 (t, J _{H-H} = 6.2 Hz)	2H	3.64 (m)	2H
H4'''			-	-	4.29 (m)	2H
H5'''					-	-
H6'''						
H5''''	6.13 (d, J _{H-H} = 7.6 Hz)	1H	6.16 (d, J _{H-H} = 7.5 Hz)	1H	6.79 (m)	1H
H6''''	7.54 (d, J _{H-H} = 7.5 Hz)	1H	7.58 (d, J _{H-H} = 7.6 Hz)	1H	8.06 (d, J _{H-H} = 7.4 Hz)	1H
-CH ₃	2.05 (s)	3H	2.07 (Brs)	3H	2.24 (s)	3H
H2''''' to H6'''''	7.36 (m)	5H	7.35 (m)	5H	7.33-7.44 (m)	5H
-O-CH ₂ -Bz	4.99 (s)	2H	5.00 (s)	2H	5.25 (s)	2H
-CH ₂ -N	4.90 (s)	2H	4.92 (s)	2H	5.05 (s)	2H
-C(CH ₃) ₃	1.43 (s)	9H	1.43 (s)	9H	1.43 (s)	9H

4.1.7. Synthesis of conjugates protected CIPSID7 to protected CIPSID11

NBocCipro-OH compounds were also coupled with veratric acid and ferulic acid to afford the desired products (protected CIPSID7 to protected CIPSID11), using HBTU as coupling reagent and DMAP as catalyst. This coupling was successful for the linkage of NBocCipro-OH derivatives with veratric acid, however, with medium to low yields (ranging from 6% to 37%) - Table 13. These results may be explained by the low nucleophilicity of NBocCipro-OH intermediates or/and the diminished acidity of veratric acid ($pK_a = 4.14$) [143, 144].

Table 13 - Reaction yields of protected conjugates CIPSID7 to CIPSID11 synthesis

Compound	Chemical structure	Yield
Protected CIPSID7		12%
Protected CIPSID8		6%
Protected CIPSID9		29%
Protected CIPSID10		37%
Protected CIPSID11		-

Attempts were performed to enhance the yields of the veratric acid conjugates, by the synthesis of a veratric acid-OH derivative (VA-PropOH, see point 4.1.4.3), that was further coupled with NBocCipro to afford protected CIPSID9 conjugate via an esterification reaction using HBTU and DMAP, with a yield of 38% - Figure 42. Although this coupling reaction had a slightly higher yield than the one associated with the coupling between NBocCipro-OH₃ and veratric acid (39% vs 28%), it was decided to continue using the synthetic methodology based on the coupling of NBocCipro-OH with veratric acid. This is due to the fact that, that the synthesis of NBocCipro-OH presented 2.2-fold higher yields than the VA-PropOH precursor (98% vs 44%).

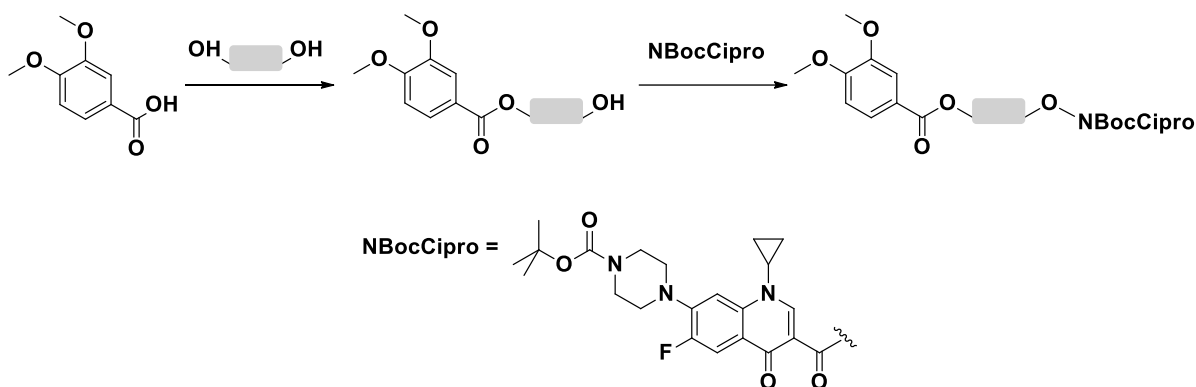


Figure 42 - Alternative synthetic route for the synthesis of conjugates protected CIPSID7 to protected CIPSID10

NBocCipro-OH₃ coupling with ferulic acid via an esterification reaction with HBTU and DMAP, to attain protected CIPSID 11 was performed, but it was unsuccessful, perhaps due to the poor reactivity of ferulic acid [147, 148]. So, following the same synthetic approach described above, ferulic acid was linked to propane-1,3-diol followed by the coupling to NBocCipro (see point 4.1.4.3). It was possible to successfully couple the ferulic acid to propane-1,3-diol, with a yield of 55%. Unfortunately, the coupling of the resulting compound (FA-PropOH) with NBocCipro, via an esterification reaction employing HBTU and DMAP, also revealed to be ineffective, probably due to the electron withdrawing nature of acrylate moiety of FA-PropOH [147, 148].

4.1.7.1. NMR analysis of conjugates protected CIPSID7 to protected CIPSID10

The structural elucidation of conjugates protected CIPSID7 to protected CIPSID10 (attachments 31-39) by ¹H NMR are summarized in Tables 14 and 15. Protected CIPSID9 was also analysed by COSY, ¹³C, DEPT, HSQC and HMBC NMR techniques – Tables 14 and 15 [110-114].

Table 14 - NMR analysis of the conjugates protected CIPSID7 to protected CIPSID10 in CDCl₃

Position							
	δ ^1H (mult, J, Hz)	δ ^1H (mult, J, Hz)	δ ^1H (mult, J, Hz)	δ ^{13}C	COSY	HMBC correlations	δ ^1H (mult, J, Hz)
2	8.53 (s)	8.56 (s)	8.50 (s)	<u>148.2</u>	-	<u>34.7</u> (C1'), 138.1 (C8a), 165.7 (COO -C1'') and 173.0 (C4)	8.49 (s)
3	-	-	-	110.0	-	-	-
4	-	-	-	173.0	-	-	-
4a	-	-	-	123.1	-	-	-
5	8.05 (d, $J_{\text{H-F}}$ = 13.3 Hz)	8.05 (d, $J_{\text{H-F}}$ = 13.1 Hz)	8.04 (d, $J_{\text{H-F}}$ = 13.1 Hz)	<u>113.6</u> (d, $J_{\text{C-F}}$ = 23 Hz)	-	138.1 (C8a), 144.6 (C7), 153.4 (C6), 173.1 (C4)	8.02 (d, $J_{\text{H-F}}$ = 13.2 Hz)
6	-	-	-	153.4 (d, $J_{\text{C-F}}$ = 242 Hz)	-	-	-
7	-	-	-	144.6 (d, $J_{\text{C-F}}$ = 10 Hz)	-	-	-
8	6.89 (d, $J_{\text{H-F}}$ = 8.4 Hz)	7.31 (d, $J_{\text{H-F}}$ = 7.0 Hz)	7.27 (d, $J_{\text{H-F}}$ = 7.4 Hz)	<u>105.1</u> (d, $J_{\text{C-F}}$ = 2.0 Hz)	-	123.1 (C4a), 138.1(C8a),144.6 (C7), 153.4 (C6)	7.25 (d, $J_{\text{H-F}}$ = 7.1 Hz)
8a	-	-	-	138.1	-	-	-
1'	3.45 (m)	3.45 (m)	3.41 (m)	<u>34.7</u>	1.12;1.32 (H2'/H3')	-	3.70 (m)
2' and 3'	1.12 and 1.31 (m)	1.12 and 1.33 (m)	1.12 and 1.32 (m)	<u>8.2</u>	3.41 (H1')	-	1.09 and 1.27 (m)
2'' and 6''	3.24 (t, $J_{\text{H-H}}$ = 4.9 Hz)	3.24 (t, $J_{\text{H-H}}$ = 5.0 Hz)	3.23 (t, $J_{\text{H-H}}$ = 5.0 Hz)	<u>50.0</u>	3.65 (H3'' and H5'')	49.97(C2''/C6'')	3.22 (t, $J_{\text{H-H}}$ = 5.1 Hz)
3'' and 5''	3.66 (t, $J_{\text{H-H}}$ = 4.8 Hz)	3.66 (t, $J_{\text{H-H}}$ = 5.8 Hz)	3.65 (t, $J_{\text{H-H}}$ = 5.8 Hz)		3.22 (H2'' and H6'')	49.97(C2''/C6'') and 152.96 (-COO-N)	3.65 (t, $J_{\text{H-H}}$ = 5.0 Hz)

Table 15 – NMR analysis of the conjugates protected CIPSID7 to protected CIPSID10 in CDCl₃ (continuation of Table 14)

Position							
	δ ¹ H (mult, J, Hz)	δ ¹ H (mult, J, Hz)	δ ¹ H (mult, J, Hz)	δ ¹³ C	COSY	HMBC correlations	δ ¹ H (mult, J, Hz)
1'''	4.65 (m)	4.46 (m)	4.50 (t, J _{H-H} = 6.3 Hz)	<u>61.8</u>	2.26 (H2''')	<u>28.4 (C2''')</u> , <u>61.7 (C1'''/C3''')</u> , <u>165.7 (COO-C1''')</u>	4.48 (m)
2'''		3.85 (m)	2.26 (m)	<u>28.4</u>	4.50 (H1''' and H3''')	<u>61.8 (C1'''/C3''')</u>	3.91 (m)
3'''	-	3.74 (m)	4.50 (t, J _{H-H} = 6.3 Hz)	<u>61.8</u>	2.26 (H2''')	<u>28.4 (C2''')</u> , <u>61.7 (C1'''/C3''')</u> , <u>165.7 (COO-C1''')</u>	3.91 (m)
4'''		3.85 (m)	-				4.48 (m)
5'''							-
6'''							-
1''''		4.46 (m)					-
2''''	7.30 (d, J _{H-H} = 6.8 Hz)	7.52 (d, J _{H-H} = 2.0 Hz)	7.53 (d, J _{H-H} = 1.9 Hz)	<u>112.0</u>	7.67 (H6''')	<u>123.7 (C6''')</u> , <u>148.6 (C3''')</u> , <u>153.0 (C4''')</u> , <u>166.3 (COO-C1''')</u>	7.51 (d, J _{H-H} = 2.0 Hz)
3''''	-	-	-	148.6	-	-	-
4''''	-	-	-	153.0	-	-	-
5''''	7.46 (dd, J _{H-H} = 8.8 Hz)	6.86 (dd, J _{H-H} = 8.5 Hz)	6.82 (d, J _{H-H} = 8.4 Hz)	<u>110.2</u>	7.53 (H2'''), 7.67 (H6''')	122.8 (C1'''), 148.6 (C3'''), 153.0 (C4''')	6.81 (dd, J _{H-H} = 8.4 Hz)
6''''	7.73 (dd, J _{H-H} = 8.8 Hz)	7.67 (dd, J _{H-H} = 8.5 and 4.1 Hz)	7.67 (dd, J _{H-H} = 8.4 and 1.9 Hz)	<u>123.7</u>	6.82 (H5''')	<u>112.0 (C2''')</u> and <u>153.0 (C4''')</u>	7.66 (dd, J _{H-H} = 8.4 and 2.0 Hz)
-OCH ₃	3.93 (s)	3.90 and 3.92 (s)	3.90 (s)	<u>56.0</u>	-	153.0 (C4''')	3.89 (s)
-C(CH ₃) ₃	1.68 (s)	1.50 (s)	1.50 (s)	<u>28.4</u>	-	-	1.50 (s)
-C(CH ₃) ₃	-	-	-	80.3	-	-	-
-COO-N			-	153.0	-	-	
-COO-C1''''			-	166.3	-	-	
-COO-C1'''			-	165.7	-	-	

4.1.8. Synthesis of final compounds

The compounds, entitled protected CIPSIDs, possess two types of protecting groups:

The N-Boc protecting group of the amine at the piperazine ring of ciprofloxacin.

The protecting O-benzyl and O-methyl groups of the hydroxyl moiety(ies) at the siderophore mimetic part of the conjugate.

The removal of the protecting groups will enable the retrieval of the ciprofloxacin antibiotic and the hydroxyl groups, providing the structural features for antibacterial and iron-chelating activity. N-Boc deprotection of the ciprofloxacin conjugates occurred with TFA in toluene, whose mechanism is explained in Figure 43 [149-151]. After N-Boc deprotection, hydrogenolysis reactions with catalytic Pd/C were carried out to remove the O-benzyl group from precursors CIPSID1 to CIPSID 6, resulting in desired compounds with the overall yields ranging from 8 to 12% [152]. These low yields can somehow be explained by parallel ester hydrolysis reactions promoted by the acidic conditions of Boc deprotection [149, 153-156].

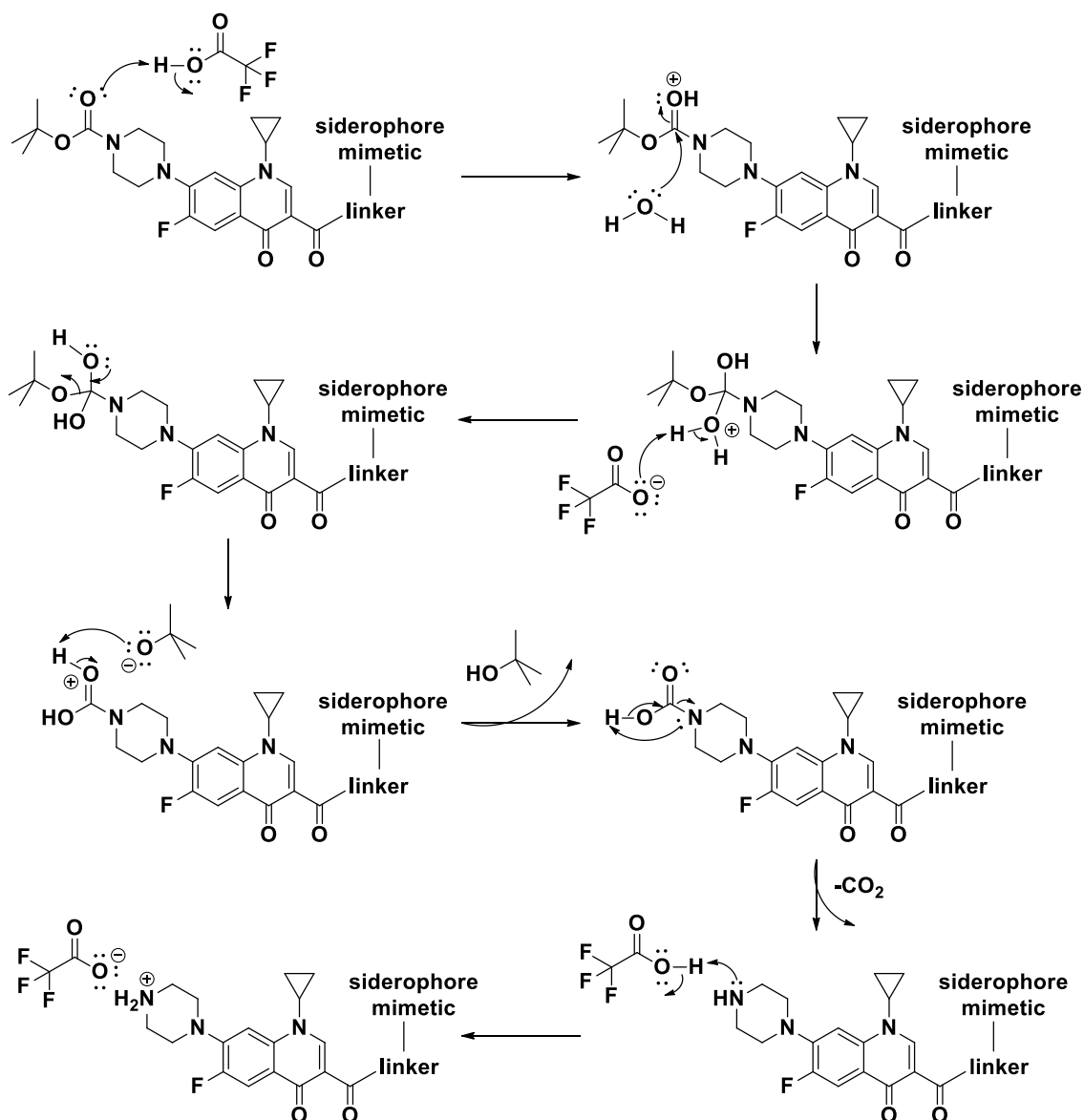


Figure 43 - Acid catalysed Boc deprotection mechanism

The deprotecting reactions for the removal of O-methyl group from compounds protected CIPSID7 to protected CIPSID10, were performed using a BBr_3 solution to recover the catechol moiety, which may display a high influence on the iron chelating properties of the final compounds [157, 158]. Unfortunately, these O-demethylation reactions were unsuccessful and ineffective. NMR analysis of the attained products suggested the occurrence of an acid hydrolysis reaction that led to the rupture of the ester bond between the carboxyl group of ciprofloxacin and the alcohol linker. This ester hydrolysis may probably occur due to the formation of hydrobromic acid during the demethylation process [149, 153-156].

The hydrolysis occurred preferentially in the ester bond established between the carboxyl group of ciprofloxacin and the linker (alcohol) since it is more unstable than the ester group involving the linker (alcohol) and protocatechuic acid moiety [153, 159-161].

4.1.8.1. NMR analysis of final compounds

The structural elucidation of compounds CIPSID1 to CIPSID6 (attachments 40-55) by ^1H NMR, ^{13}C NMR and DEPT are summarized in Tables 16 and 17 [110-114].

Table 16 - ^1H , ^{13}C and DEPT NMR data of CIPSID1, CIPSID2 and CIPSID3 in DMSO- d_6

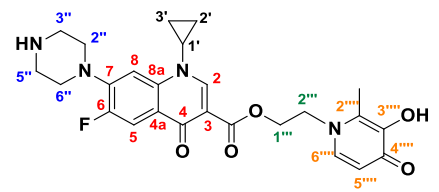
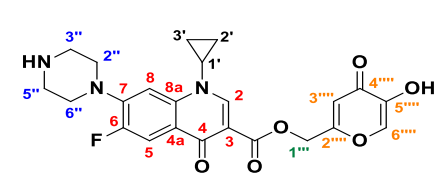
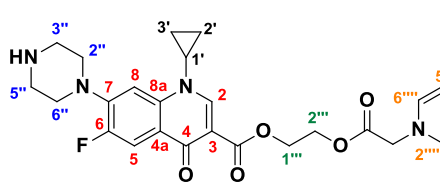
Position						
	δ ^1H (mult, J, Hz)	δ ^{13}C (mult, J, Hz)	δ ^1H (mult, J, Hz)	δ ^{13}C (mult, J, Hz)	δ ^1H (mult, J, Hz)	δ ^{13}C (mult, J, Hz)
2	8.36 (s)	<u>149.2</u>	8.51 (s)	<u>149.5</u>	8.44 (s)	<u>148.9</u>
3	-	108.6	-	108.5	-	109.2
4	-	172.0	-	174.2	-	172.1
4a	-	123.0 (d, $J_{\text{C-F}} = 7.0$ Hz)	-	123.1	-	123.0
5	7.81 (d, $J_{\text{H-F}} = 13.1$ Hz)	<u>112.3</u> (d, $J_{\text{C-F}} = 22.9$ Hz)	7.82 (d, $J_{\text{H-F}} = 13.0$ Hz)	<u>112.4</u> (d, $J_{\text{C-F}} = 14$ Hz)	7.83 (d, $J_{\text{H-F}} = 13.1$ Hz)	<u>112.3</u> (d, $J_{\text{C-F}} = 22.0$ Hz)
6	-	153.00 (d, $J_{\text{C-F}} = 245$ Hz)	-	153.0 (d, $J_{\text{C-F}} = 246$ Hz)	-	153.0 (d, $J_{\text{C-F}} = 244$ Hz)
7	-	143.3 (d, $J_{\text{C-F}} = 10.0$ Hz)	-	143.3 (d, $J_{\text{C-F}} = 11$ Hz)	-	143.3
8	7.48 (d, $J_{\text{H-F}} = 7.2$ Hz)	<u>107.3</u>	7.47 (d, $J_{\text{H-F}} = 7.2$ Hz)	<u>107.4</u>	7.49 (d, $J_{\text{H-F}} = 7.3$ Hz)	<u>107.3</u>
8a	-	138.4	-	138.5	-	138.5
1'	3.68 (m)	<u>35.4</u>	3.20 (m)	<u>35.5</u>	3.47 (m)	<u>35.2</u>
2' and 3'	1.06 and 1.28 (m)	<u>8.0</u>	1.11 and 1.26 (m)	<u>8.1</u>	1.08 and 1.24 (m)	<u>8.0</u>
2'' and 6''	3.34 (m)	<u>43.2</u>	3.29 (m)	<u>43.2</u>	3.47 (m)	<u>43.2</u>
3'' and 5''	3.47 (m)	<u>47.0</u>	3.73 (m)	<u>47.1</u>	3.70 (m)	<u>47.0</u>
1'''	4.65 (m)	<u>62.7</u>	5.12 (s)	<u>61.4</u>	4.51 (m)	<u>59.6</u> or <u>64.0</u>
2'''	4.53 (t, $J_{\text{H-H}} = 4.7$ Hz)	<u>54.2</u>	-	-	4.41 (t, $J_{\text{H-H}} = 4.7$ Hz)	<u>59.6</u> or <u>64.0</u>
2''''	-	123.0 (d, $J_{\text{C-F}} = 7.0$ Hz)	-	162.8	-	118.7
3''''	-	144.0	6.63 (Brs)	<u>128.6</u>	-	144.4
4''''	-	164.2	-	172.0	-	168.1
5''''	6.97 (d, $J_{\text{H-H}} = 6.8$ Hz)	<u>110.9</u>	-	146.5	6.69 (d, $J_{\text{H-H}} = 7.1$ Hz)	<u>111.2</u>
6''''	8.26 (d, $J_{\text{H-H}} = 6.9$ Hz)	<u>139.5</u>	8.09 (Brs)	<u>140.2</u>	7.92 (d, $J_{\text{H-H}} = 7.3$ Hz)	<u>139.7</u>
-CH ₃	2.58 (s)	<u>12.9</u>	-	-	2.29 (s)	<u>12.4</u>
-OH	9.21 (Brs)	-	4.95 (Brs)	-	9.03 (Brs)	-
-NH			-			
-COO-C3	-	162.0	-	164.3	-	164.4
-COO-C2'''		-		-	-	158.7
-CH ₂ -N		-		-	5.24 (s)	<u>55.4</u>

Table 17 - ^1H , ^{13}C and DEPT NMR data of CIPSID4, CIPSID5 and CIPSID6 (* in MeOD- d_4 and ** in DMSO- d_6)

Position					
	δ ^1H (mult, J, Hz)*	δ ^{13}C (mult, J, Hz)*	δ ^1H (mult, J, Hz)**	δ ^{13}C (mult, J, Hz)**	δ ^1H (mult, J, Hz)*
2	8.70 (s)	<u>150.4</u>	8.47 (s)	<u>148.8</u>	8.73 (s)
3	-	110.2	-	109.5	-
4	-	175.4	-	172.3	-
4a	-	124.3	-	122.9	-
5	7.84-7.87 (d, J _{H-H} = 13.1 Hz)	<u>113.4</u> (d, J _{C-F} = 24.0 Hz)	7.79 (d, J _{H-F} = 13.2 Hz)	<u>112.2</u> (d, J _{C-F} = 22.0 Hz)	7.98 (d, J _{H-F} = 13.1 Hz)
6	-	154.8 (d, J _{C-F} = 246 Hz)	-	153.0 (d, J _{C-F} = 245 Hz)	-
7	-	145.1 (d, J _{C-F} = 11.1 Hz)	-	143.3 (d, J _{C-F} = 8.9 Hz)	-
8	7.55 (d, J _{H-F} = 7.2 Hz)	<u>108.0</u>	7.55 (d, J _{H-F} = 7.3 Hz)	<u>107.2</u>	7.59 (d, J _{H-F} = 7.2 Hz)
8a	-	139.8	-	138.5	-
1'	3.64 (m)	<u>36.4</u>	3.59 (m)	<u>36.3</u>	3.81 (m)
2' and 3'	1.17 and 1.37 (m)	<u>8.7</u>	1.08 and 1.90 (m)	<u>8.0</u>	1.17 and 1.36 (m)
2'' and 6''	3.43 (m)	<u>44.8</u>	3.42 (t, J _{H-H} = 4.9 Hz)	<u>43.2</u>	3.47 (m)
3'' and 5''	3.57 (m)	<u>48.2</u>	3.53 (t, J _{H-H} = 4.8 Hz)	<u>47.0</u>	3.57 (m)
1'''	4.44 (m)	<u>64.9</u>	4.28 (t, J _{H-H} = 4.8 Hz)	<u>72.7</u>	4.42 (m)
2'''	3.83 (m)	<u>69.7</u>	3.59 (m)	<u>68.8</u>	3.81 (m)
3'''	3.70 (m)	<u>71.6</u>	4.28 (t, J _{H-H} = 4.8 Hz)	<u>70.3</u>	3.81 (m)
4'''	3.64 (m)	<u>73.7</u>	-	-	4.42 (m)
5'''	3.70 (m)	<u>70.3</u>			
6'''	4.33 (m)	<u>66.4</u>			
2''''	-	119.6			
3''''	-	145.7			
4''''	-	168.4			
5''''	6.79 (d, J _{H-H} = 7.1 Hz)	<u>112.1</u>	6.19 (d, J _{H-H} = 7.1 Hz)	<u>111.1</u>	6.92 (d, J _{H-H} = 7.1 Hz)
6''''	7.84-7.87 (d, J _{H-H} = 7.3 Hz)	<u>140.5</u>	7.47 (m)	<u>139.2</u>	8.05 (d, J _{H-H} = 7.1 Hz)
-CH ₃	2.38 (s)	<u>12.6</u>	2.17 (s)	<u>11.9</u>	2.45 (s)
-OH	-	-	4.93 (Brs)	-	-
-COO-C3		166.0	-	169.5	
-COO-CH ₂ -N		158.7 (d, J _{C-F} = 31 Hz)	-	164.7	
-CH ₂ -N	5.14 (s)	56.9	4.77 (s)	55.1	5.28 (s)

4.2. Antimicrobial activity results

Compounds CIPSID1 to CIPSID6 (Figure 44) were screened, in collaboration with CO-ADD (Community for Open Antimicrobial Drug Discovery), against a panel of some of the most serious microbial threats to human health, including *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, as well as the fungal pathogens *Cryptococcus neoformans* and *Candida albicans*.

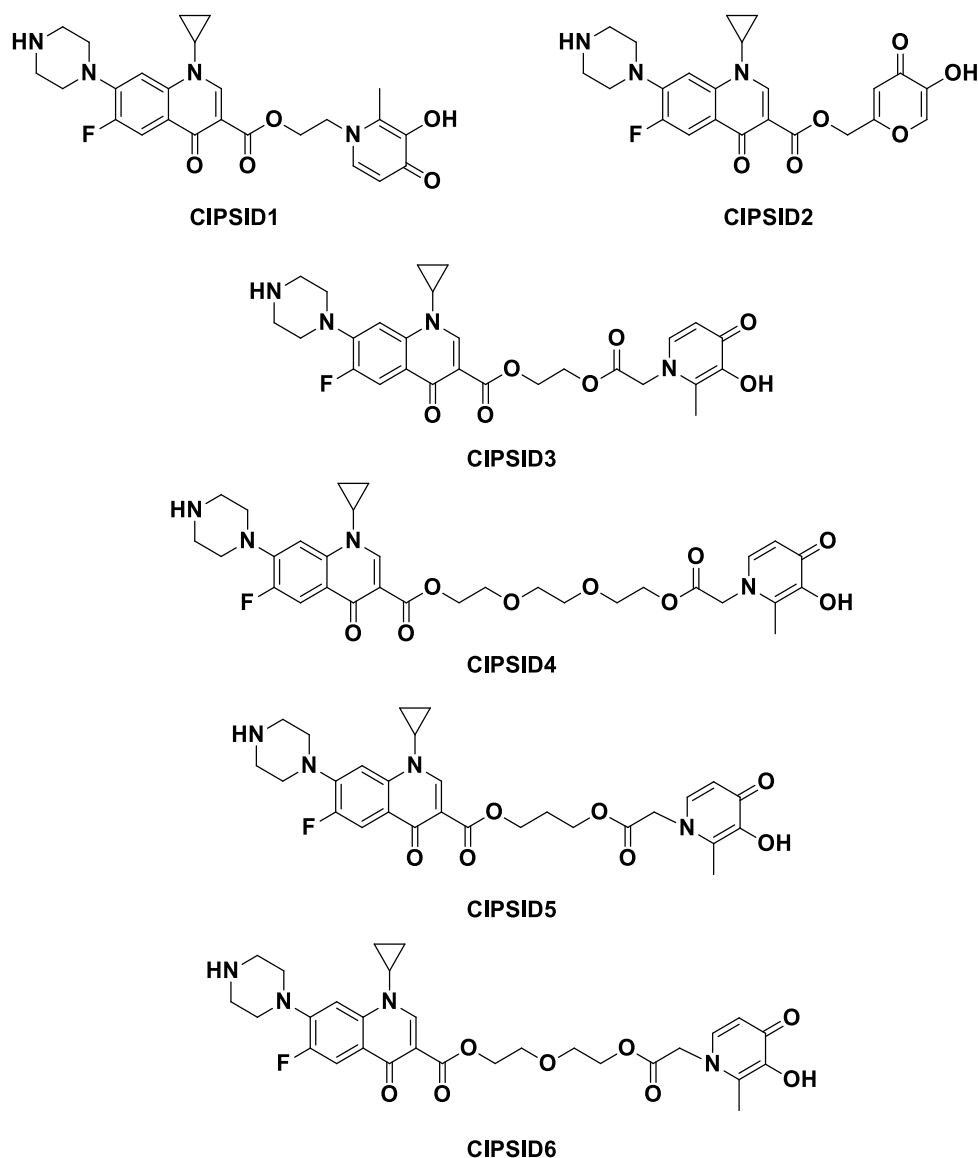


Figure 44 - Chemical structure of tested compounds (CIPSID1 to CIPSID6)

These preliminary and biased antibacterial high-through screening (HTS) was performed with a single concentration of 32 µg/ml, revealing that all the tested conjugates possessed antimicrobial activity. These results were further confirmed by whole-cell growth inhibition assays to determine the Minimum Inhibitory Concentration (MIC). These assays were conducted as an 8-point dose-response curve, and the MIC value was determined as the lowest concentration at which the growth was fully inhibited, defined by an inhibition $\geq 80\%$ [162]. The results are outlined in Table 18. These assays involved turbidity measurements of bacterial growth at 600 nm. The percentage of inhibition growth was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) as references.

Table 18 - MIC values of CIPSID1 to CIPSID6 conjugates (in µg/mL).

Compound	Sa	Ec	Kp	Pa	Ab	Ca
CIPSID1	4	≤ 0.25	4	≤ 0.25	>32	>32
CIPSID2	4	≤ 0.25	4	≤ 0.25	16	>32
CIPSID3	>32	≤ 0.25	>32	>32	>32	>32
CIPSID4	>32	≤ 0.25	>32	>32	>32	>32
CIPSID5	>32	≤ 0.25	>32	>32	>32	>32
CIPSID6	>32	≤ 0.25	>32	>32	>32	>32

Sa: *Staphylococcus aureus* (MRSA, ATCC 43300); Ec: *Escherichia coli* (FDA control, ATCC 25922);
Kp: *Klebsiella pneumoniae* (MDR, ATCC 700603); Pa: *Pseudomonas aeruginosa* (ATCC 27853);
Ab: *Acinetobacter baumannii* (ATCC 19606); Ca: *Candida albicans* (CLSI reference, ATCC 90028);

The assessment of the MIC values showed that all compounds inhibited the gram-negative bacteria *Escherichia coli* with a MIC ≤ 0.25 µg/mL and that CIPSID1 and CIPSID2 also inhibited the Gram-negative bacteria *Pseudomonas aeruginosa* (MIC ≤ 0.25 µg/mL) and *Klebsiella pneumoniae* (MIC= 4 µg/mL). Moreover, these two compounds also showed antibacterial activity against the methicillin-resistant gram-positive bacteria *Staphylococcus aureus* (MIC= 4 µg/mL).

It is reported that under iron restriction conditions, the siderophore uptake system is upregulated, and consequently, usually, an increase in the antibacterial potency of the antibiotic-siderophore conjugate is observed [12, 23, 63, 101]. Hence, to study the

possible influence of bacterial iron uptake system on the antibacterial activity of the conjugates, the MIC and MBC (minimum bactericidal concentration) values of the best compounds (CIPSID1 and CIPSID2) were determined in iron-repleted (MHB) and iron-depleted media (ID-MHB), in collaboration with the LEPABE's group coordinated by Prof. Manuel Simões [104].

These studies were performed with three bacterial strains that produce natural siderophores: *Pseudomonas aeruginosa* PAO1, *Escherichia coli* ATCC 19138 and *Staphylococcus aureus* CECT 976. The antibacterial activity of these conjugates was also compared with the parent drug (ciprofloxacin) and to the deferiprone derivatives Def2 and Def3. The results are shown in Table 19.

Table 19 - MIC and MBC values under iron-repleted (MHB) and iron-depleted media (ID-MHB). All results are in µg/mL

Compounds	<i>Pseudomonas aeruginosa</i> PAO1				<i>Escherichia coli</i> ATCC 191				<i>Staphylococcus aureus</i> CECT 976			
	MHB		ID-MHB		MHB		ID-MHB		MHB		ID-MHB	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ciprofloxacin	0.125	1	0.125	1	0.016	0.25	0.016	0.25	0.25	1	0.25	1
Def 2	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Def 3	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
CIPSID1	8	32	8	32	4	32	4	32	16	>32	8	>32
CIPSID2	2	16	2	16	2	8	1	8	4	32	2	32
CIPSID3	8	>32	8	>32	4	32	2	32	16	>32	4	>32

The gathered data revealed that the synthesized conjugates have lower antibacterial activity than the parent drug, in line with the reported literature regarding SACs based on fluoroquinolone antibiotics [12, 23, 57, 59, 101]. Nevertheless, CIPSID2 showed MIC values for *Staphylococcus aureus* and *Escherichia coli*, which were two-fold lower in iron-depleted conditions, and CIPSID3 also showed the same tendency with MIC values that were two-fold and four-fold lower under iron-depleted conditions for *Staphylococcus aureus* and *Escherichia coli*, respectively. Therefore, it can be inferred that these two conjugates may use bacterial iron uptake systems to exert antibacterial activity [12, 23].

4.3. Assessment of the toxicological profile

The conjugates CIPSID1 to CIPSID6 were assayed for their toxicological profile, taking into account two parameters: cytotoxicity in human embryonic kidney cells and hemolysis of human red blood cells. The results are shown in Table 20.

Table 20 - Cytotoxicity and Haemolytic activity of conjugates CIPSID1 to CIPSID6

Compounds	H _k (cytotoxicity) in µg/mL	H _m (haemolytic activity) in µg/mL
CIPSID1	>32	9.2
CIPSID2	>32	>32
CIPSID3	>32	2.3
CIPSID4	>32	>32
CIPSID5	>32	19.7
CIPSID6	>32	13.9

Hk: Human embryonic kidney cells HEK-293 (ATCC CRL-1573)

Hm: Human red blood cells (RNC)

All the tested conjugates unveil an excellent toxicological profile, with none showing cytotoxicity or hemolytic activity at MIC values, and CIPSID 2 stands out as the most promising one, with MIC values from 1 and 16 µg/mL and cytotoxicity concentrations (HEK-293 and RNC) (>32 µg/mL).

4.4. Ferrozine assay results

The Ferrozine (3-(2-Pyridyl)-5,6-bis(4-sulfophenyl)-1,2,4-triazine disodium salt) assay relies on the formation of a stable purple-colored chromophore between ferrozine and free Fe(II) ions, with strong absorbance at 562 nm – Figure 45 and equation 1. Compounds that possess iron chelating properties reduce the amount of free Fe(II) in solution, thereby lowering the formation of the ferrozine-Fe(II) complex, which leads to a decrease in absorbance values at 562 nm (equation 2). Thus, low absorbance values are associated with compounds with great chelating properties [105-107, 163, 164].

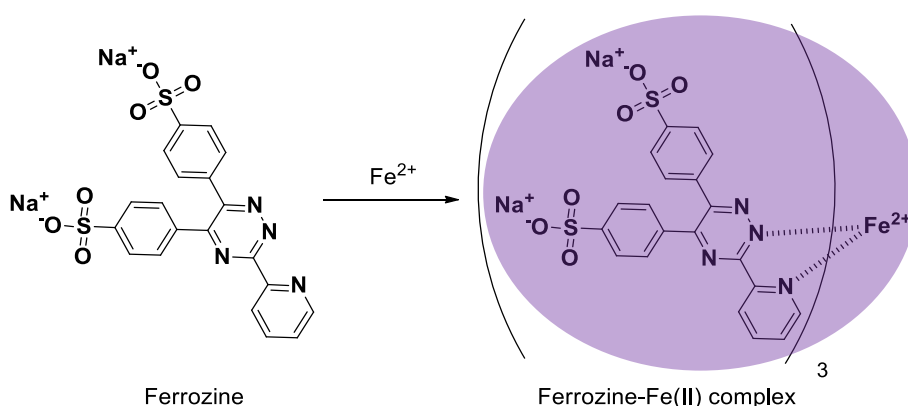
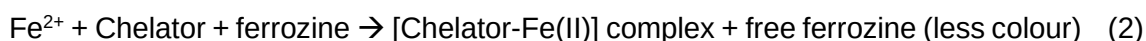


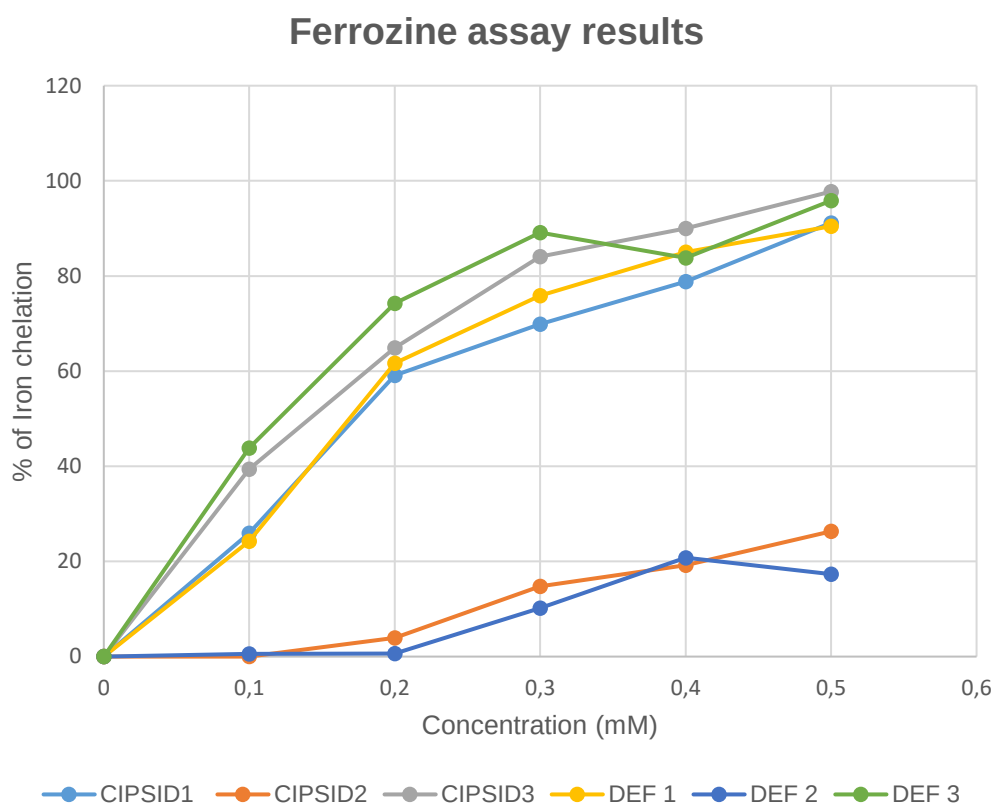
Figure 45 - Fundamentals of the ferrozine method for evaluation of the iron chelating activity



The iron chelating properties of the tested compounds were assessed by the decrease in absorbance at 562 nm, and the assay planned, taking into account iron's two oxidation states (Fe(II) and Fe(III)) and ferrozine's inability to form complexes with ferric iron. Thus, to avoid interference of Fe(III) in chelating activity determinations, hydroxylamine was used as a reducing agent to prevent iron oxidation [106, 107, 163-166].

Moreover, the absorbance spectra, within the wavelength of 350-700 nm, of all tested compounds were recorded, showing that none exhibited absorbance at 563 nm, ensuring the applicability of the ferrozine methodology for the compound's iron chelation measurements. EDTA was used as a positive control, showing at 0.1 mM concentration 100% of iron chelation, underscoring the robustness and reliability of this method. The results also revealed that ciprofloxacin showed no iron chelation ability even at the

highest tested concentration (0.5 mM). The results for the other compounds tested are depicted in Graph 1.



Graph 1 - Assessment of the Iron Chelation Properties of tested compounds

According to overall data, it can be concluded that the iron-chelating properties of the conjugates are correlated with its siderophore mimetics moieties (Def1, Def2, and Def3), since ciprofloxacin, by itself, didn't show any iron chelating activity. As illustrated in Graph 1, the siderophore mimetic system that displays the lowest iron chelation profile is Def2 (kojic acid), a bio isostere of deferiprone derivatives (Def1 and Def3). These results are consistent with the data obtained for the conjugates since CIPSID2 presents the lowest iron chelation activity (26% at the highest concentration tested (0.5 Mm)). In contrast, CIPSID1 and CIPSID3 are the ones with the highest iron chelating profile, exhibiting iron chelation values surpassing 90% at the highest concentration tested, which demonstrate their ability to retain the iron chelation ability of their deferiprone derivatives moieties (Def1 and Def3 respectively).

These results are in accordance with the literature showing that both deferiprone and kojic acid possess iron-chelating properties. Although both possess the same mechanism of iron chelation through bidentate complexes, between iron (II) and

deprotonated oxo and hydroxyl groups [167-169]. Deferiprone derivatives show better iron chelating properties than kojic acid derivatives. This evidence can be attributed to the differences in their chemical structures, namely how easily is the delocalization of the lone pair of electrons of the nitrogen or oxygen and alkoxide stability, which allows iron binding to iron (Figure 46) [167, 168].

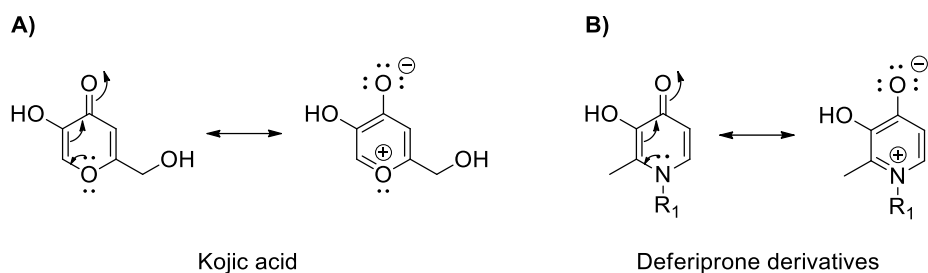


Figure 46 - Resonance structures of: a) kojic acid and b) deferiprone derivatives

Chapter 5 – Conclusion and future perspectives

The discovery and development of antibiotics, followed by their integration into clinical practice, played a vital role in the prevention and treatment of infectious diseases. This contributed to a significant reduction in mortality and morbidity rates, as well as an enhancement in life expectancy and quality of life. However, the misuse and overuse of antibiotics prompted the rapid emergence of increasingly resistant bacterial strains, posing a significant challenge to existing antibiotic efficacy [1, 8, 10-17].

Therefore, to combat this emergent public worldwide threat, it is mandatory to discover and develop new antibiotics with novel mechanisms of action to tackle AMR. In this sense, bacteria metabolic processes are being studied to explore them as potential new drug targets. The Trojan Horse strategy, for example, exploits the intrinsic siderophore bacterial pathway to transport the antibiotic to its target without it being recognized by the bacteria. The success of cefiderocol (Fetroja^(R)) revealed the potential of this strategy to overcome the AMR crisis. However, to date, the compounds developed based on this strategy revealed difficulties in being incorporated into bacterial cytoplasm, only being able to act on membrane targets [6, 11].

The project herein depicted is based on the design and synthesis of new ciprofloxacin-siderophore mimetics conjugates that potentially reach bacterial cytoplasm. In this work, deferiprone derivatives were employed as mimetic systems of 3-hydroxypyridin-4-ones, kojic acid as 3-hydroxypyrrone mimetic, and protocatechuic and caffeic acids as catechol mimetics. The synthesized conjugates consisted of the linkage of these siderophore mimetics to the carboxyl group of ciprofloxacin directly or through linkers (polyethylene glycols and diols).

Considering the synthetic strategy outlined in Figure 28, it can be said that all the starting materials, namely NBocCipro, protected Def1, protected Def2, and protected Def3, were successfully obtained in medium to good yields (NBocCipro 91%, protected Def1 41%, and protected Def3 60%). Several optimization procedures were used to successfully synthesize protected Def3, including pH adjustment, modifying the reaction time and temperature, and adjusting stoichiometric proportions.

The NBocCipro-OH derivatives were also successfully synthesized with yields ranging from 29% to 98%. The lower yields observed for some of these compounds can somehow be explained by the need for column chromatography purification and the associated loss of the desired compound.

The NBocCipro and NBocCipro-OH derivatives were linked with the corresponding siderophore mimetics (protected Def1, protected Def2 or protected Def3) to attain compounds Protected CIPSID1 to Protected CIPSID6, with yields ranging from 19% to 49%. The reversibility nature of an esterification reaction and the formation of

bulky and/or low nucleophilicity intermediates can be pointed out as some of the reasons for the low to medium yields observed. It was seen that, in an esterification reaction, an excess of alcohol typically produced better results when dealing with bulky acids (NBocCipro), whereas, for bulky alcohols (NBocCipro-OH), an excess of acid led to a higher yield of the ester product. The results also suggest that the tertiary amine (DIPEA or TEA) plays a crucial role in the esterification reaction due to steric hindrance reasons.

Moreover, the use of veratric acid derivatives as siderophore mimetics led to the synthesis of the Protected CIPSID7 to protected CIPSID10 compounds, with low to medium yields (between 6% and 37%). The attempt to enhance these yields (Chapter 4 - Discussion and Results, point 4.1.7) did not yield significant results, so it was chosen to maintain the initial procedure.

Unfortunately, the synthesis of ciprofloxacin-cinnamic acid conjugates (protected CIPSID11) failed, perhaps due to the poor reactivity of ferulic acid justified by the presence of an electron-withdrawing acrylate moiety. All the trials undertaken to obtain ciprofloxacin-cinnamic acid conjugates (Chapter 4 - Discussion and Results, point 4.1.7) proved to be unproductive. In the future, another attempt of this coupling could be performed employing PTSA as a catalyst. The use of PTSA can make this esterification reaction successful as the formed intermediate is less bulky than that observed during the HBTU/DMAP reaction mechanism, which allows a more efficient interaction with alcohols [135, 136].

The synthesis of the final compounds CIPSID1 to CIPSID6 was successfully achieved. However, the yields were low, possibly due to some amount of the compound undergoing ester hydrolysis under acidic conditions (TFA) during the Boc deprotection step.

The final compounds CIPSID7 to CIPSID10 were not obtained perhaps due to a rupture of the ester bond between the carboxyl group of ciprofloxacin and the alcohol linker.

Other compounds synthesized in this work include Def1 and Def3, obtained by O-debenzylation reactions from the protected counterparts. This deprotection reactions were achieved with yields of 68% and 98% for Def1 and Def3, respectively. It wasn't necessary to carry out the deprotection reaction of the protected Def2, since the resulting compound would correspond to kojic acid, a commercially available compound.

All the obtained final compounds (CIPSID1 to CIPSID6) were screened against multiple microbial strains. The results showed a good antibacterial profile of the six conjugates for *Escherichia coli* with MIC values $\leq 0.25 \mu\text{g/mL}$, and CIPSID1/CIPSID2 were the ones that presented the lower MIC values and, in turn, the most significant

antimicrobial activity, showing additional MIC values of 4 µg/mL against *Staphylococcus aureus* and *Klebsiella pneumoniae*. Moreover, gathered data suggest that the tested compound's antibacterial activity is somehow connected to the bacterial iron uptake system. For instance, CIPSID2 and CIPSID3 showed MIC values for *Escherichia coli*, two-fold lower in iron-depleted conditions than in iron-normal ones.

Additionally, the tested conjugates demonstrate a favourable cytotoxicity profile, with none exhibiting cytotoxic effects against the HEK-293 cell line and hemolytic activity at MIC values. For instance, CIPSID 2 stands out for its MIC range of 1 to 16 µg/mL and cytotoxicity values above a concentration of 32 µg/mL.

The ferrozine assay showed that the iron chelating properties of the conjugates (CIPSID1 to CIPSID3) can be correlated with its siderophore mimetics moieties (Def1, Def2, and Def3), since ciprofloxacin, by itself, didn't show any iron chelating activity. CIPSID1 and CIPSID3 are the conjugates that displayed better iron chelating properties, surpassing 90% at the highest concentration tested. These two conjugates exhibited better iron chelating activity than CIPSID2 because deferiprone derivatives moieties (Def1 and Def3) present a higher binding affinity for iron than kojic acid (Def2).

This project has shown that it is possible to attain new siderophores/ciprofloxacin conjugates (CIPSID) with siderophore mimetics, namely deferiprone, instead of natural ones. This project also evidenced that this goal can be accomplished by ester linkage between the ciprofloxacin carboxylic group at position 3 and deferiprone derivatives. The attained conjugates proved to exert antibacterial activity against several strains with iron-chelating properties, and some of the collected data, namely for CIPSID 2 and 3, leads to the assumption that these two assets may be correlated.

The R&D of this MSc project opened new avenues in designing and developing innovative antibacterial chemical libraries, and allowed the in-house implementation and/or optimization of expeditious techniques to speed up the screening assays.

However, the library of CIPSID conjugates obtained in the timeframe of this project is limited in number, which negatively influences the structure-activity relationship studies, constraining the robustness of the acquired data. Thus, in the future, the chemical library must be enlarged in number and chemical diversity. Alternative synthetic methodologies should be implemented to obtain CIPSID7 to CIPSID11, and other CIPSID conjugates, such as the ones based on natural siderophores. The enlarged chemical library must also be screened for antibacterial, iron-chelating and cytotoxicity activity. Furthermore, the stability profile of the best compounds under different experimental conditions (pH variations, redox environments, mimetic physiological

fluids) should also be assessed. It is also important to study the mode of action of the top ranked compounds.

Some future perspectives also include the use of other type of linker, namely (acyloxy)alkyl ester. Some authors reported that it presents better properties than ester bond since the introduction of a bulky group, due to steric hindrance, increases conjugates stability to hydrolysis and, consequently, avoids premature cleavage [11, 50].

Besides the linkage of siderophore mimetic to carboxyl moiety of ciprofloxacin, it could be also done an siderophore attachment to piperazine moiety to investigate if adding an extra siderophore molecule to ciprofloxacin enhance the antimicrobial properties of the conjugates.

Coordination complexes of CIPSID1-CIPSID6 with gallium could also be performed since many studies report the use of this toxic metal as an iron-mimetic to improve the antibacterial activity of the conjugates. It takes the place of iron, depriving the cell from this metal, that then leads to bacterial growth inhibition or death [6, 11, 40, 41].

Chapter 6 – References

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Chapter 7 – Supplementary Data

Table S1 – Summary of published ciprofloxacin-siderophore conjugates. The employed linkers were classified as cleavable (C) or non-cleavable (NC). The conjugates were also classified according to their antibacterial activity as either H (activity higher than ciprofloxacin), L (activity equal to or lower than ciprofloxacin) or N (no activity reported for the conjugate). Siderophore moiety is highlighted in blue, ciprofloxacin in black and linker in pink.

Siderophore	Linker	Chemical structure	Activity	Year of ref
Desferrioxamine B	NC (Thiol maleimide linker)		L	2012 [42]
Desferrioxamine B	C (quinone analogue of trimethyl lock)		L	2012 [43]
Mixed ligand siderophore	C (quinone analogue of trimethyl lock)		L	2012 [43]
Desferrioxamine B	NC (succinyl linker)		L	2012 [43]
Mixed ligand siderophore	NC (succinyl linker)		L	2012 [43]
Desferrioxamine A (series of mono-, bis- and trihydroxamates)	NC (succinyl linker)		L	2013 [23]

Table S1 – Continued.

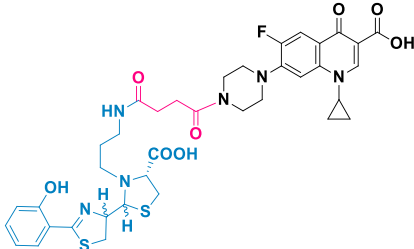
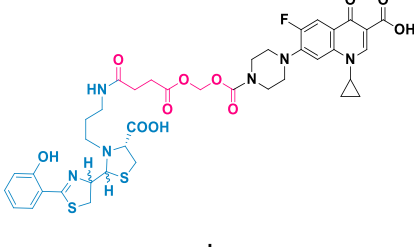
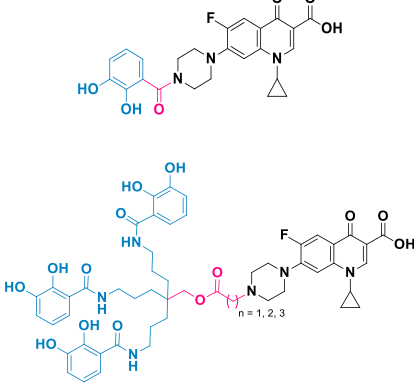
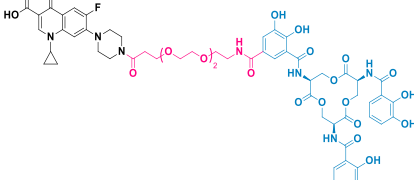
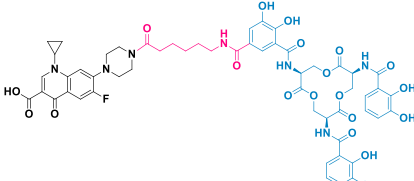
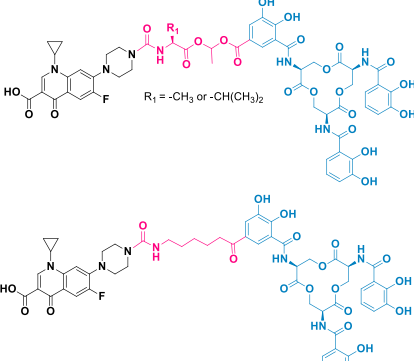
Siderophore	Linker	Chemical structure	Activity	Year of ref
Pyochelin	NC (succinyl linker)		N	2011 [13]
Pyochelin	C ("spacer arm"- in vivo hydrolysable linker)		L	2011 [13]
Catechol	NC (alkylacyl linker and amide bond)		L	2014 [26]
Enterobactin	C (PEG derivative linker)		L	2012 [56]
Enterobactin	C (alkyl chain linker)		L	2012 [56]
Enterobactin	C ((acyloxy) alkyl ester linker)		L	2015 [50]

Table S1 – Continued.

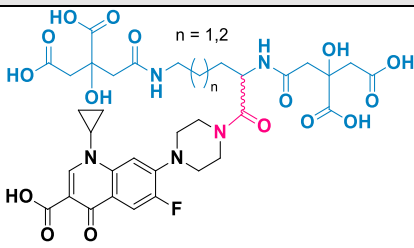
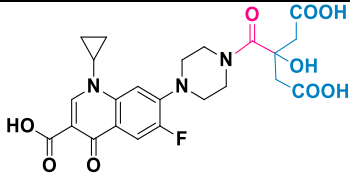
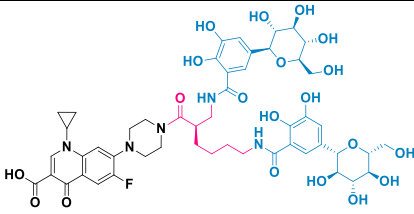
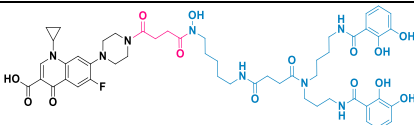
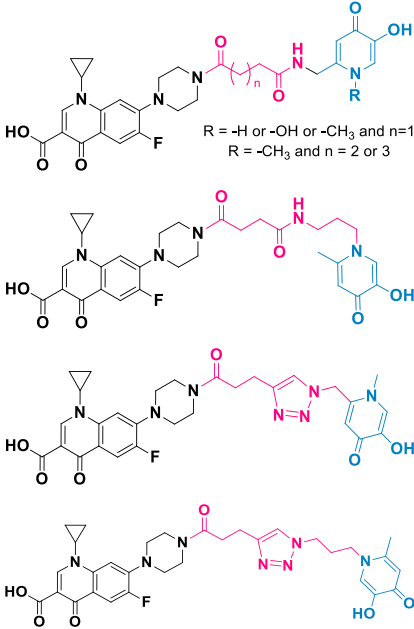
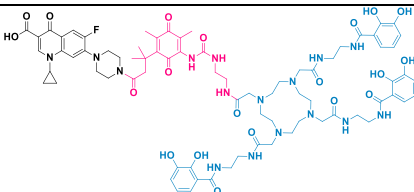
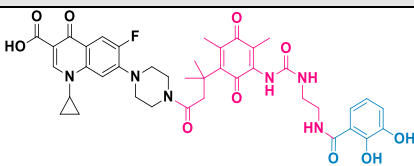
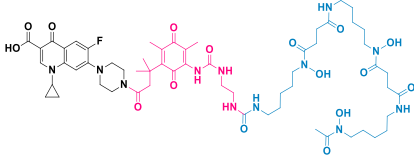
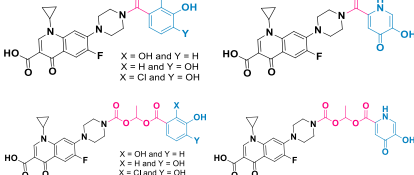
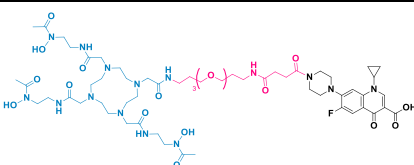
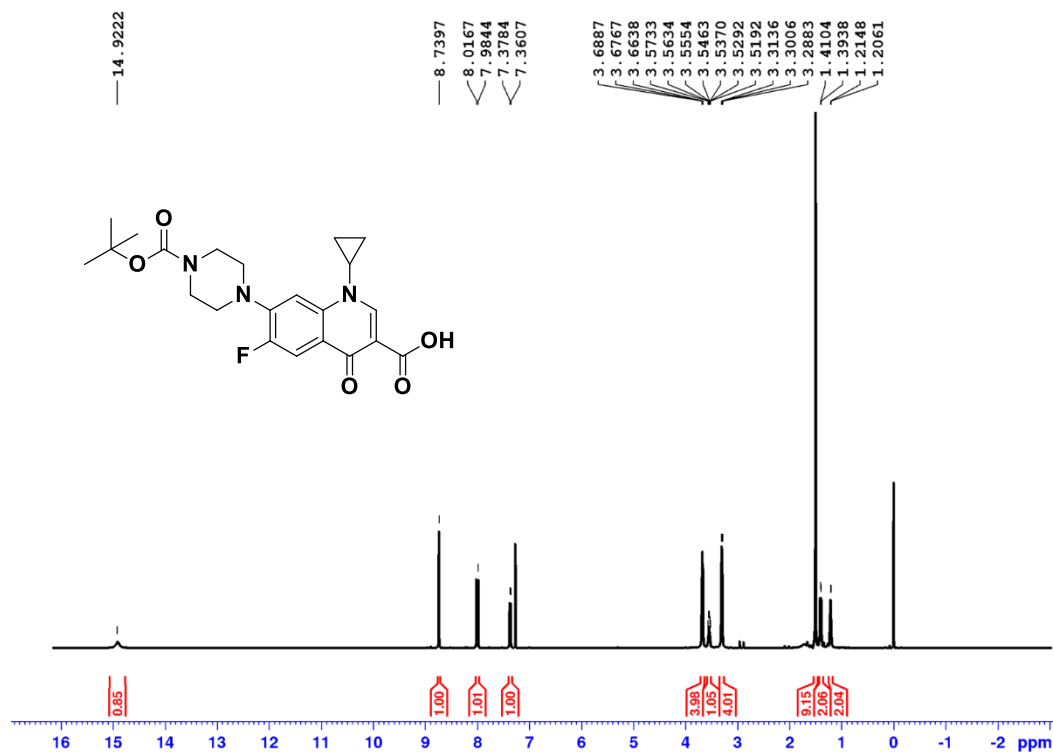
Siderophore	Linker	Chemical structure	Activity	Year of ref
Staphyloferrin A	NC (amide bond)		L	2013 [57]
Citrate	NC (amide bond)		L	2009 [58]
Salmochelin derivative	NC (aliphatic linker based on L-lysine derivative)		L	2020 [62]
Bis-catechol-hydroxamate mixed-ligand	NC (succinyl linker)		N	2013 [12]
3-Hydroxy-pyridin-4-one derivative	NC (highlighted in pink)		L	2023 [100]
DOTAM anchored triscathecol	C (trimethyl lock)		L	2023 [63]

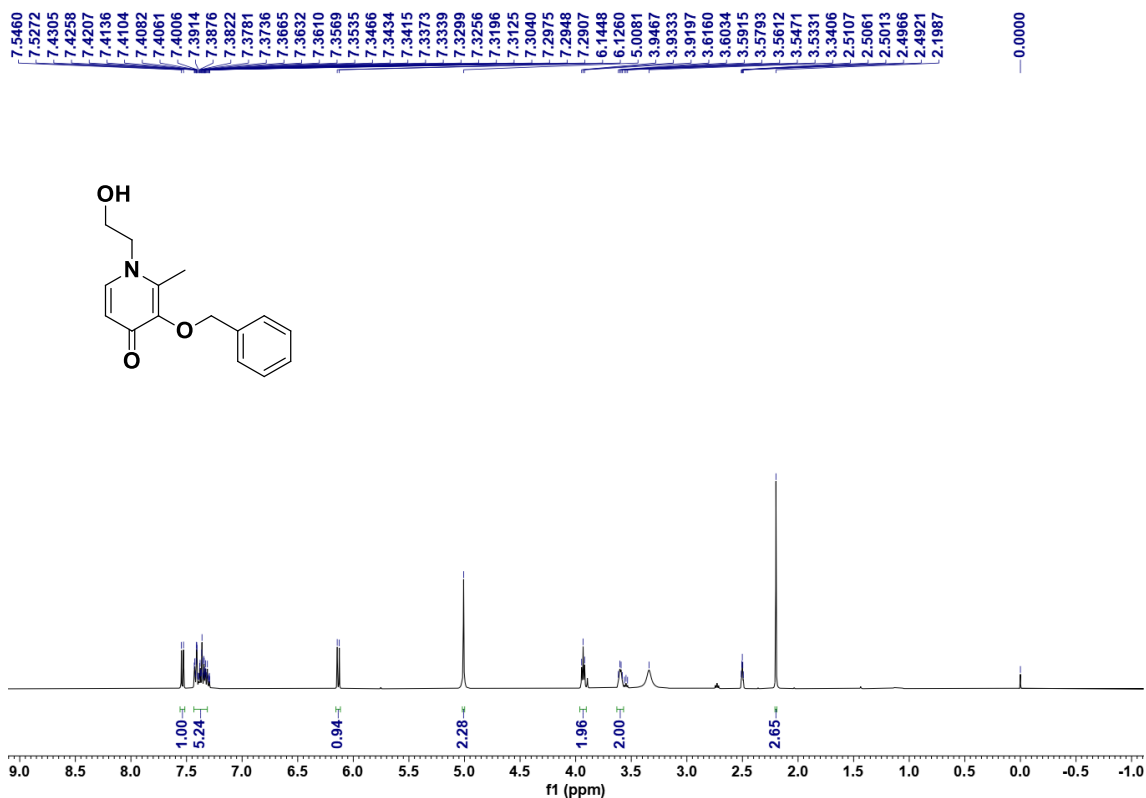
Table S1 – Continued.

Siderophore	Linker	Chemical structure	Activity	Year of ref
Catechol	C (trimethyl lock)		L	2023 [63]
Trihydroxamate	C (trimethyl lock)		L	2023 [63]
Catechol and 3-hydroxy-pyridin-4-one	NC (amide bond) and C ((acyloxy) alkyl type)	 X = OH and Y = H X = H and Y = OH X = Cl and Y = OH	L	2023 [102]
Trihydroxamate – DOTAM scaffold	NC (triple-PEG-succinate linker)		L	2023 [101]

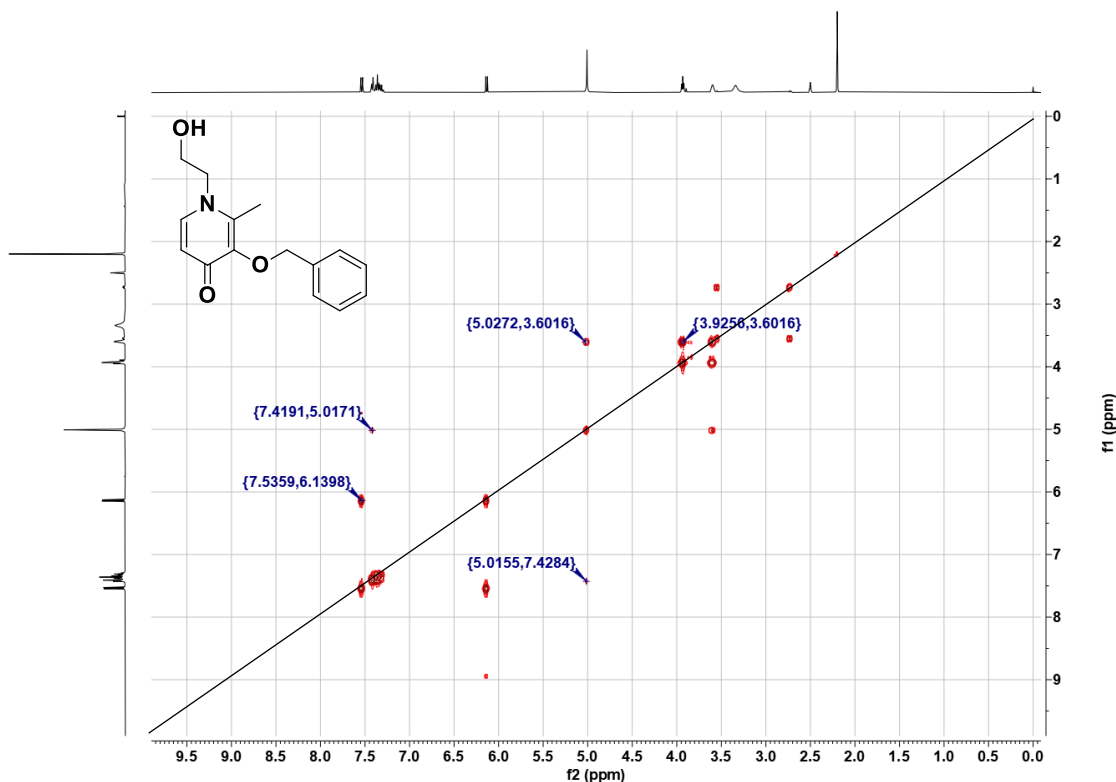
Attachment 1: ^1H NMR spectrum of NBocCipro [7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] in CDCl_3



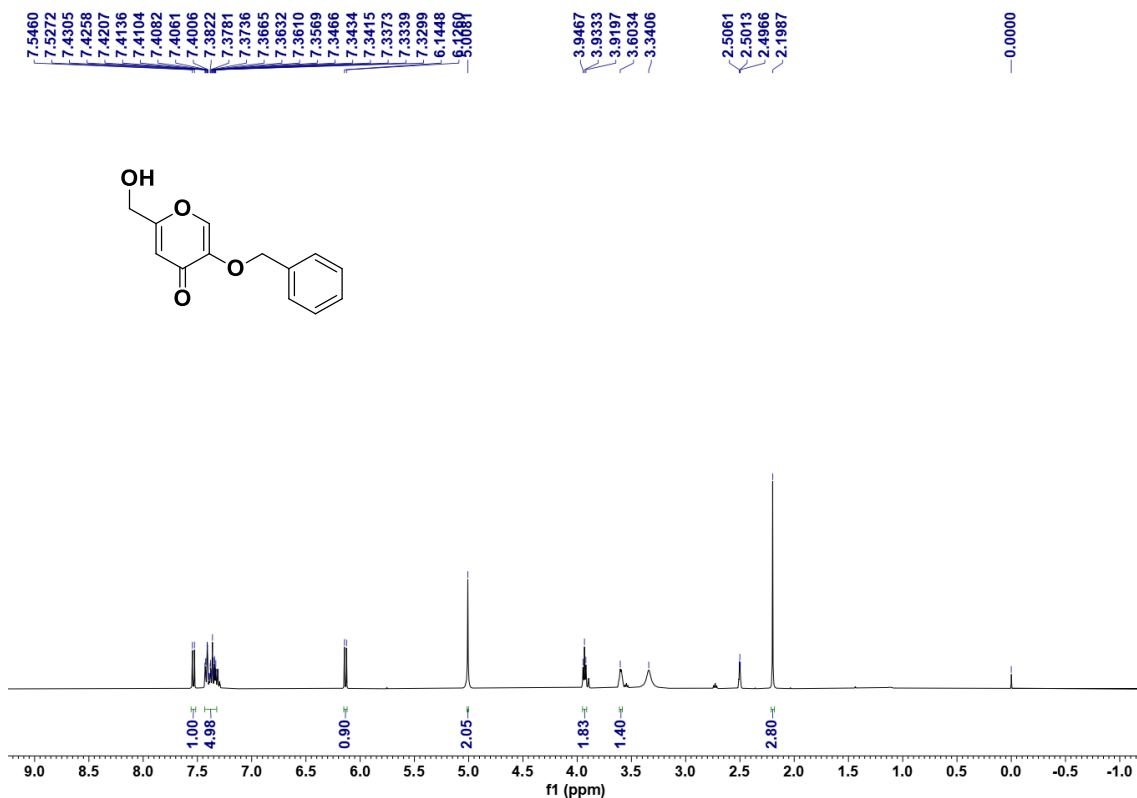
Attachment 2: ^1H NMR spectrum of Protected Def1 [3-(benzyloxy)-1-(2-hydroxyethyl)-2-methylpyridin-4(1H)-one] in DMSO-d_6



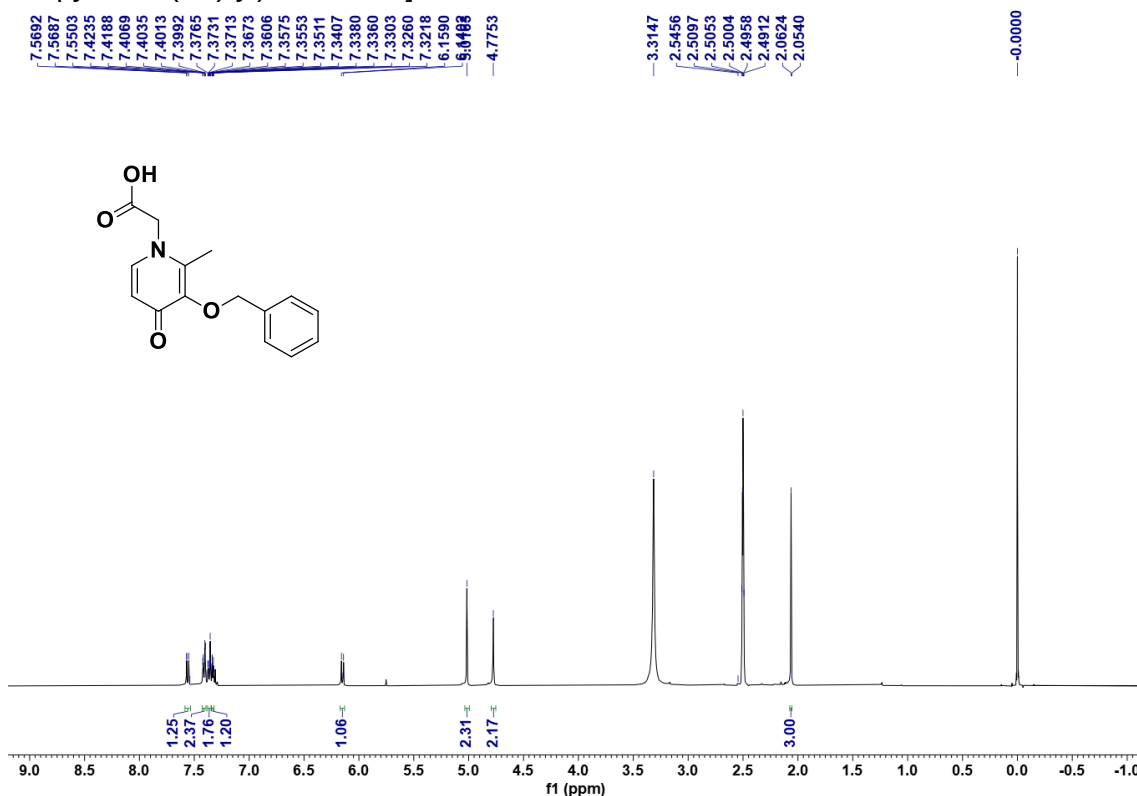
Attachment 3: ^1H - ^1H COSY NMR spectrum of Protected Def1 [3-(benzyloxy)-1-(2-hydroxyethyl)-2-methylpyridin-4(1H)-one] in in DMSO-d_6



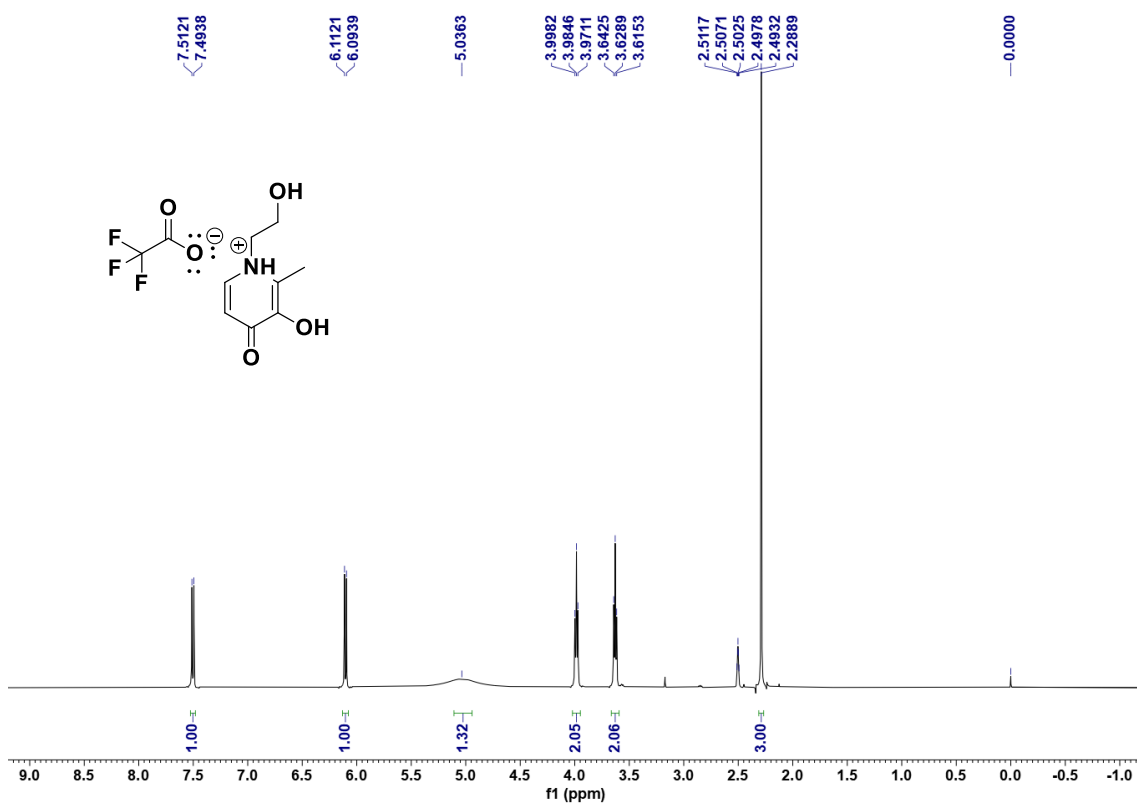
Attachment 4: ^1H NMR spectrum of Protected Def2 [5-(benzyloxy)-2-(hydroxymethyl)-4H-pyran-4-one] in DMSO-d_6



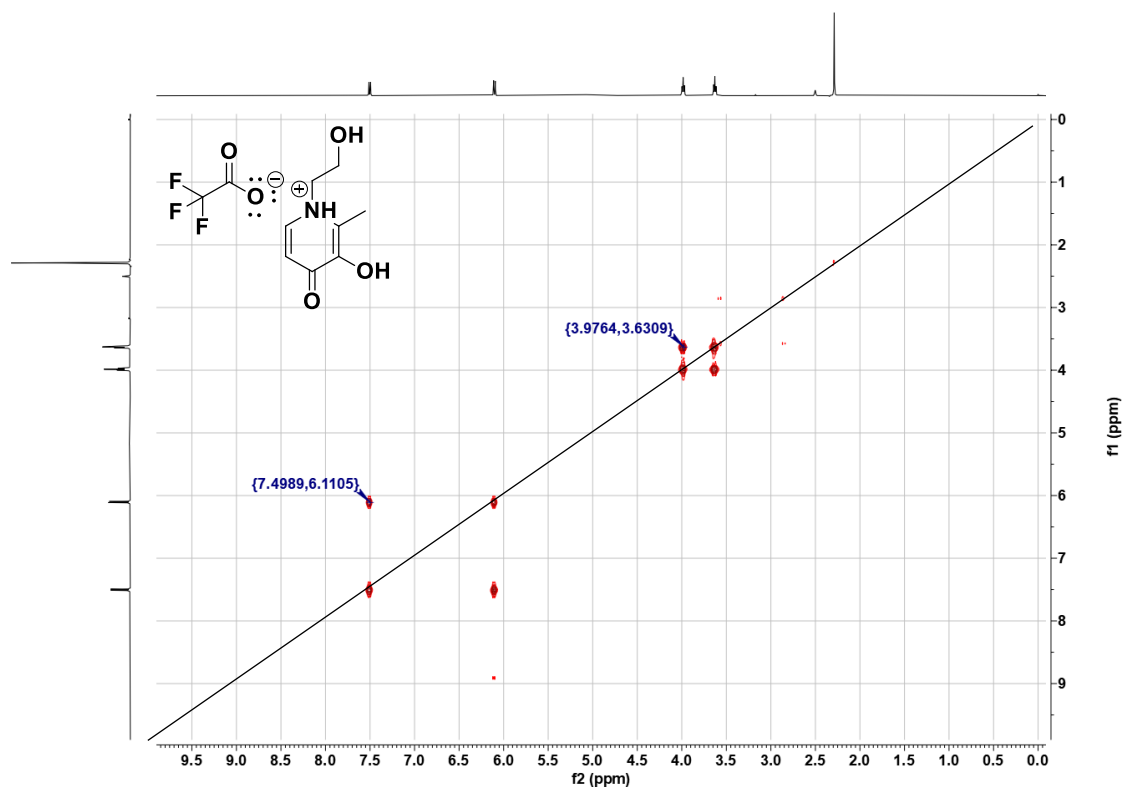
Attachment 5: ^1H NMR spectrum of Protected Def3 [2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetic acid] in DMSO-d_6



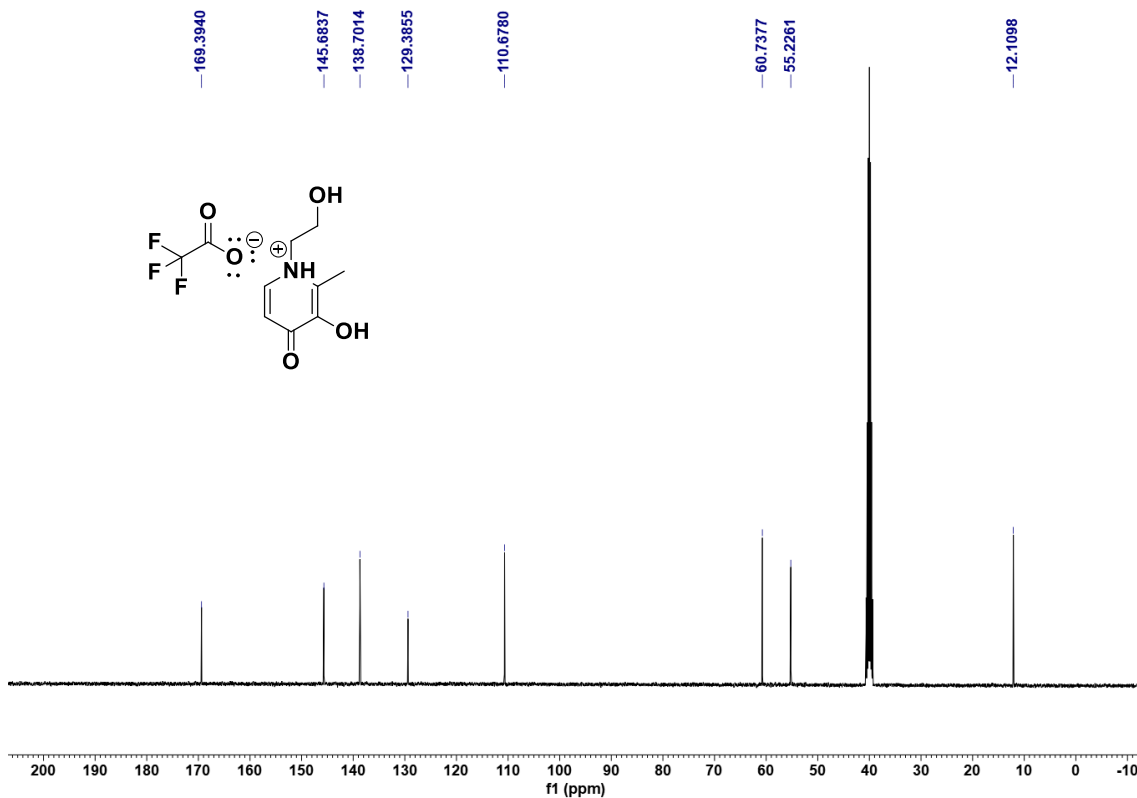
Attachment 6: ^1H NMR spectrum of Def1 [3-hydroxy-1-(2-hydroxyethyl)-2-methyl-4-oxo-1,4-dihydropyridin-1-ium-2,2,2-trifluoroacetate] in DMSO-d_6



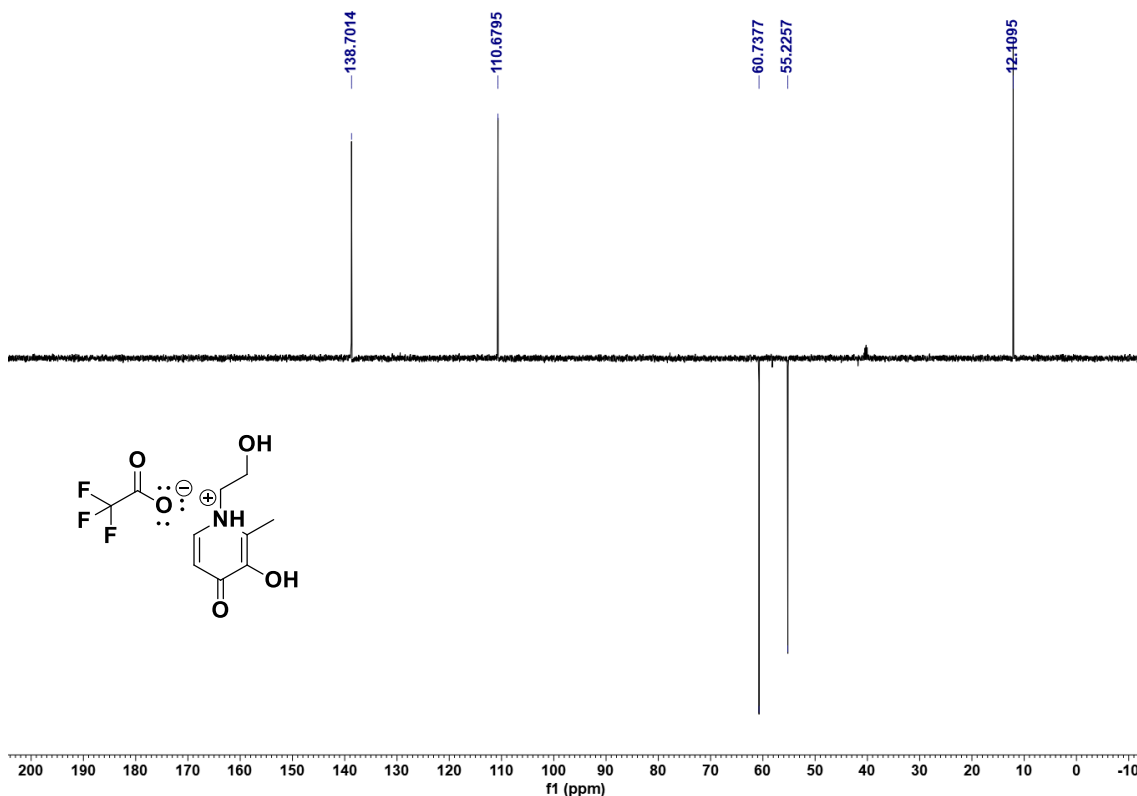
Attachment 7: ^1H - ^1H COSY NMR spectrum of Def1 in DMSO-d_6



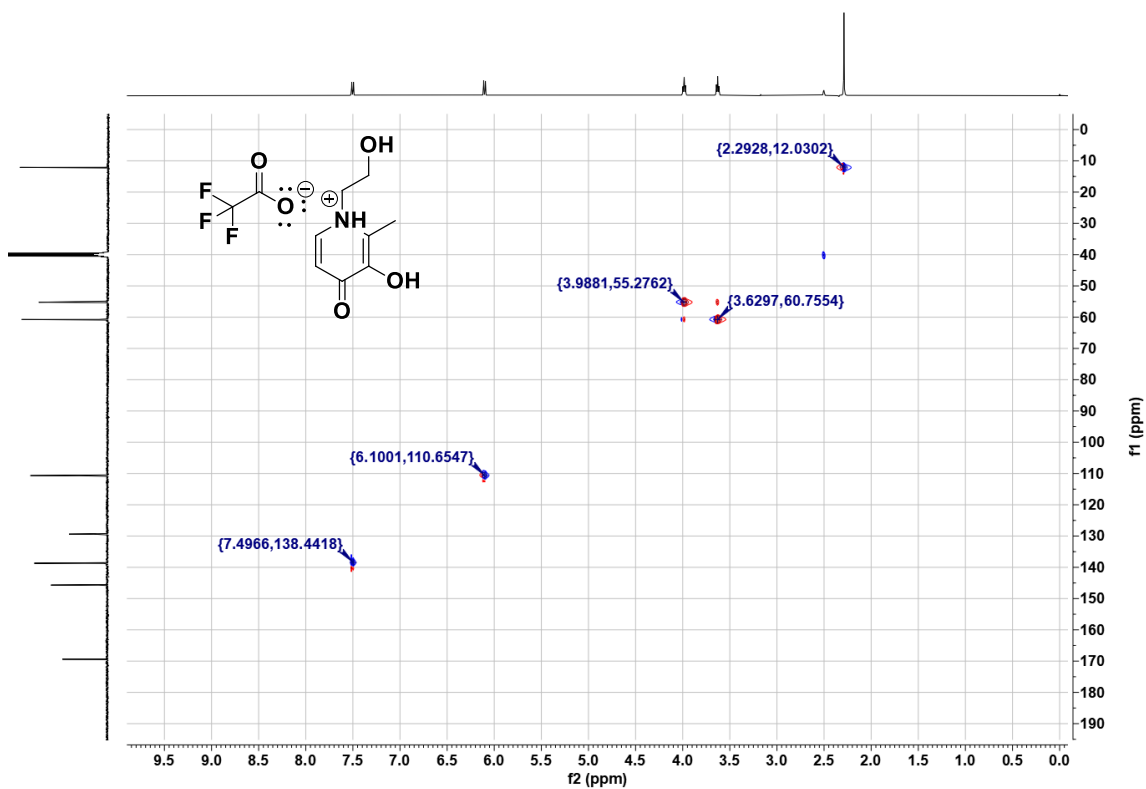
Attachment 8: ^{13}C NMR spectrum of Def1 in DMSO-d_6



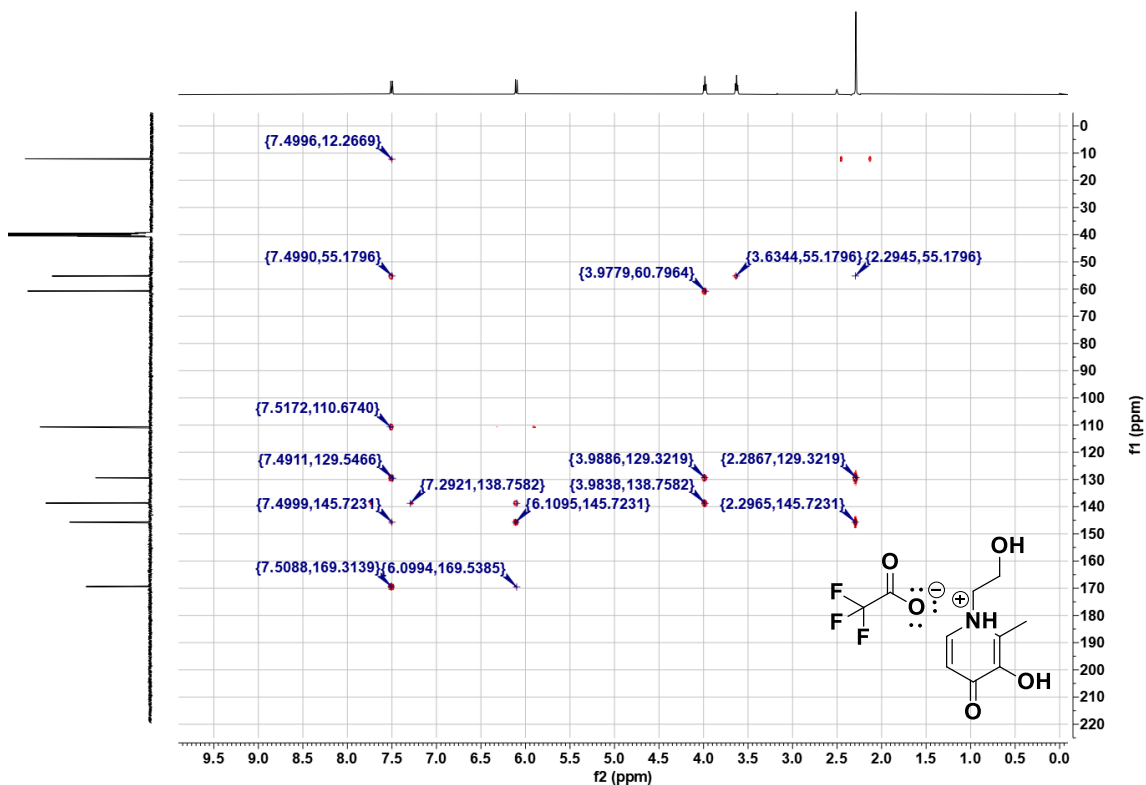
Attachment 9: DEPT-135 ^{13}C NMR spectrum of Def1 in DMSO-d_6



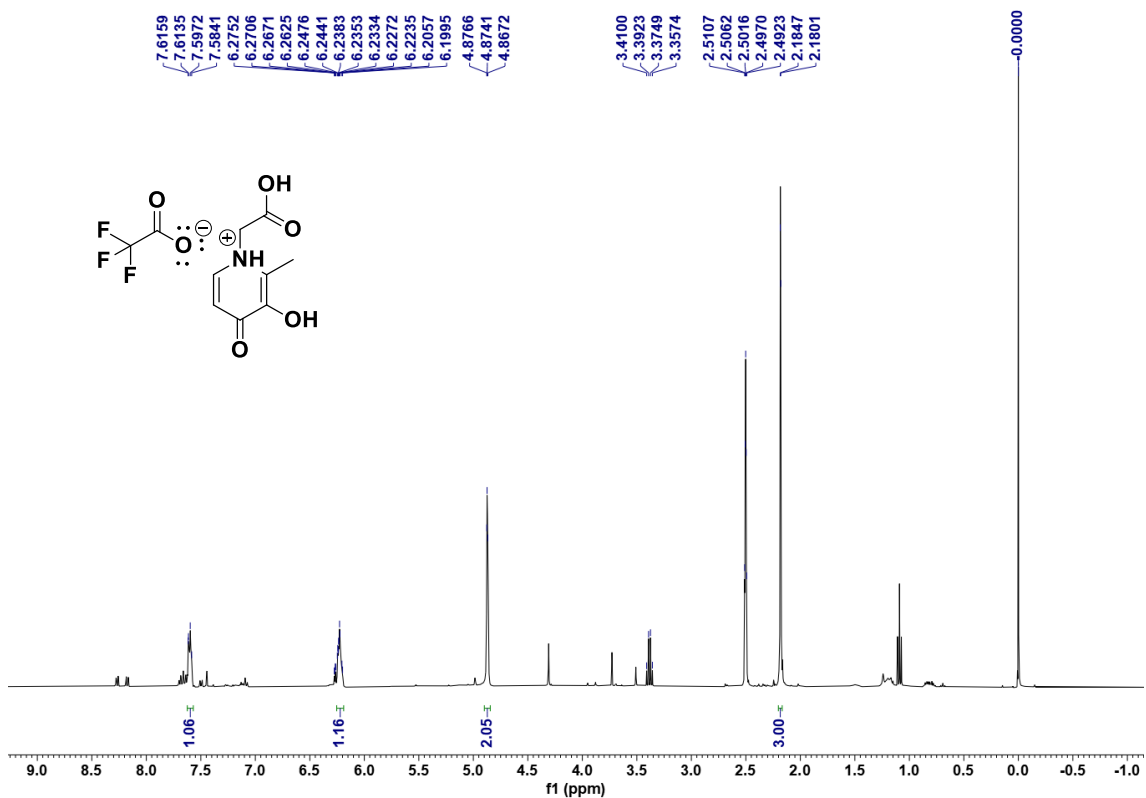
Attachment 10: ^1H - ^{13}C HSQC NMR spectrum of Def1 in DMSO-d_6



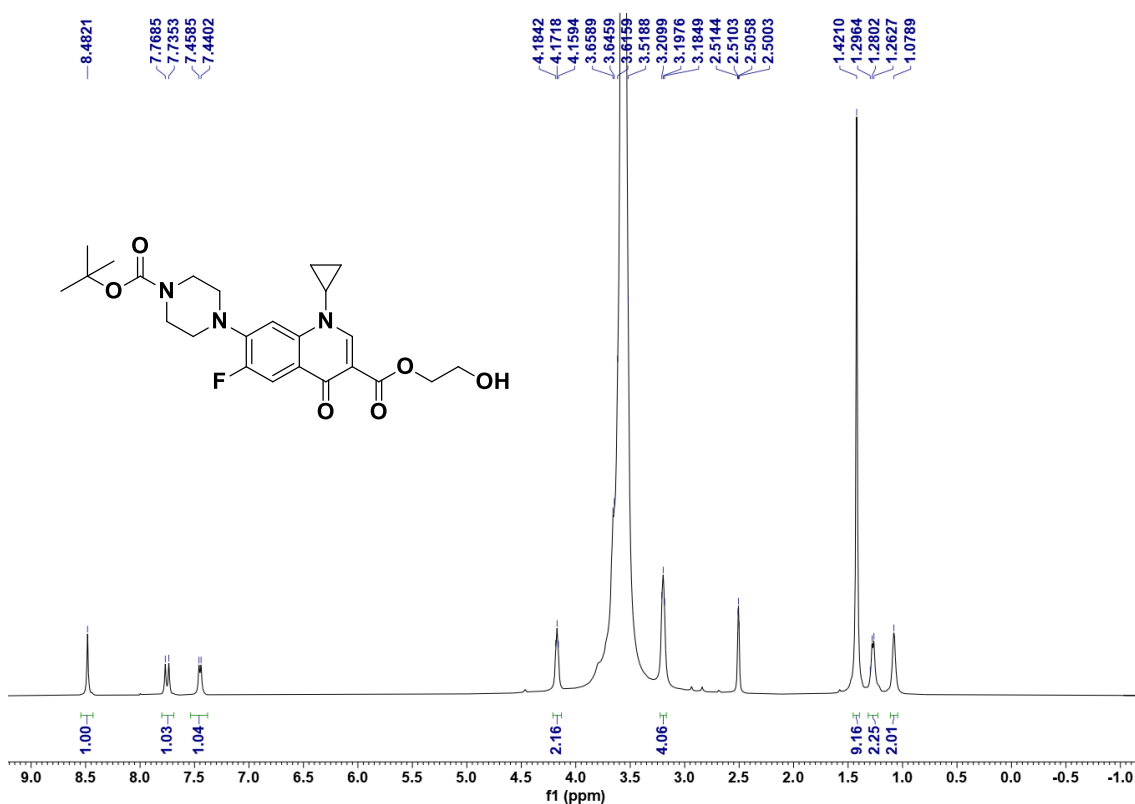
Attachment 11: ^1H - ^{13}C HMBC NMR spectrum of Def1 in DMSO-d_6



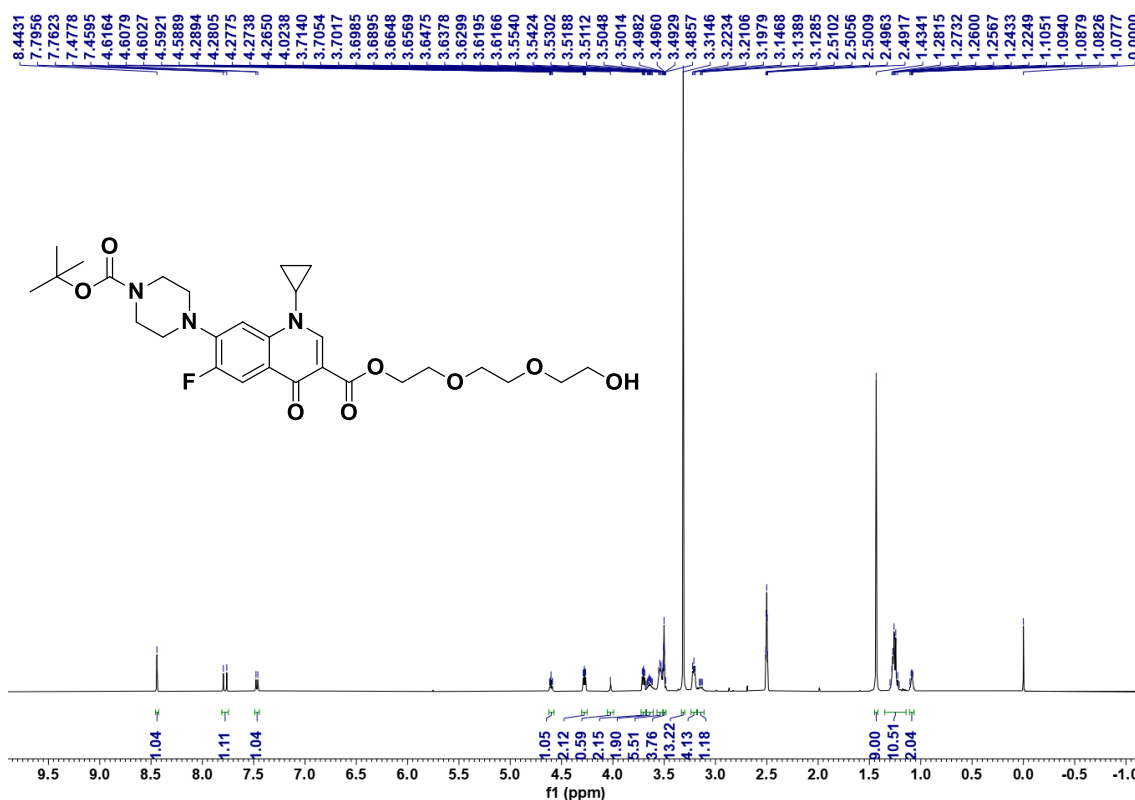
Attachment 12: ^1H NMR spectrum of Def3 [1-(carboxymethyl)-3-hydroxy-2-methyl-4-oxo-1,4-dihydropyridin-1-ium-2,2,2-trifluoroacetate] in DMSO-d_6



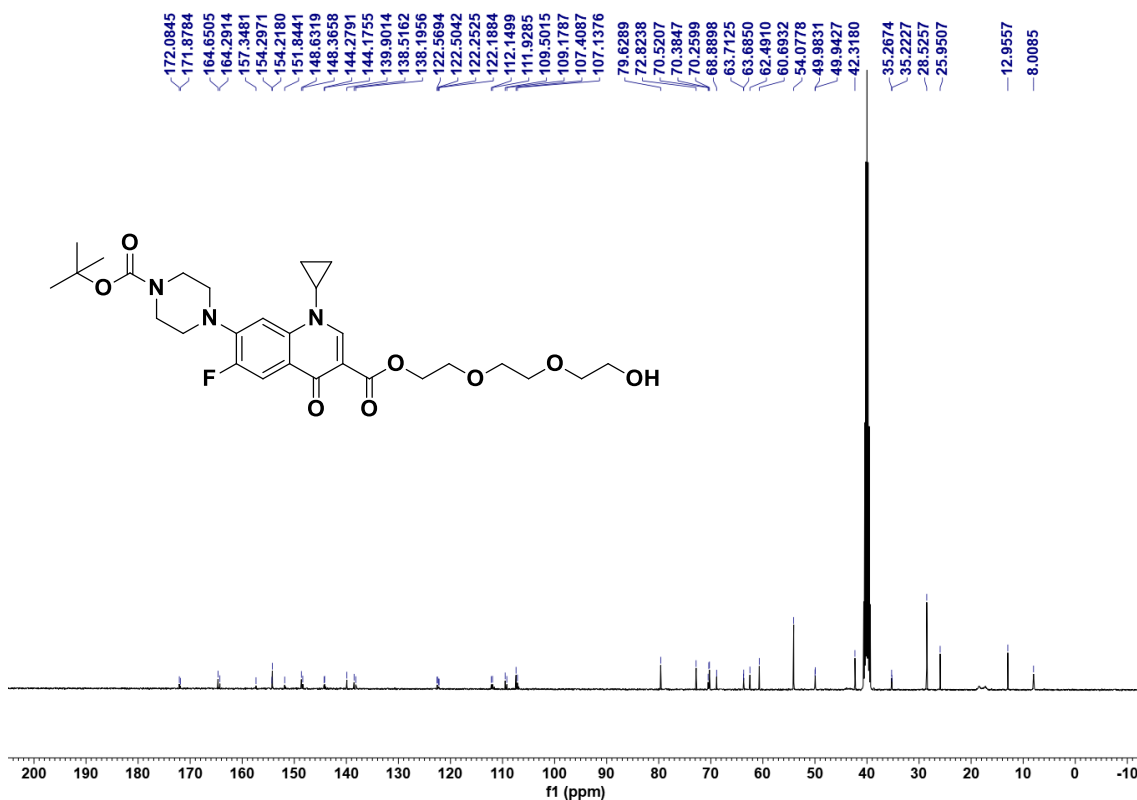
Attachment 13: ^1H NMR spectrum of NBocCipro-OH1 [2-hydroxyethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in DMSO-d_6



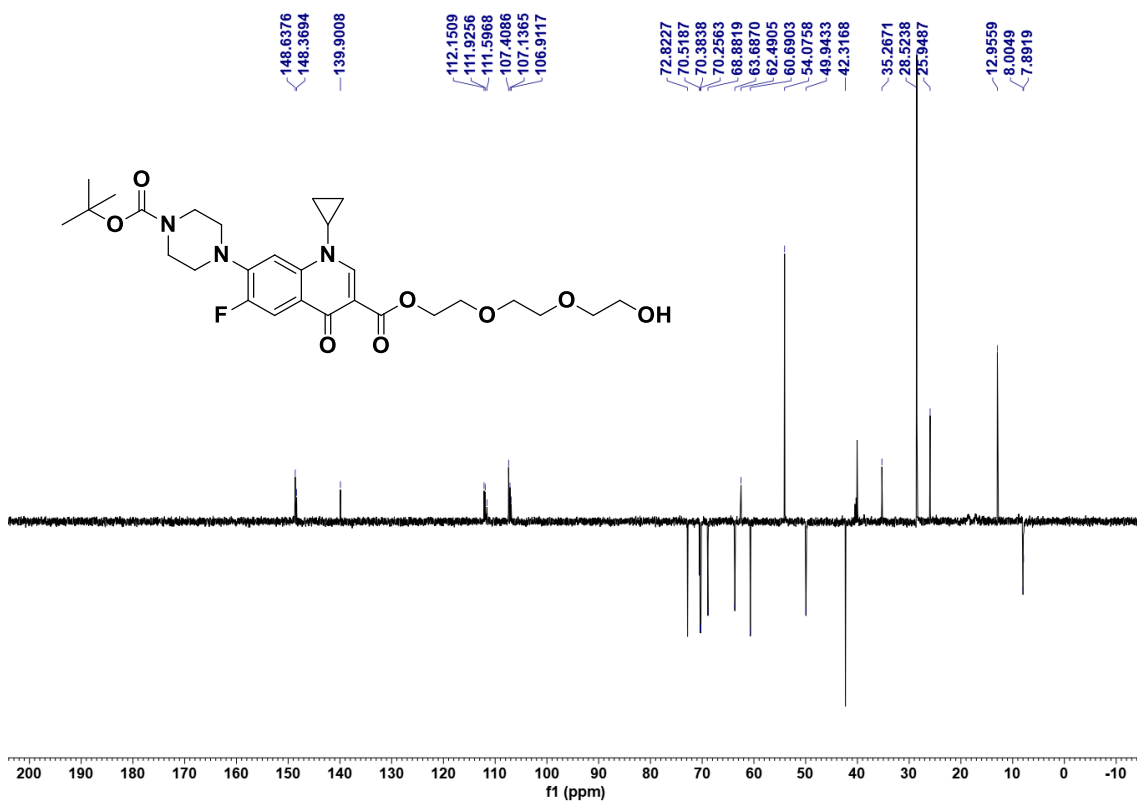
Attachment 14: ^1H NMR spectrum of NBocCipro-OH2 [2-(2-(2-hydroxyethoxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in DMSO-d_6



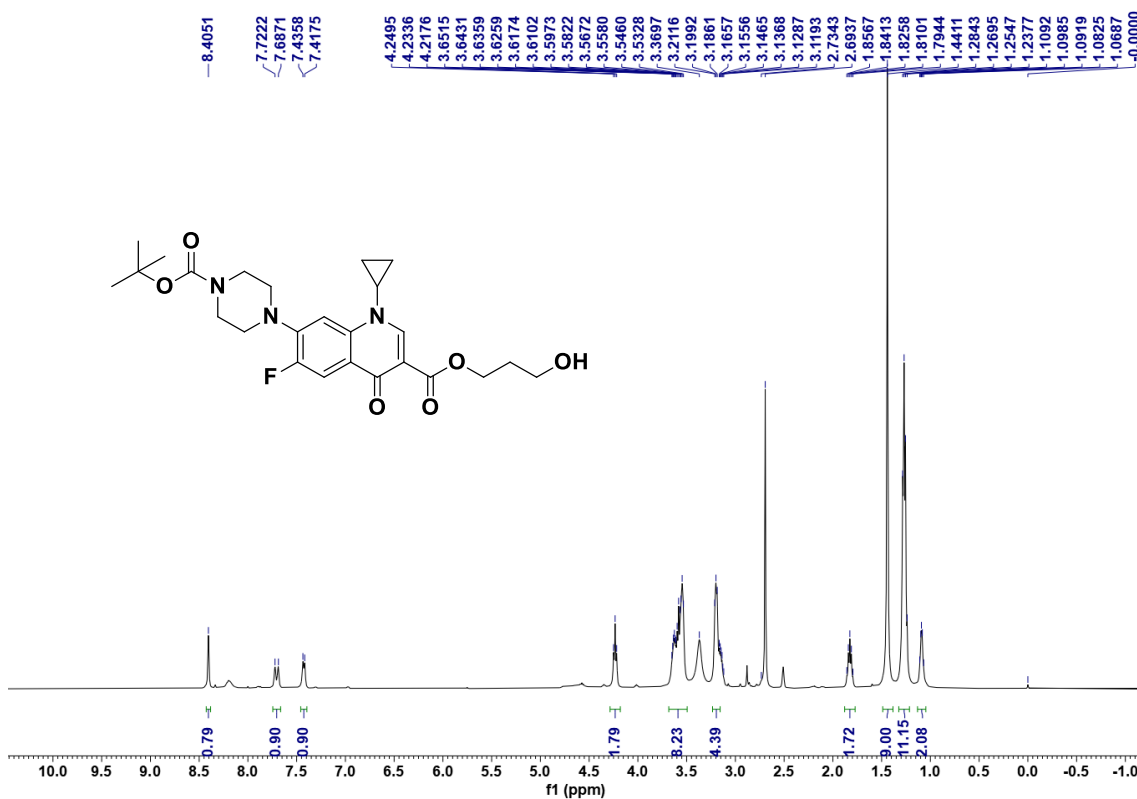
Attachment 15: ^{13}C NMR spectrum of NBocCipro-OH2 in DMSO- d_6



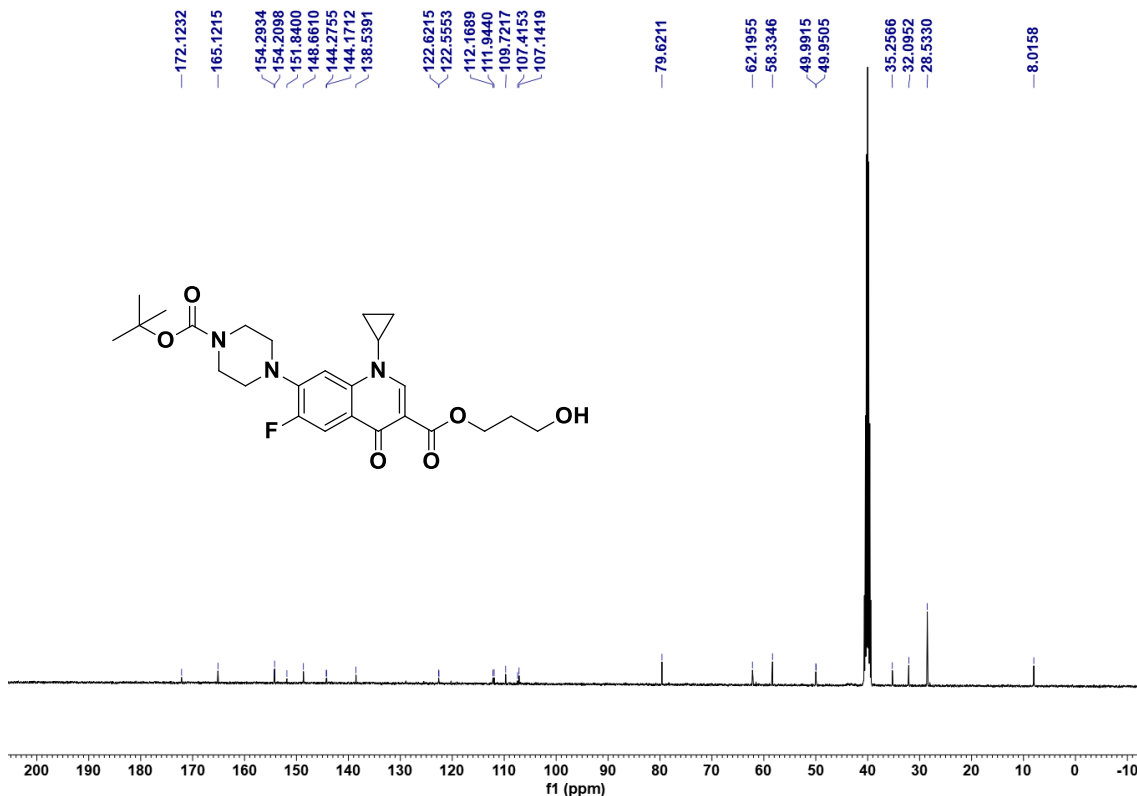
Attachment 16: DEPT-135 ^{13}C NMR spectrum of NBocCipro-OH2 in DMSO- d_6



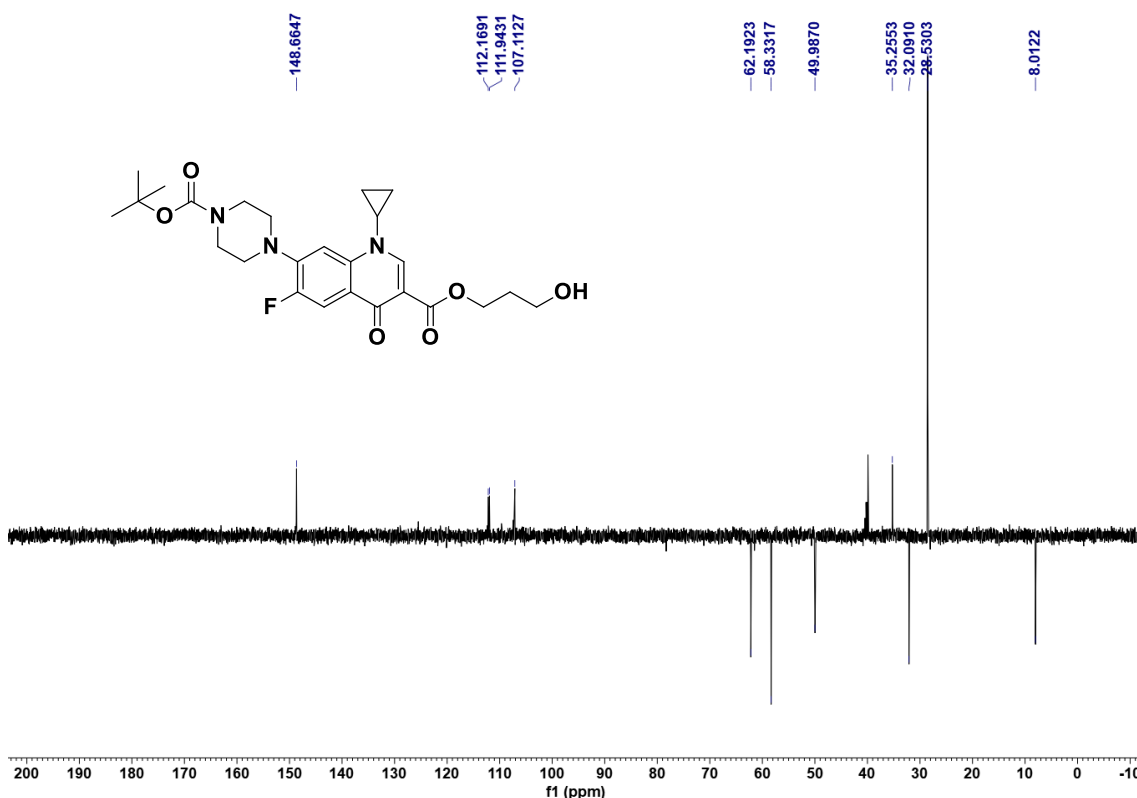
Attachment 17: ^1H NMR spectrum of NBocCipro-OH3 [3-hydroxypropyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in DMSO-d_6



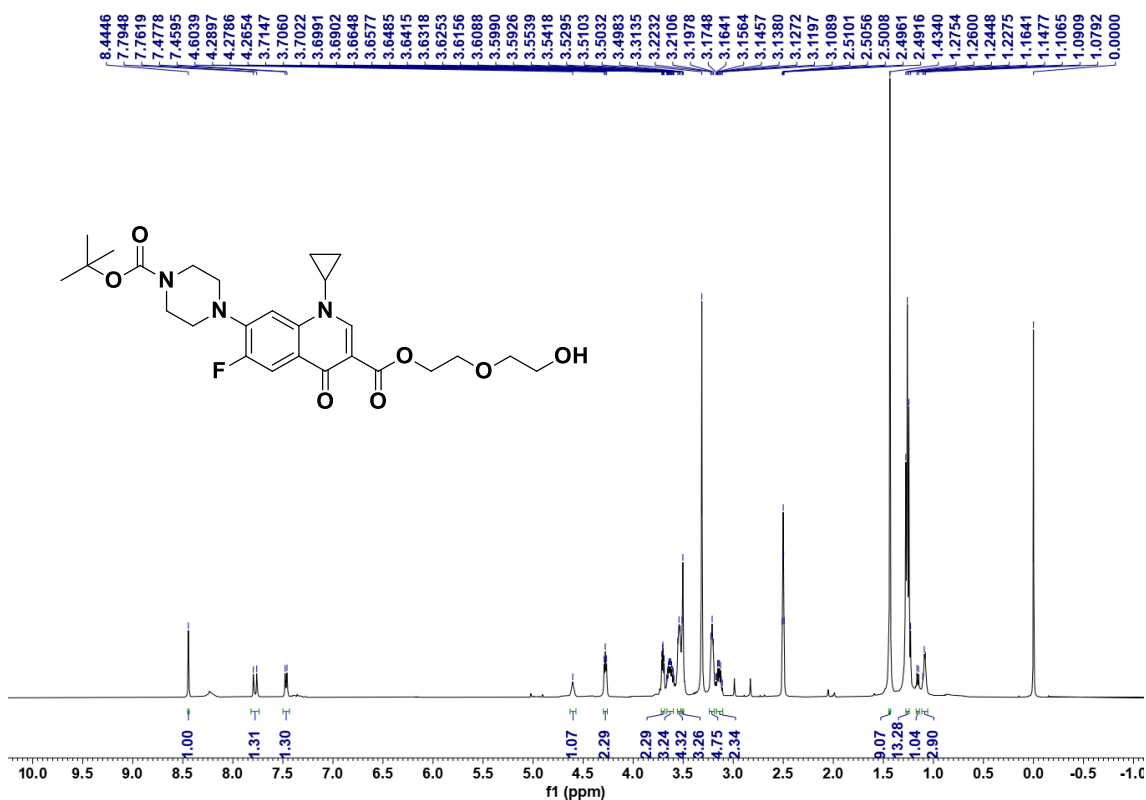
Attachment 18: ^{13}C NMR spectrum of NBocCipro-OH3 in DMSO-d_6



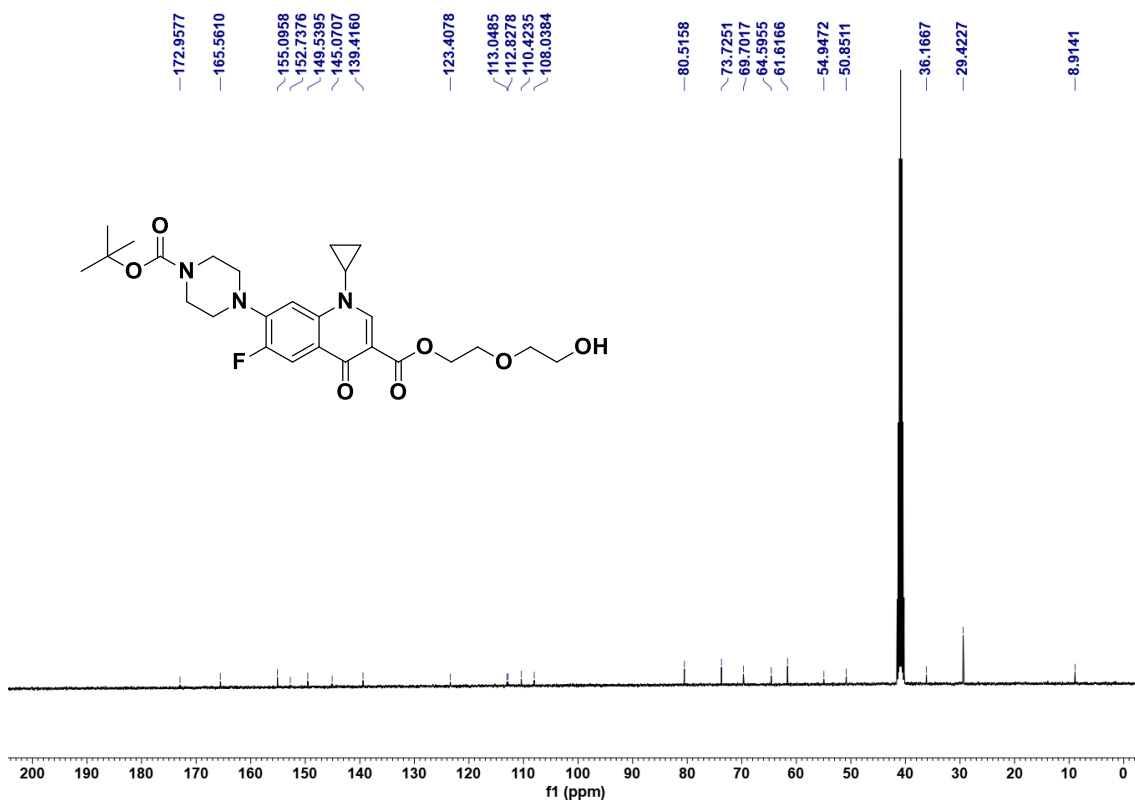
Attachment 19: DEPT-135 ^{13}C NMR spectrum of NBocCipro-OH3 in DMSO- d_6



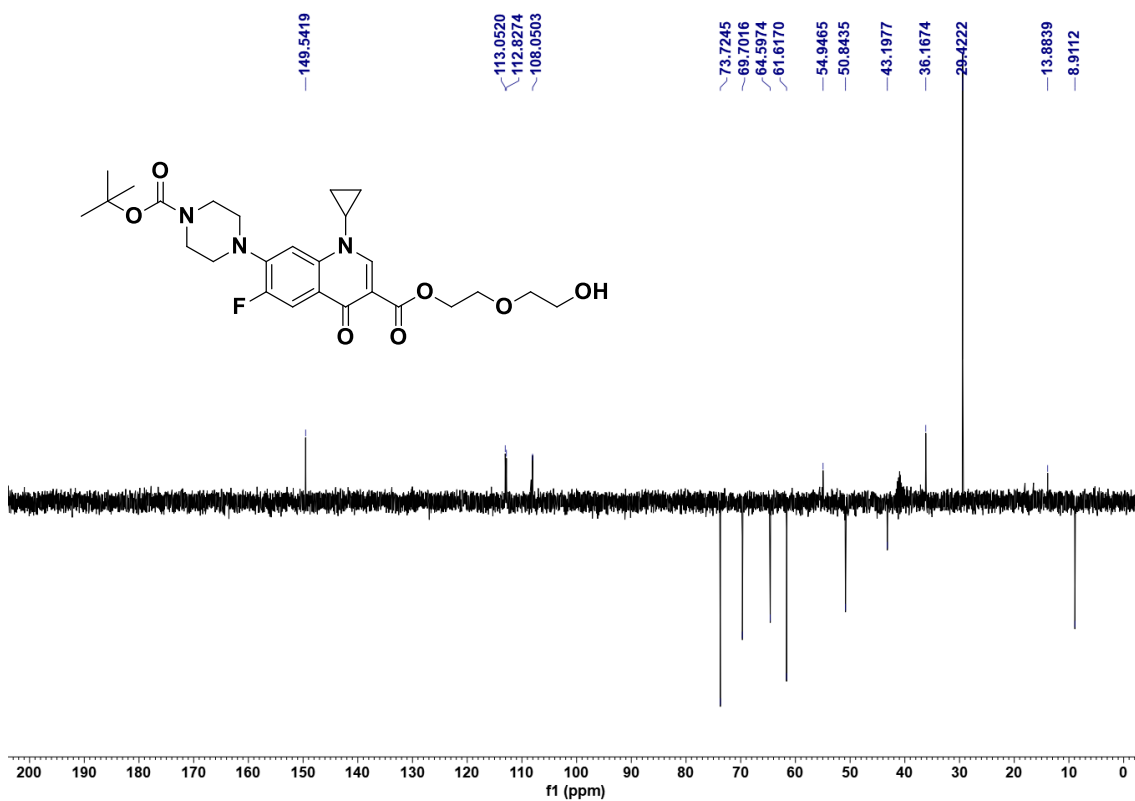
Attachment 20: ^1H NMR spectrum of NBocCipro-OH4 [2-(2-hydroxyethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in DMSO- d_6



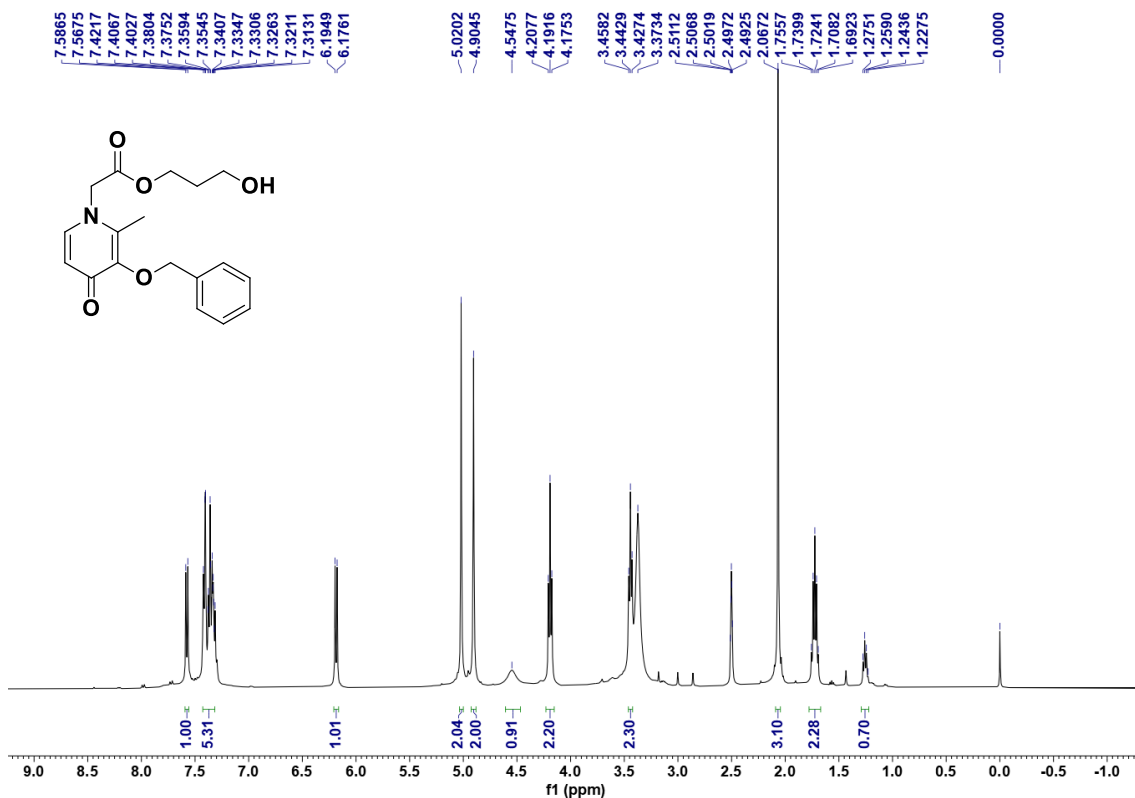
Attachment 21: ^{13}C NMR spectrum of NBocCipro-OH4 in DMSO- d_6



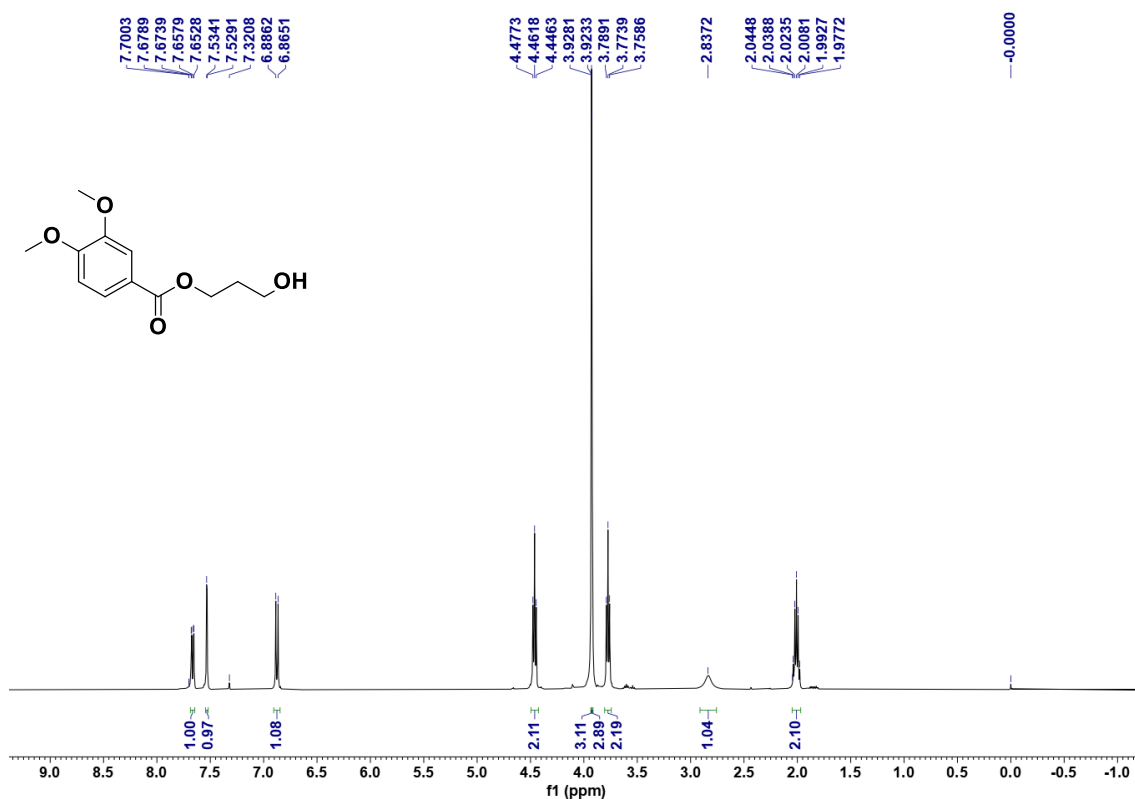
Attachment 22: DEPT-135 ^{13}C NMR spectrum of NBocCipro-OH4 in DMSO- d_6



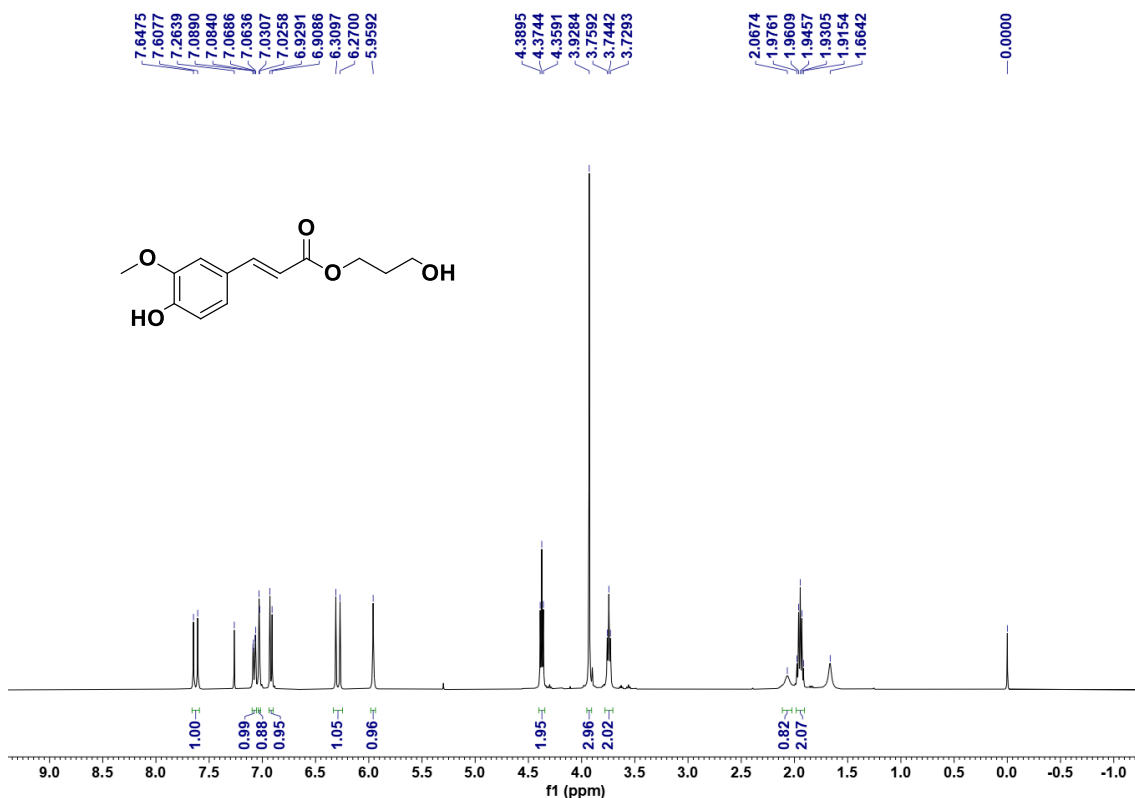
Attachment 23: ^1H NMR spectrum of Protected Def3-PropOH [3-hydroxypropyl 2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetate] in DMSO-d_6



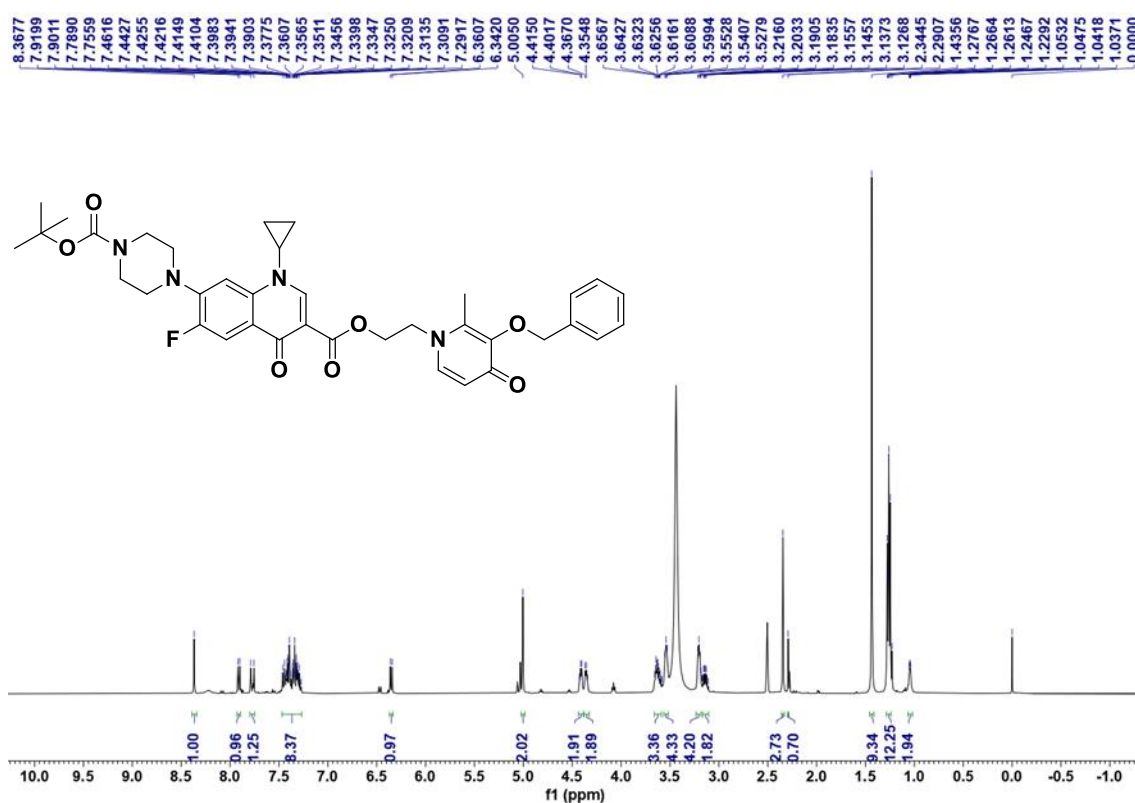
Attachment 24: ^1H NMR spectrum of VA-PropOH [3-hydroxypropyl-3,4-dimethoxybenzoate] in CDCl_3



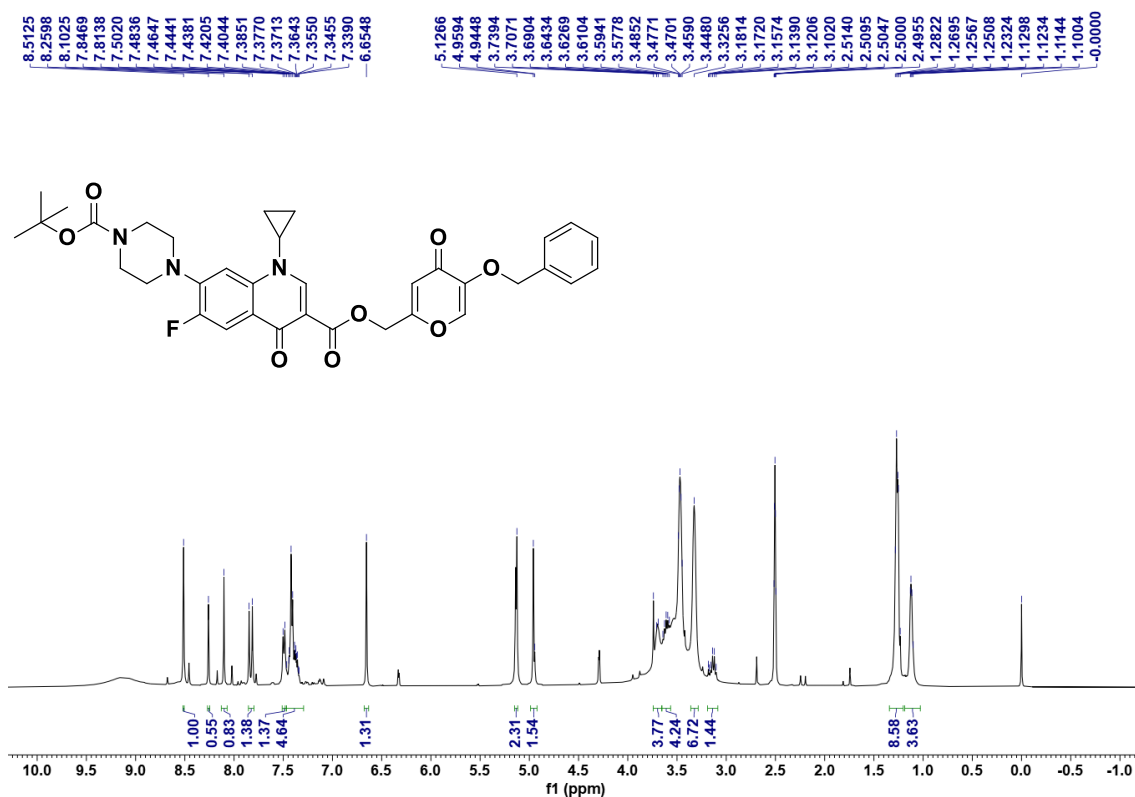
Attachment 25: ^1H NMR spectrum of FA-PropOH [(*E*)-3-hydroxypropyl-3-(4-hydroxy-3-methoxyphenyl)acrylate in CDCl_3



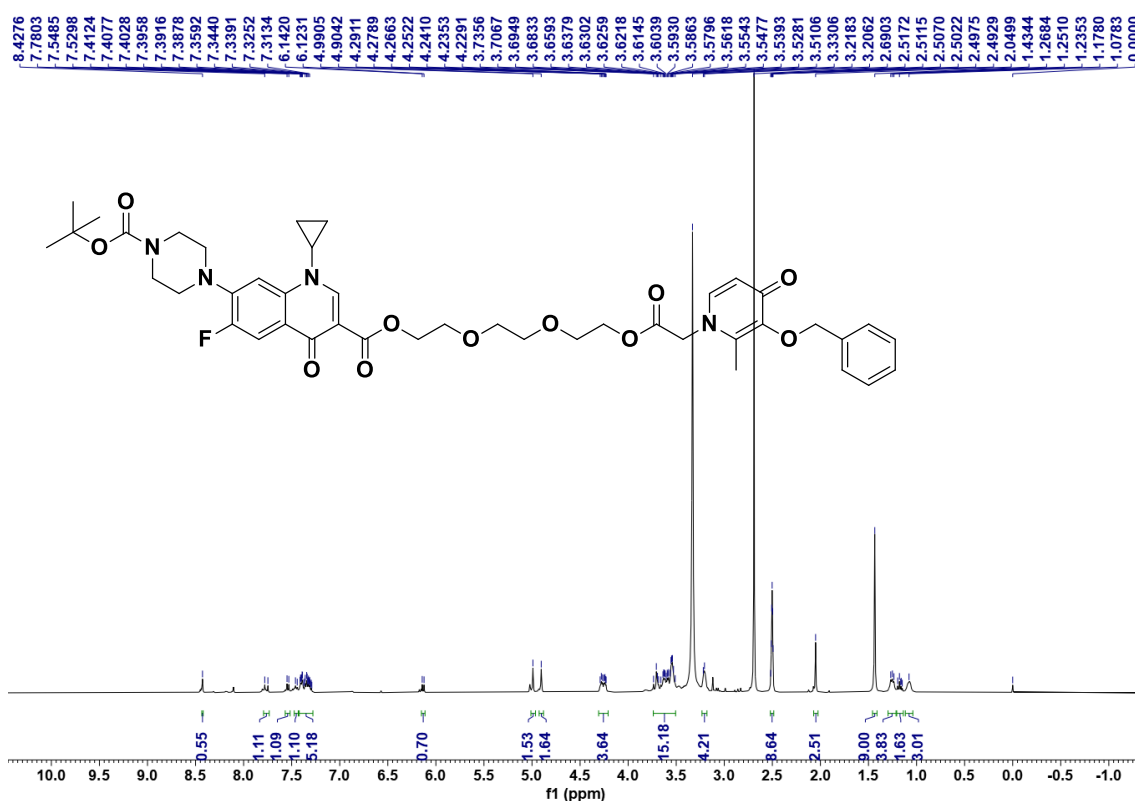
Attachment 26: ^1H NMR spectrum of Protected CIPSID1 [2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4dihydroquinoline-3-carboxylate] in $\text{DMSO}-d_6$



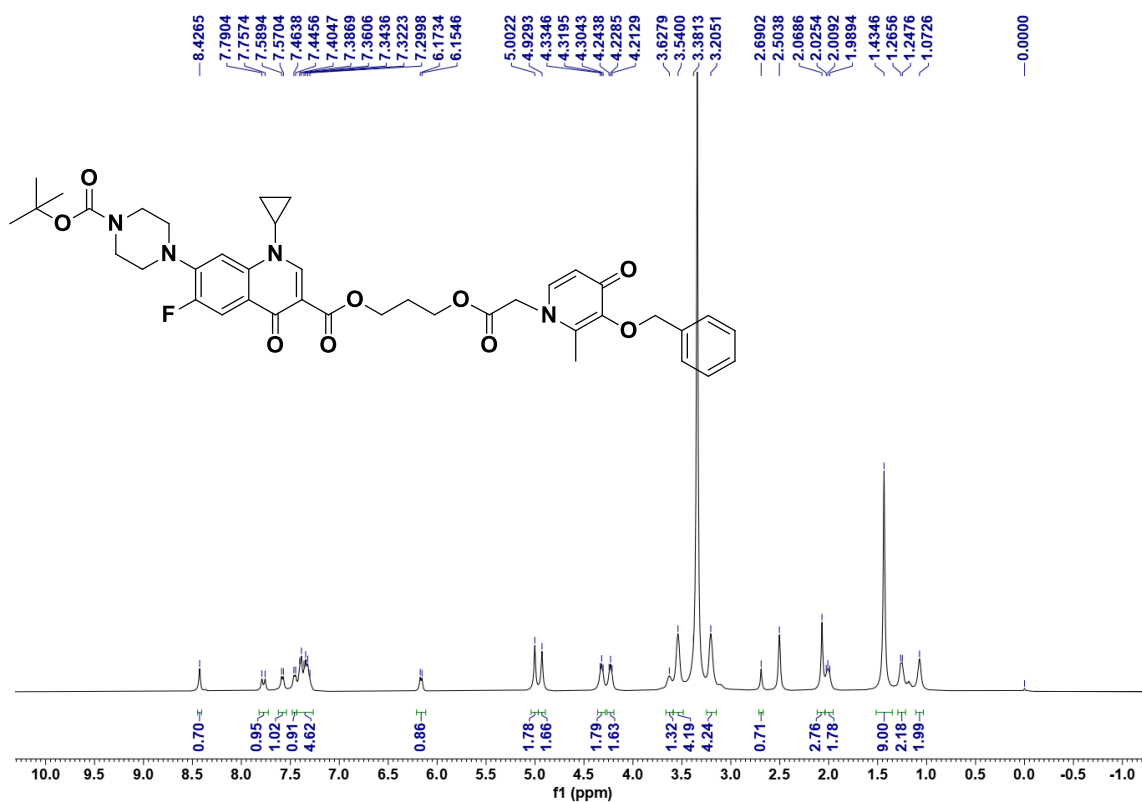
Attachment 27: ^1H NMR spectrum of Protected CIPSID2 [5-(benzyloxy-4-oxo-4H-pyran-2-yl)methyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in $\text{DMSO}-d_6$



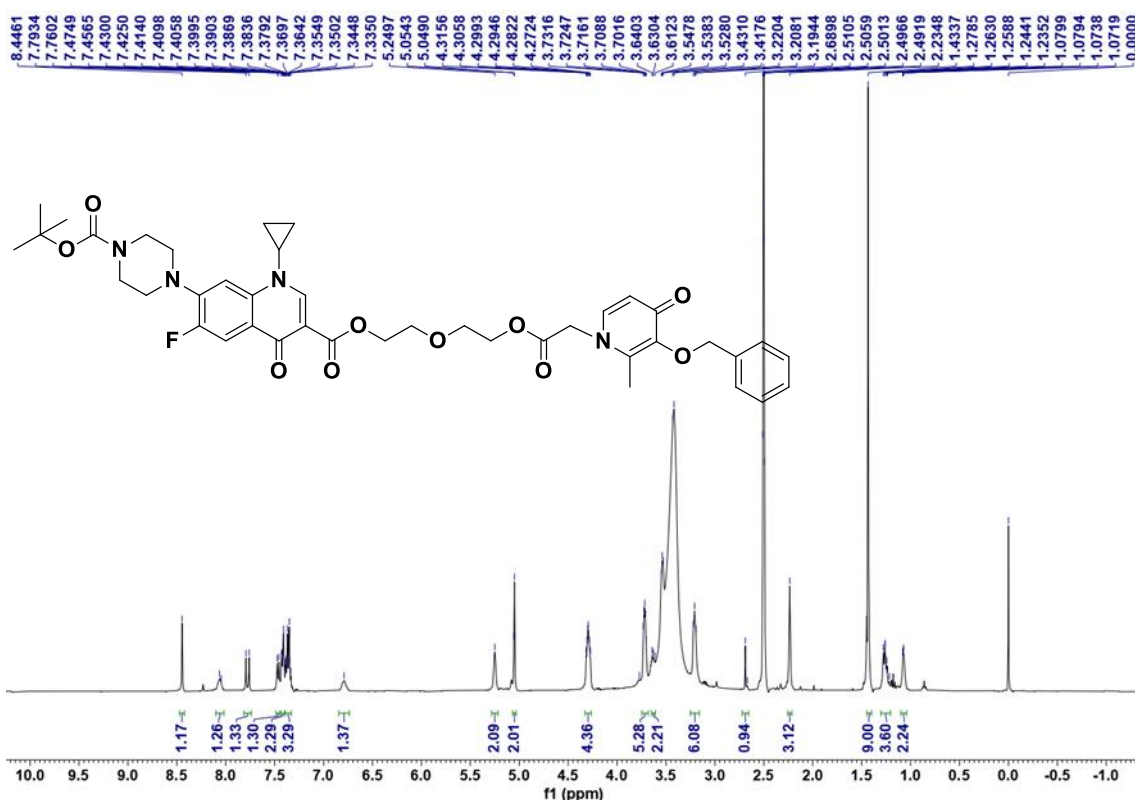
Attachment 28: ^1H NMR spectrum of Protected CIPSID4 [2-(2-(2-(2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetoxy)ethoxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] - DMSO



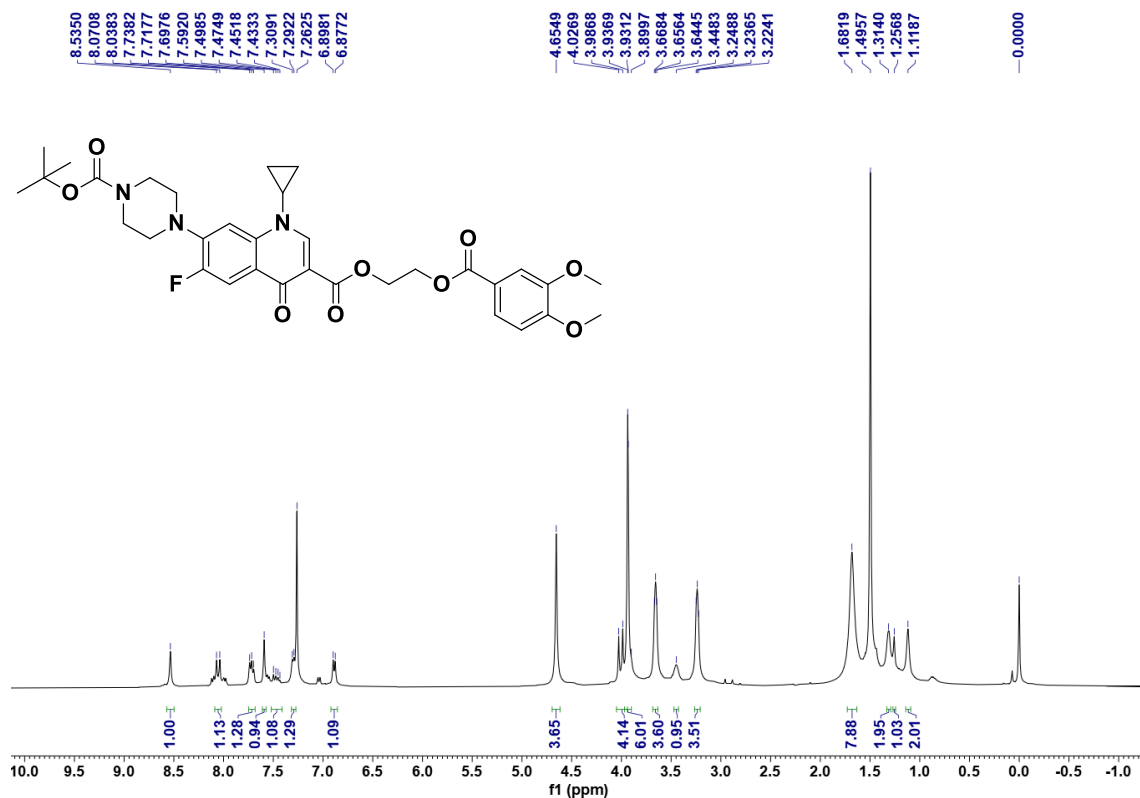
Attachment 29: ^1H NMR spectrum of Protected CIPSID5 [3-(2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetoxy)propyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in $\text{DMSO}-d_6$



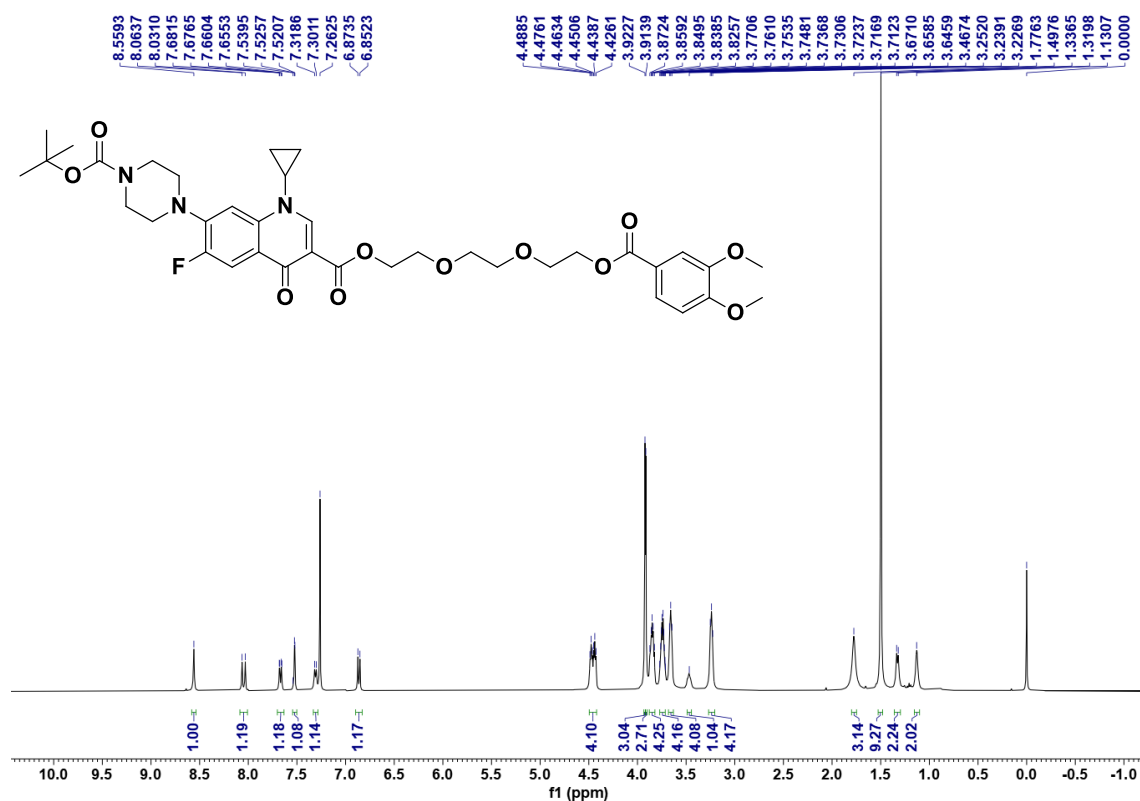
Attachment 30: ^1H NMR spectrum of Protected CIPSID6 [2-(2-(2-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4H)-yl)acetoxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in $\text{DMSO}-d_6$



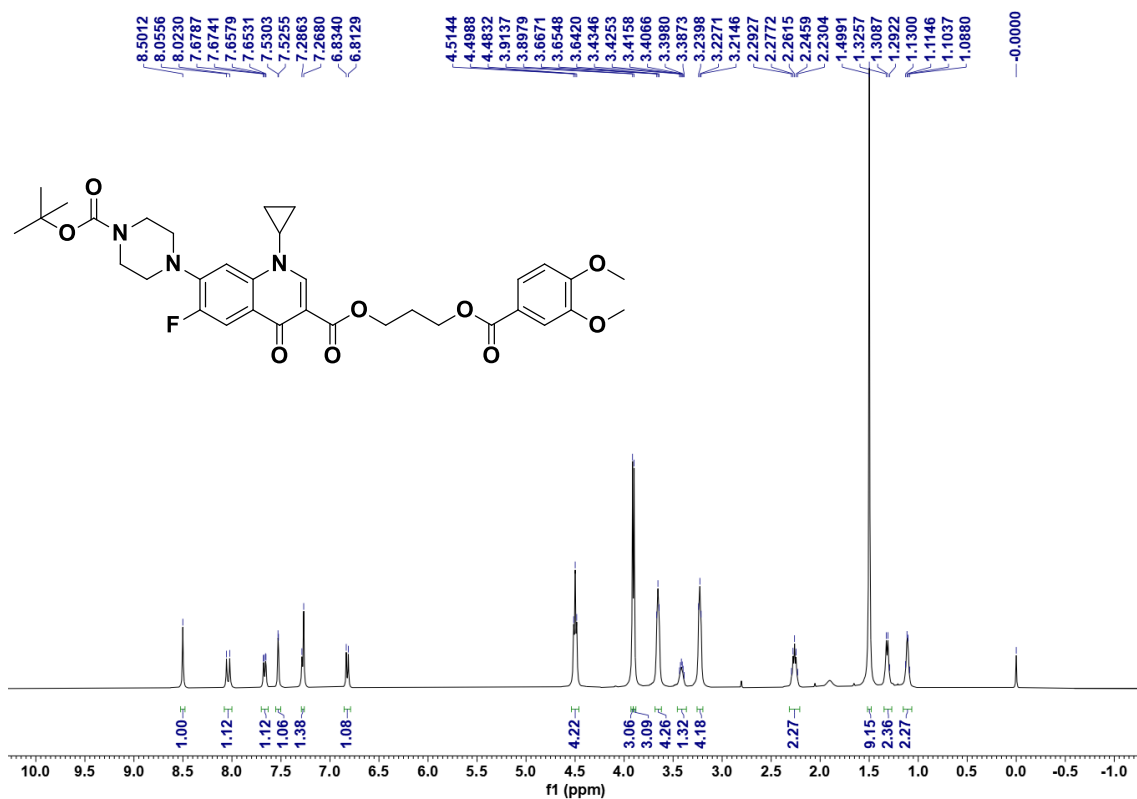
Attachment 31: ^1H NMR spectrum of Protected CIPSID7 [2-((3,4-dimethoxybenzoyl)oxy)ethyl-7-(4-(tert-butoxycarbonyl) piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in CDCl_3



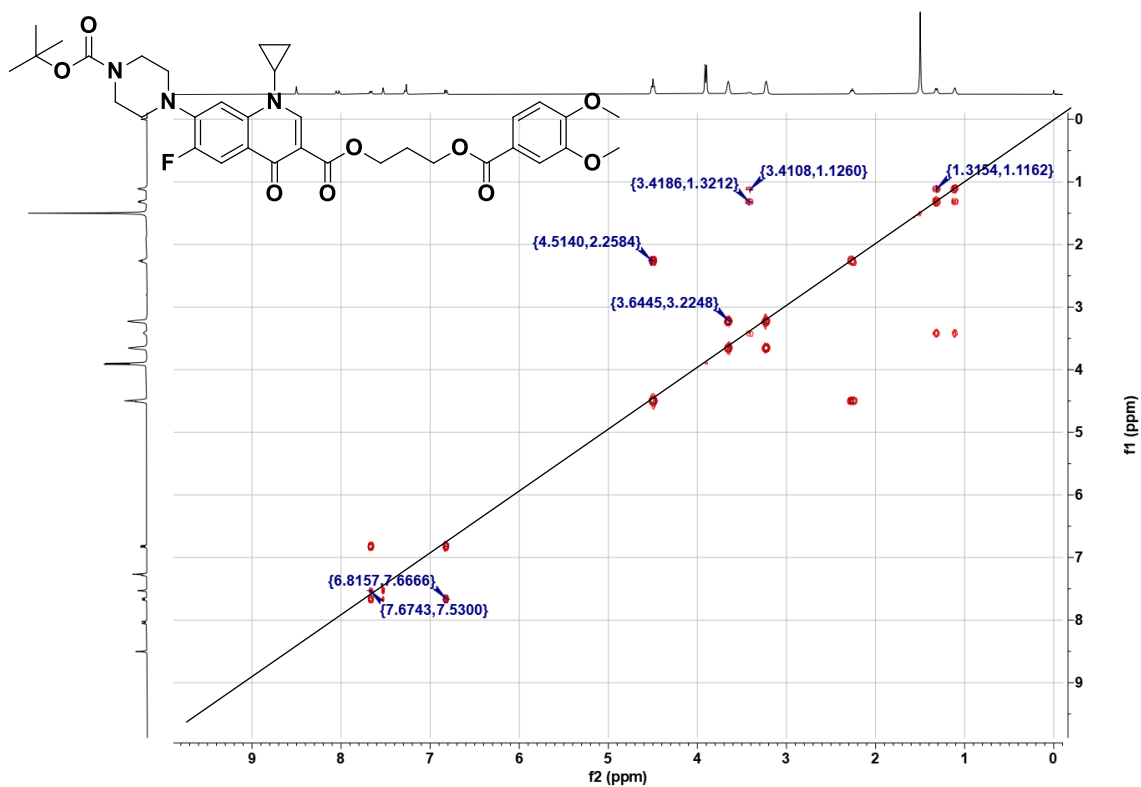
Attachment 32: ^1H NMR spectrum of Protected CIPSID8 [2-(2-(2-((3,4-dimethoxybenzoyl)oxy)ethoxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline -3-carboxylate] in CDCl_3



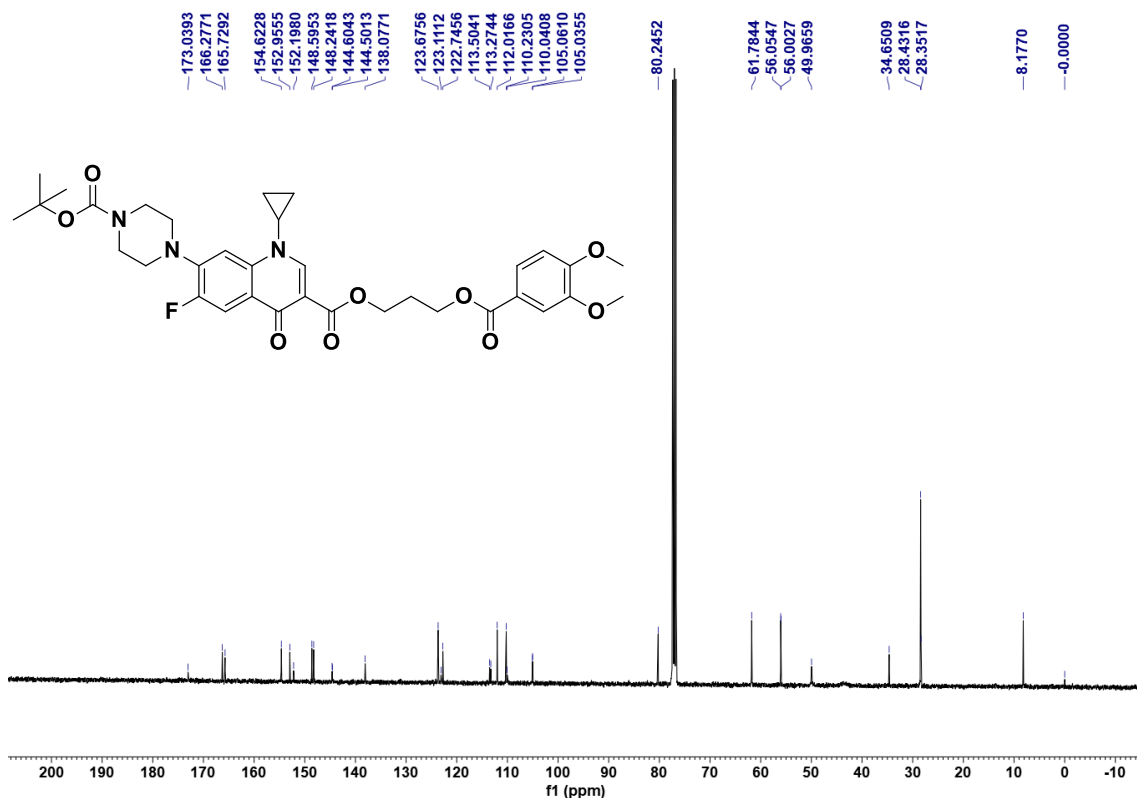
Attachment 33: ^1H NMR spectrum of Protected CIPSID9 [3-((3,4-dimethoxybenzoyl)oxy)propyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate] in CDCl_3



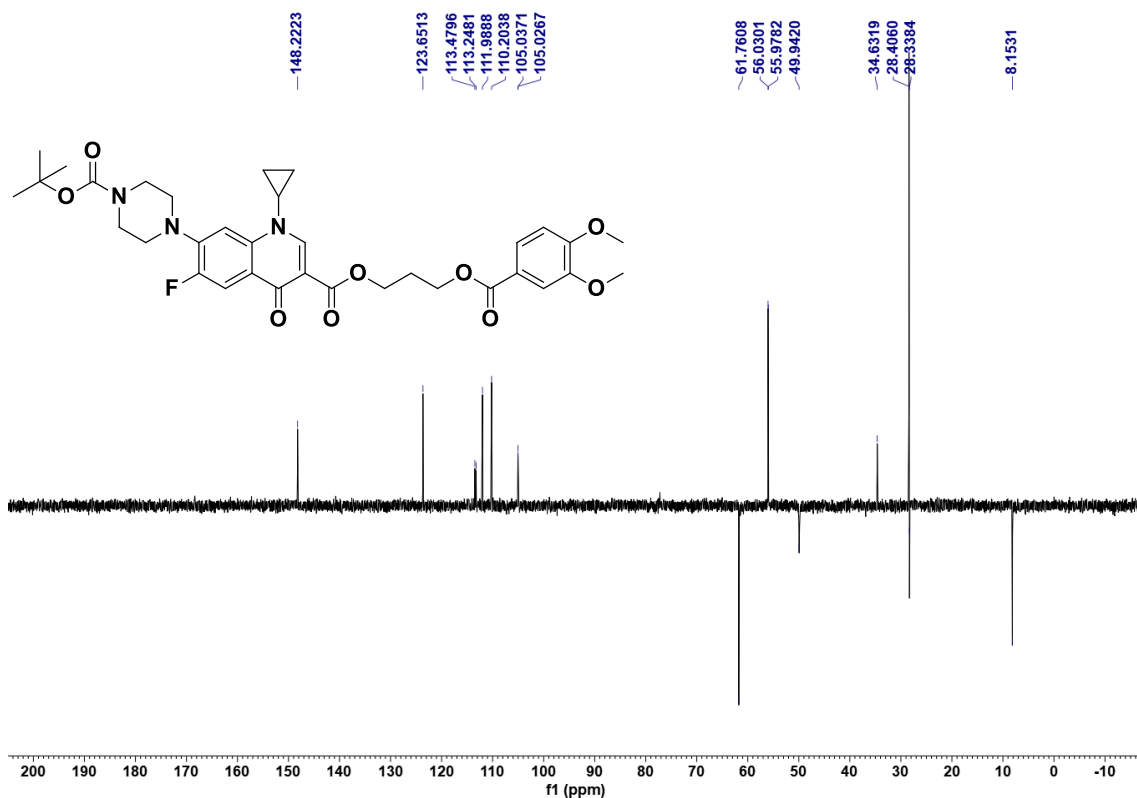
Attachment 34: ^1H - ^1H COSY NMR spectrum of Protected CIPSID9 in CDCl_3



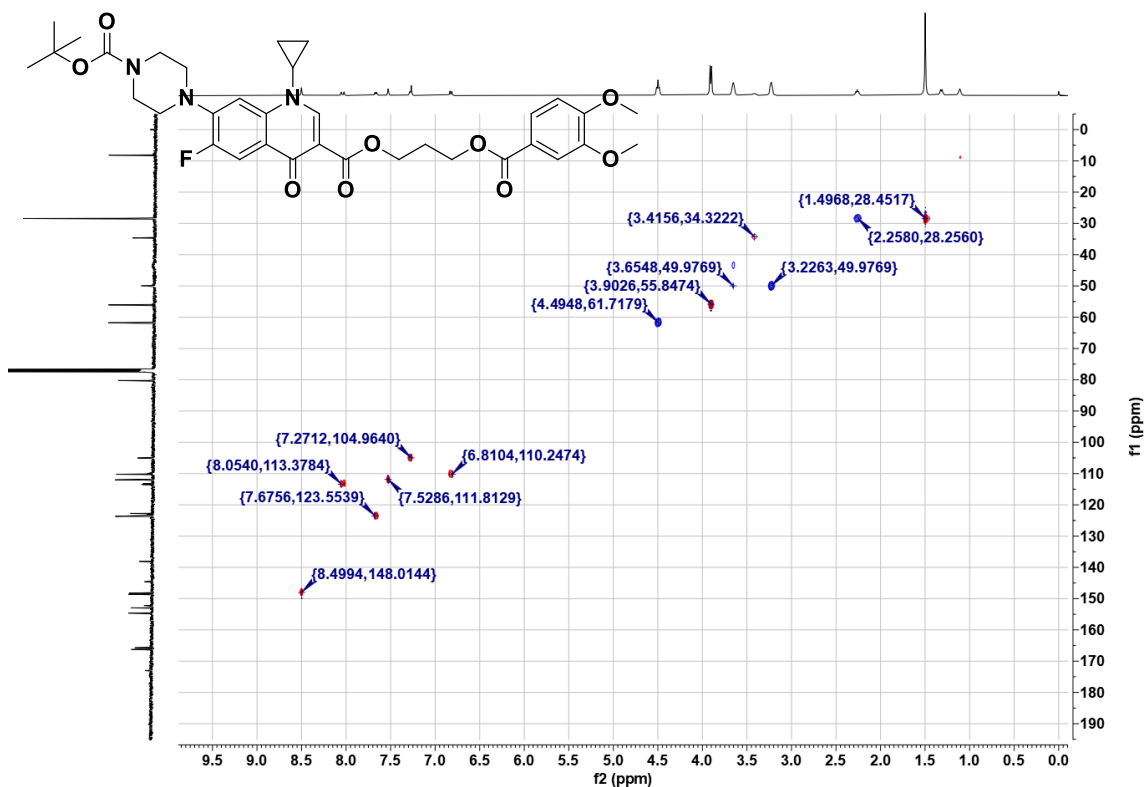
Attachment 35: ^{13}C NMR spectrum of Protected CIPSID9 in CDCl_3



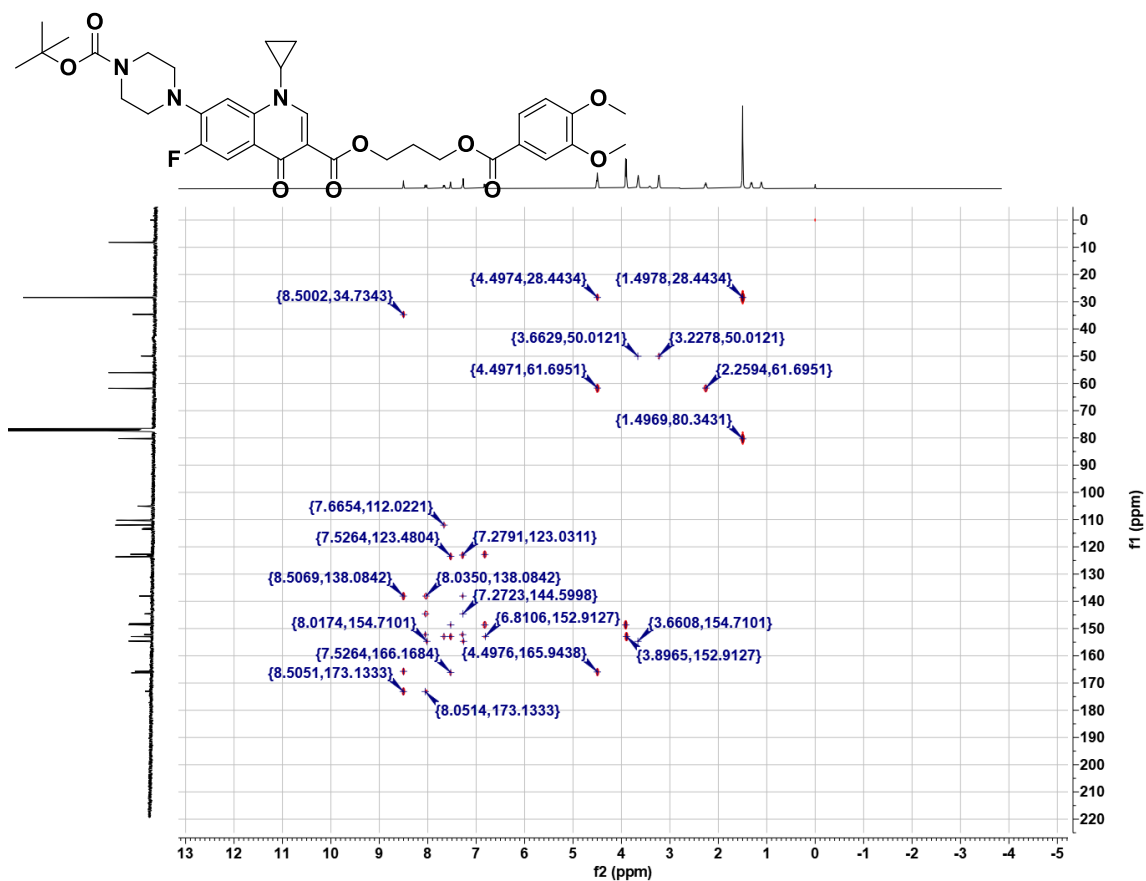
Attachment 36: DEPT-135 ^{13}C NMR spectrum of Protected CIPSID9 in CDCl_3



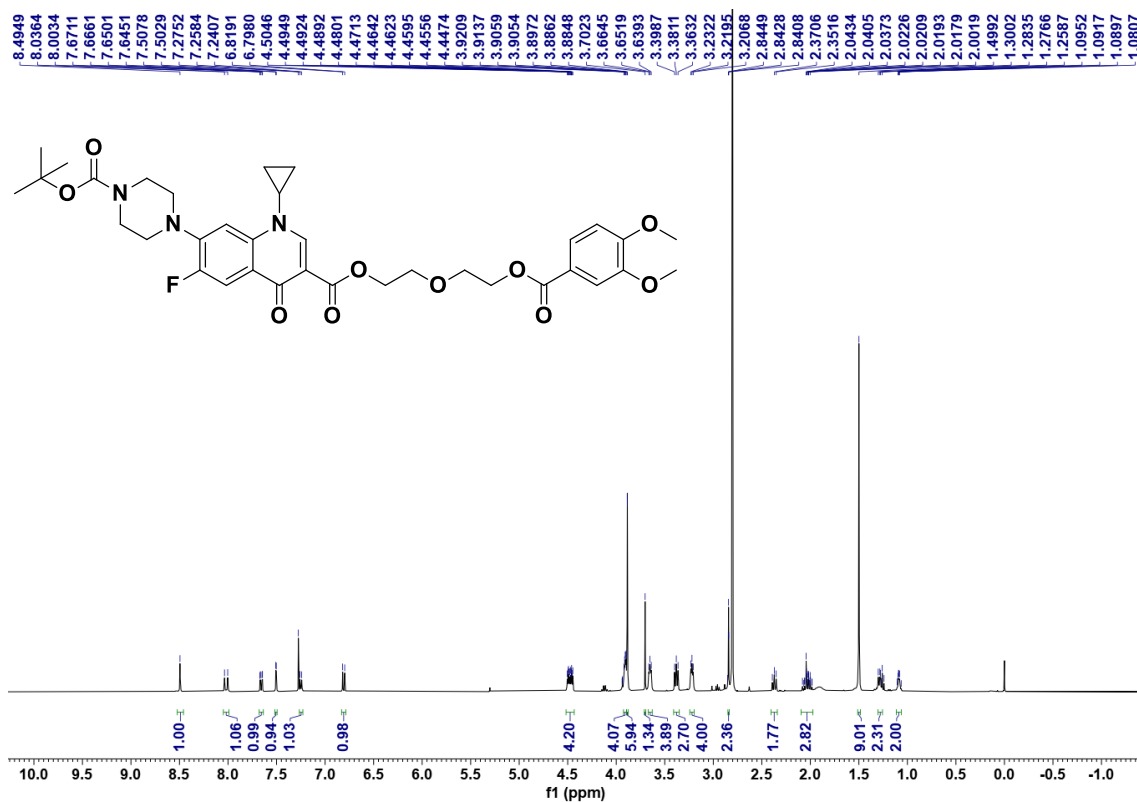
Attachment 37: ^1H - ^{13}C HSQC NMR spectrum of protected CIPSID9 in CDCl_3



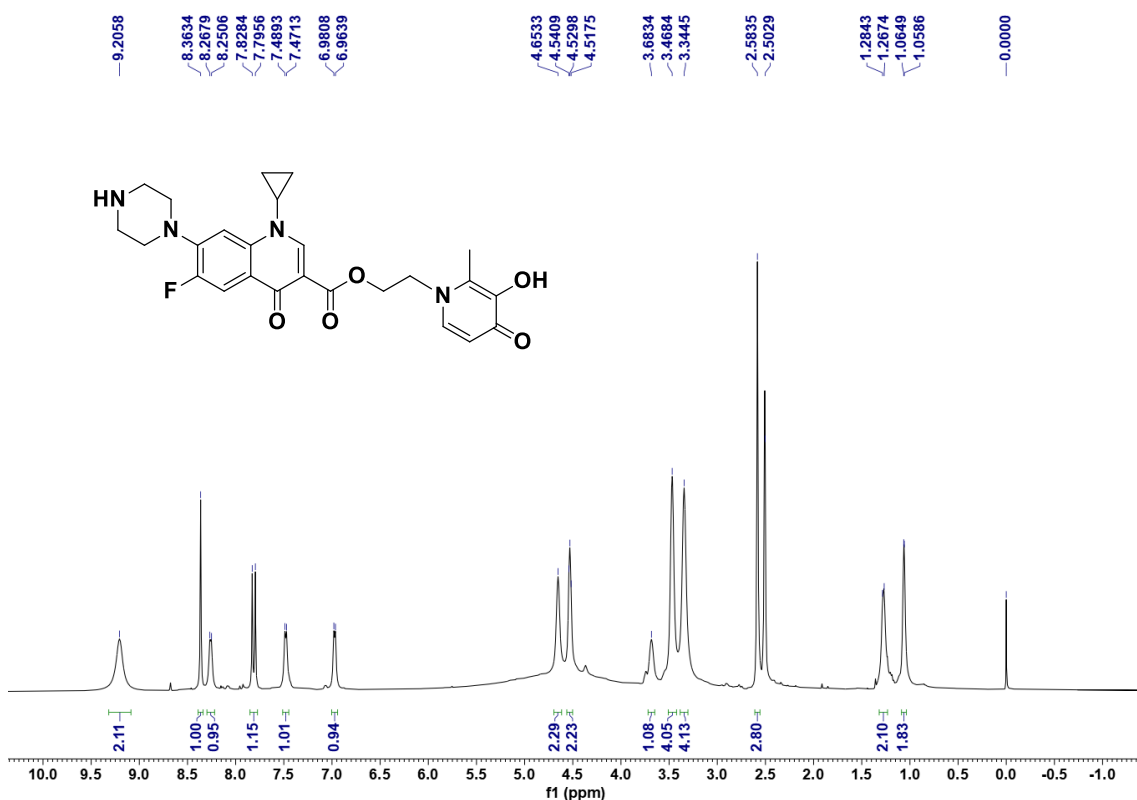
Attachment 38: ^1H - ^{13}C HMBC NMR spectrum of protected CIPSID9 in CDCl_3



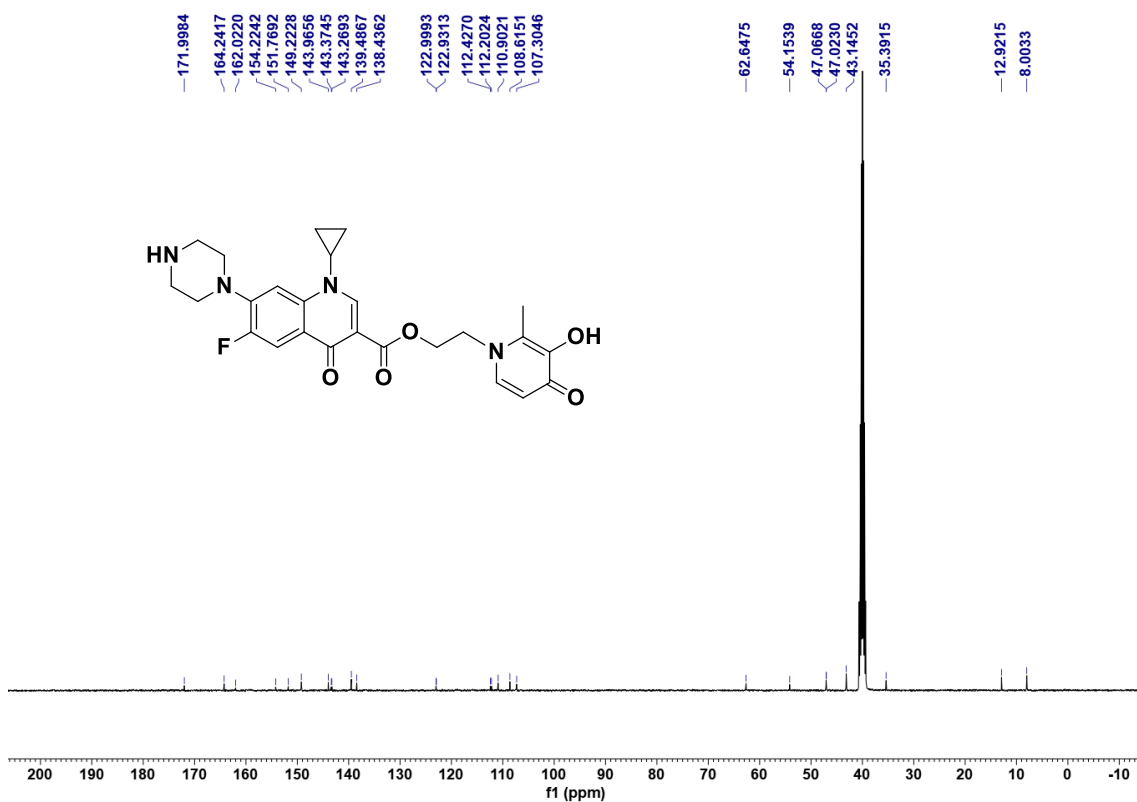
Attachment 39: ^1H NMR spectrum of Protected CIPSID10 [2-(2-((3,4-dimethoxybenzoyl)oxy)ethoxy)ethyl-7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline -3-carboxylate] in CDCl_3



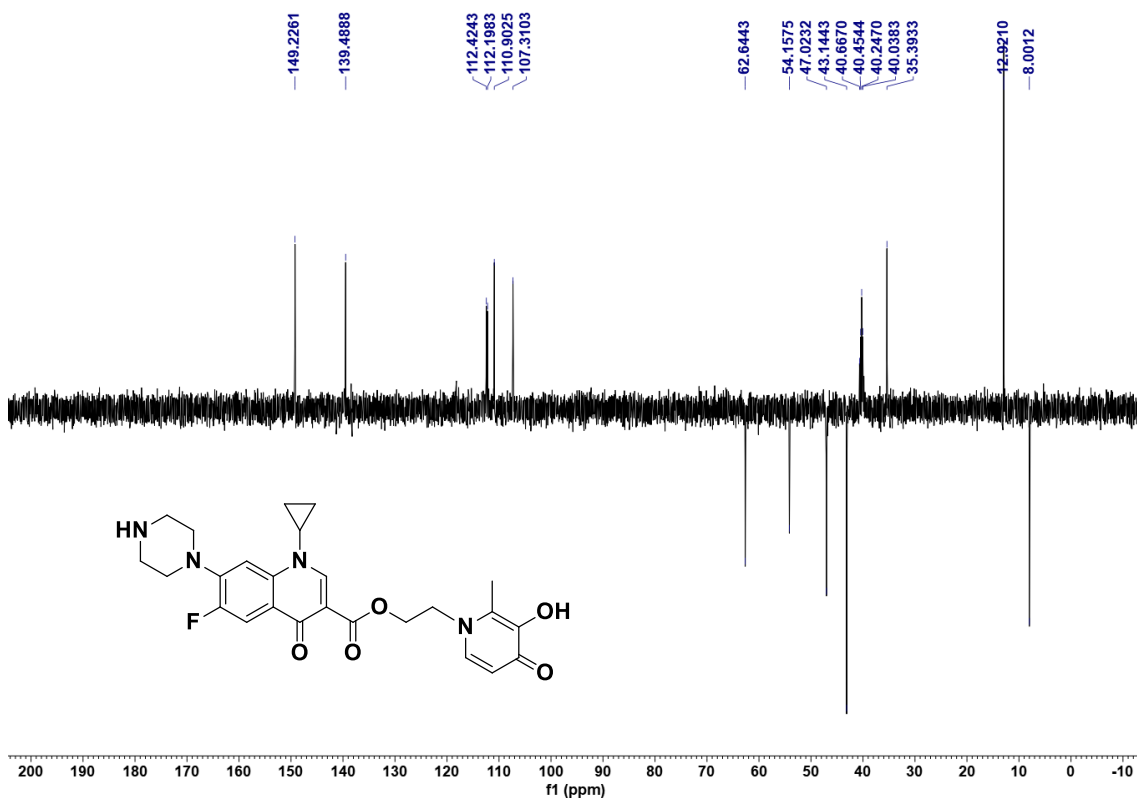
Attachment 40: ^1H NMR spectrum of CIPSID1 [4-(1-cyclopropyl-6-fluoro-3-((2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl) piperazin-1-ium 2,2,2-trifluoroacetate] in $\text{DMSO}-d_6$



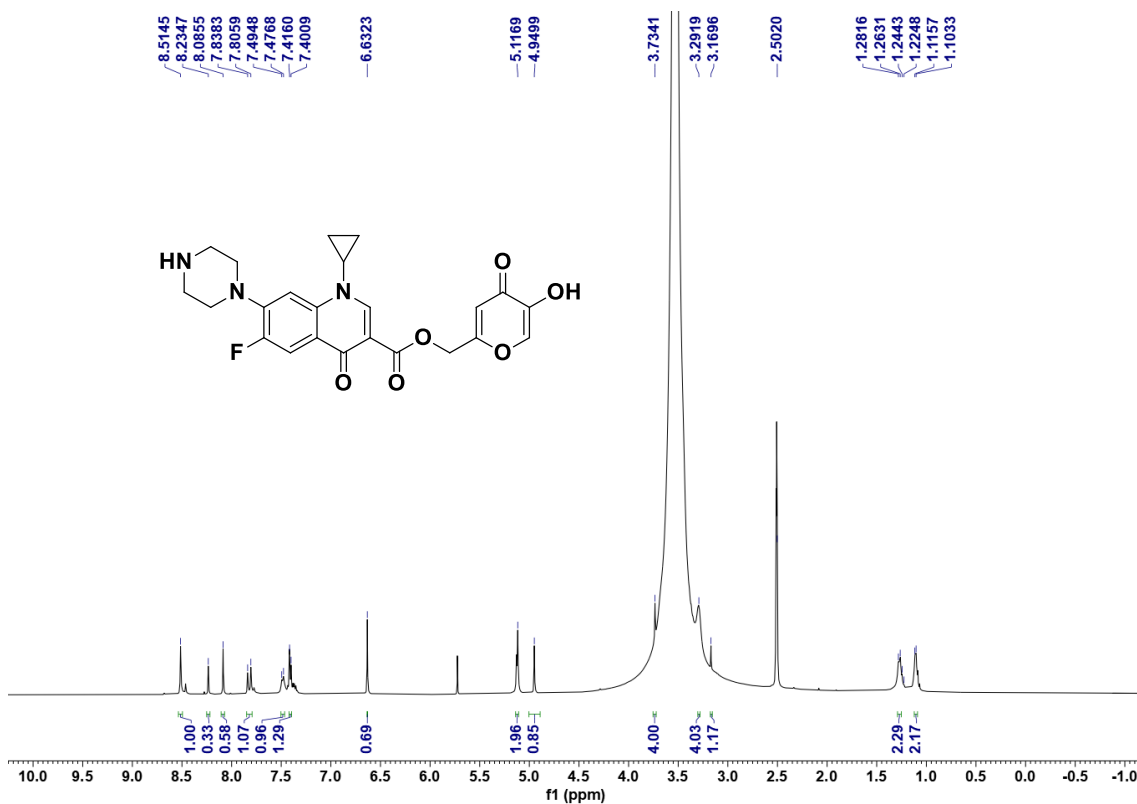
Attachment 41: ^{13}C NMR spectrum of CIPSID1 in DMSO-d_6



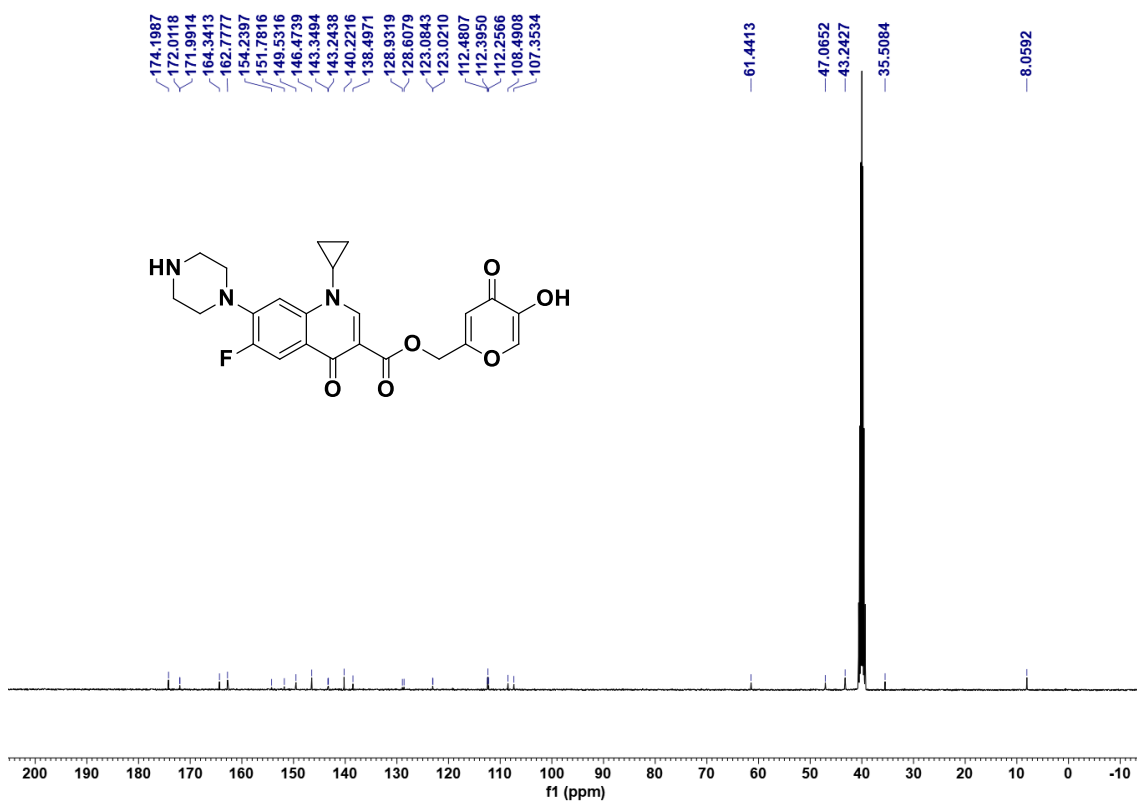
Attachment 42: DEPT-135 ^{13}C NMR spectrum of CIPSID1 in DMSO-d_6



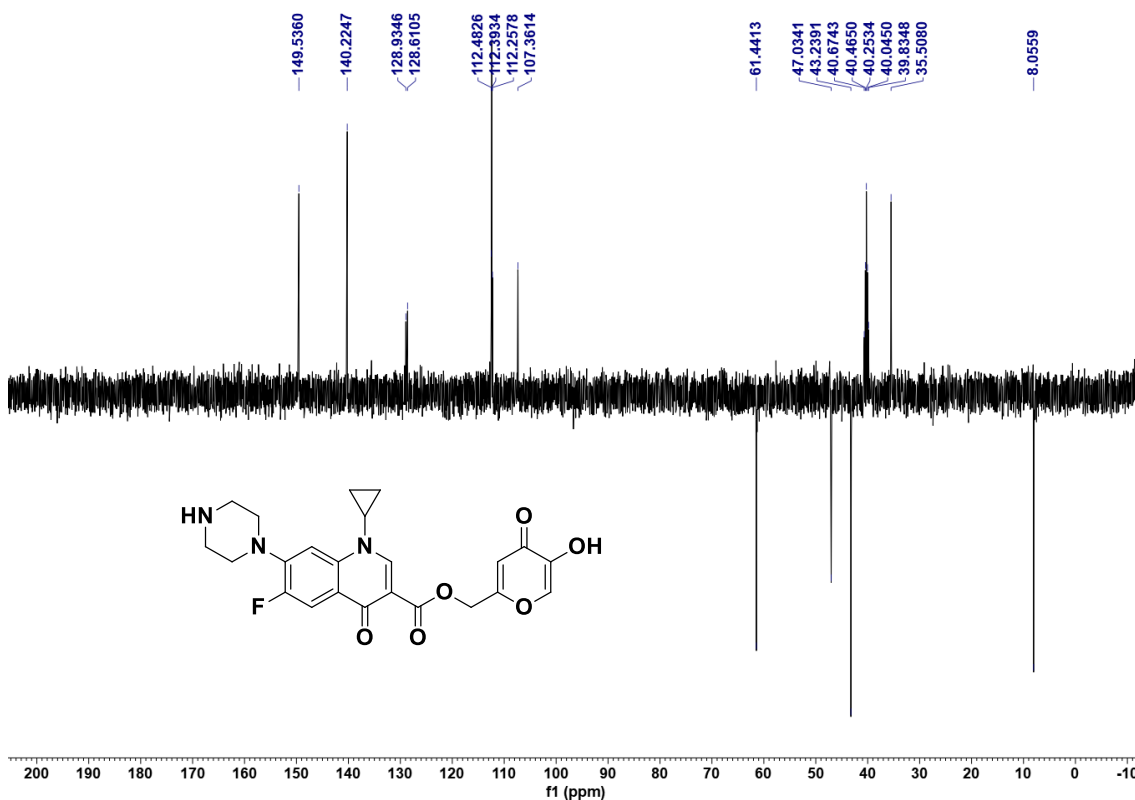
Attachment 43: ^1H NMR spectrum of CIPSID2 [4-(1-cyclopropyl-6-fluoro-3-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate] in DMSO-d_6



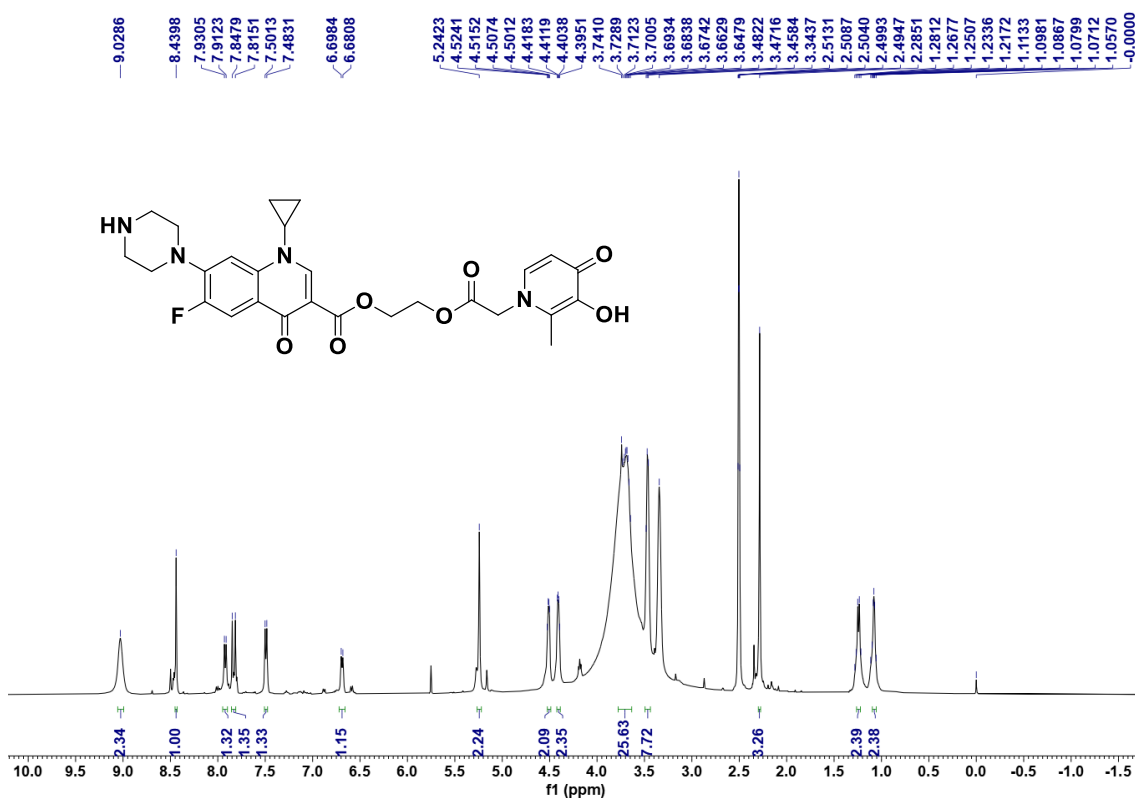
Attachment 44: ^{13}C NMR spectrum of CIPSID2 in DMSO-d_6



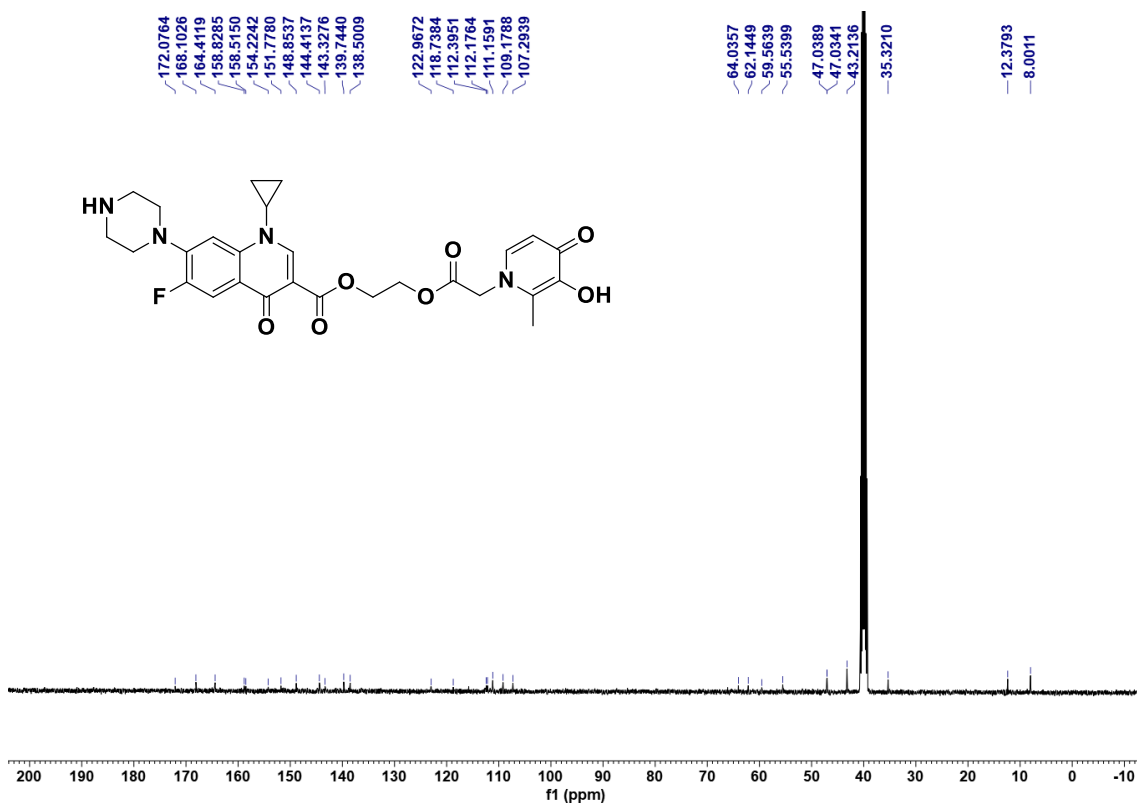
Attachment 45: DEPT-135 ^{13}C NMR spectrum of CIPSID2 in DMSO- d_6



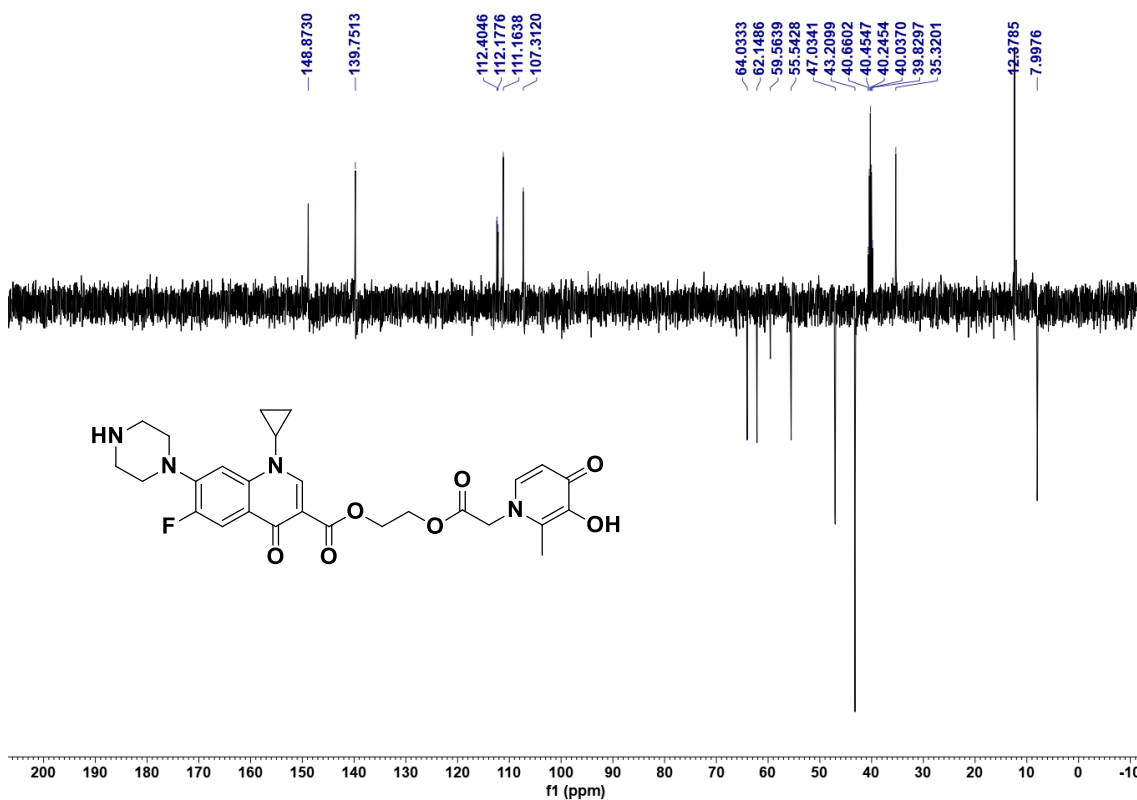
Attachment 46: ¹H NMR spectrum of CIPSID3 [4-(1-cyclopropyl-6-fluoro-3-((2-(2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)acetoxymethoxy)ethoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium 2,2,2-trifluoroacetate] in DMSO-d₆



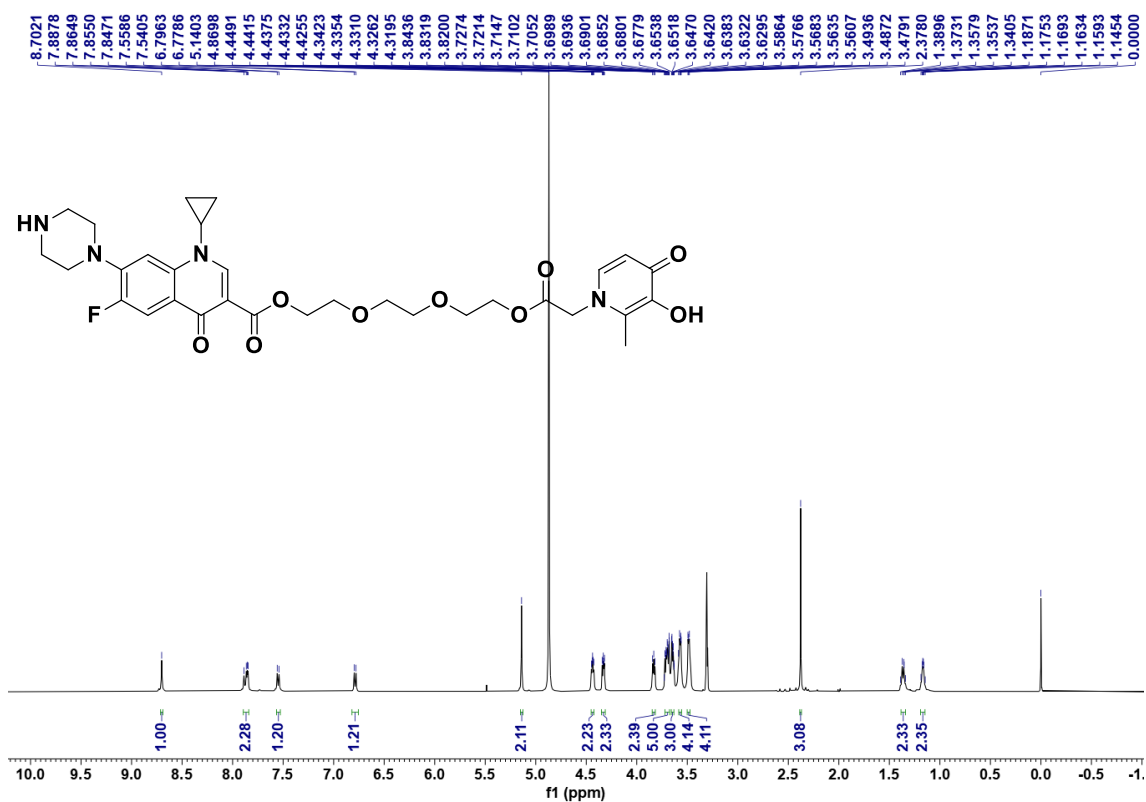
Attachment 47: ^{13}C NMR spectrum of CIPSID3 in DMSO-d_6



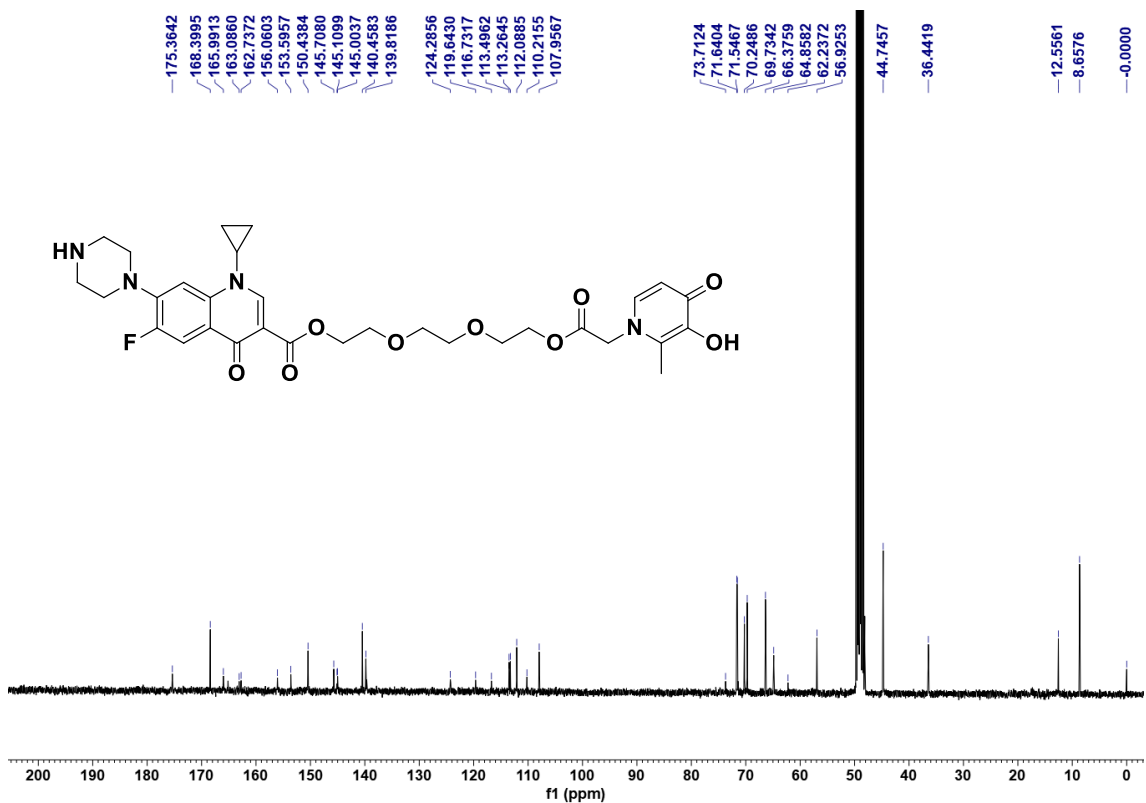
Attachment 48: DEPT-135 ^{13}C NMR spectrum of CIPSID3 in DMSO-d_6



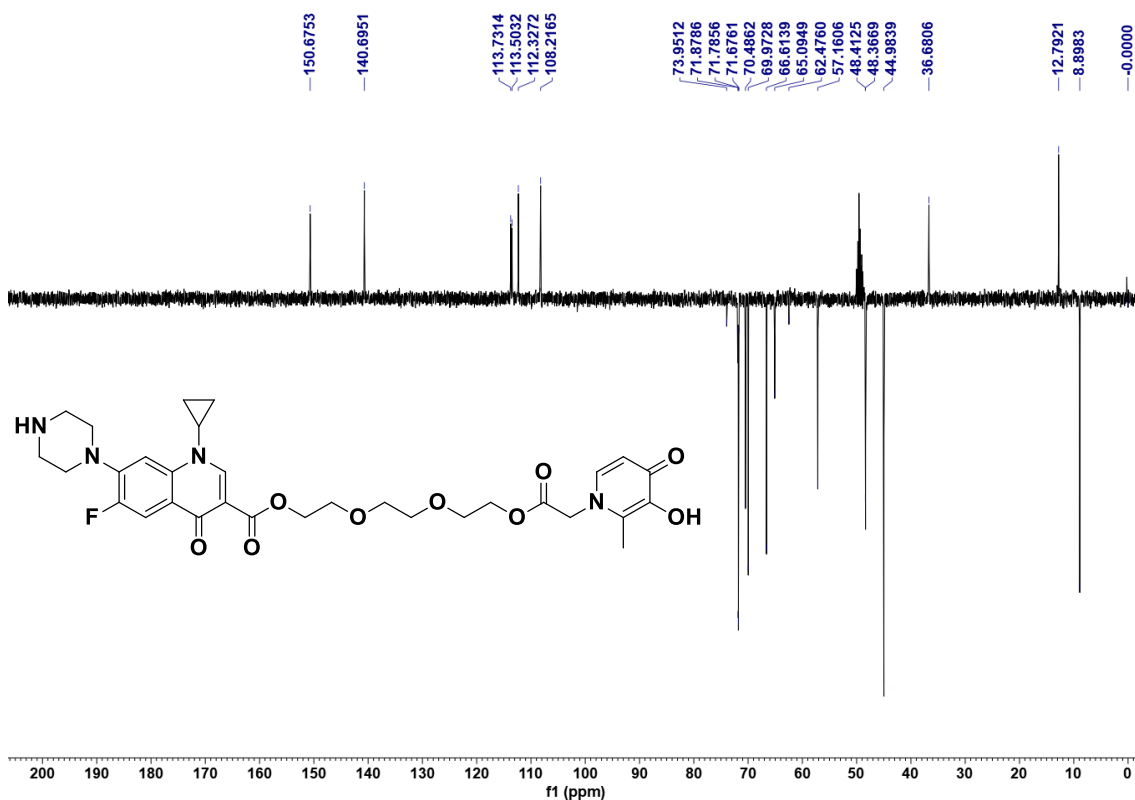
Attachment 49: ^1H NMR spectrum of CIPSID4 [4-(1-cyclopropyl-6-fluoro-3-(13-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)-12-oxo-2,5,8,11-tetraoxatridecan-1-oyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate] in MeOD-d_4



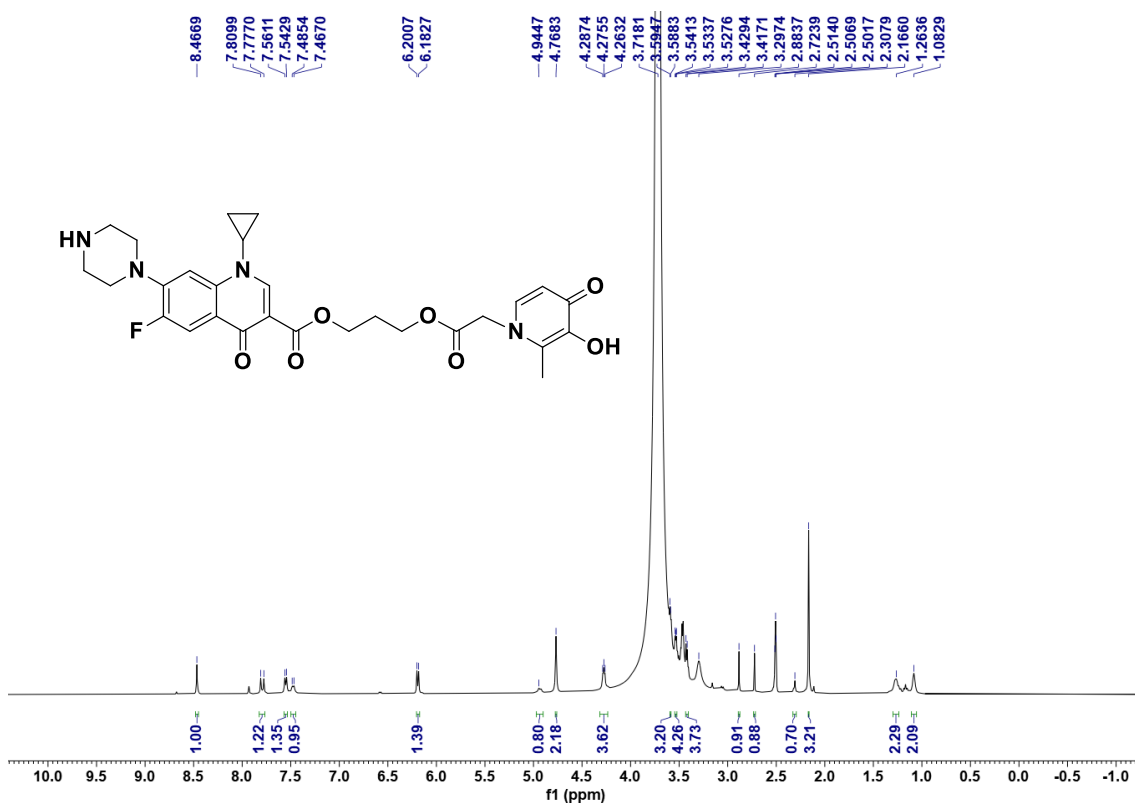
Attachment 50: ^{13}C NMR spectrum of CIPSID4 in MeOD-d_4



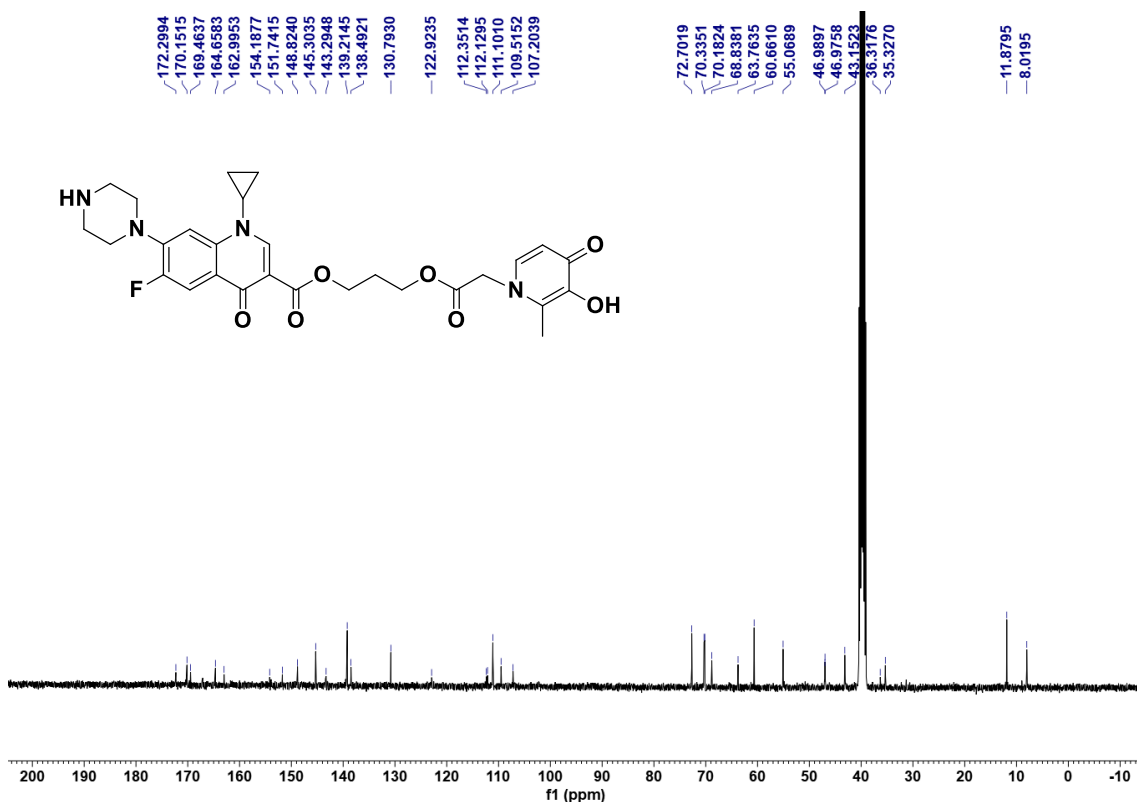
Attachment 51: DEPT-135 ^{13}C NMR spectrum of CIPSID4 in MeOD-d_4



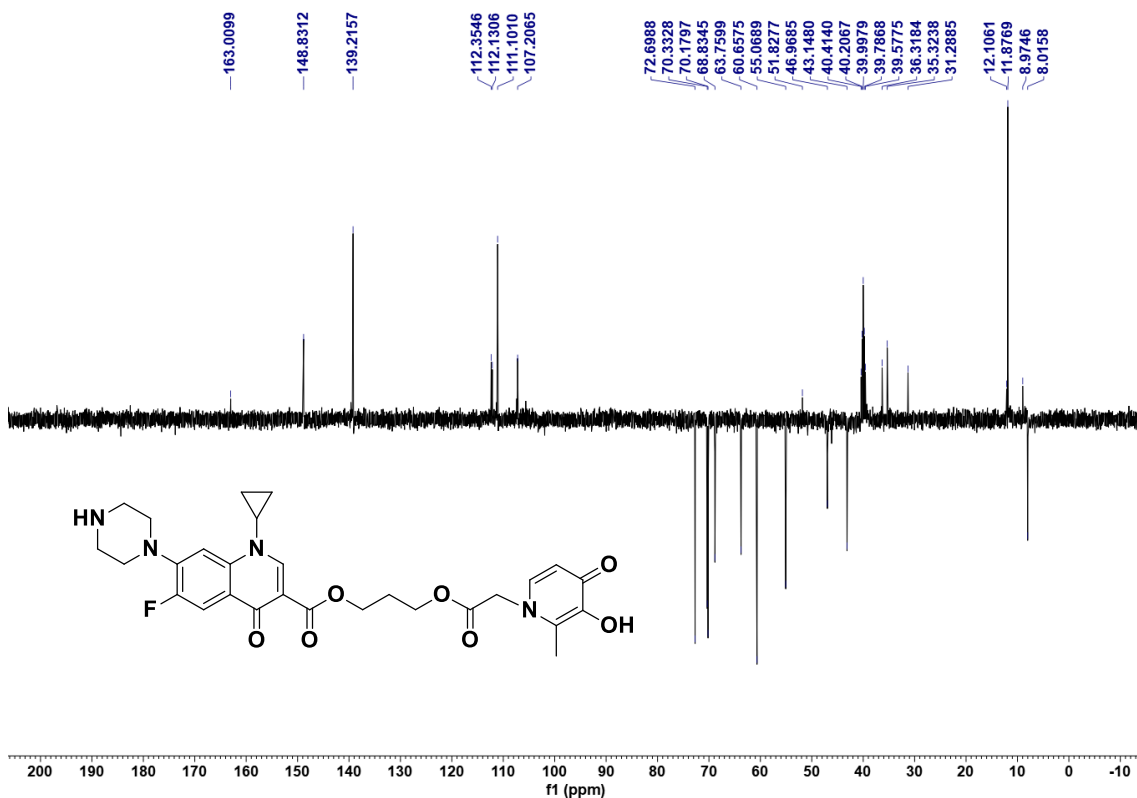
Attachment 52: ^1H NMR spectrum of CIPSID5 [4-(1-cyclopropyl-6-fluoro-3-((3-(2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)acetoxy)propoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate] in DMSO-d_6



Attachment 53: ^{13}C NMR spectrum of CIPSID5 in $\text{DMSO}-d_6$



Attachment 54: DEPT-135 ^{13}C NMR spectrum of CIPSID5 in $\text{DMSO}-d_6$



Attachment 55: ^1H NMR spectrum of CIPSID6 [4-(1-cyclopropyl-6-fluoro-3-((2-(2-(2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)acetoxy)ethoxy)ethoxy)carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium-2,2,2-trifluoroacetate] in MeOD-d_4

