Studies on structure/antimicrobial activity relationship of cyclam derivatives

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Abstract

In this work, cyclam derivatives of the type $H_4[H_3BnCyclam]Cl_4$, $H_2[H_2Bn_2Cyclam](CH_3COO)_2$, $H_4[H_2Bn_2Cyclam]Cl_4$ and $H_4[H_4Cyclam]Cl_4$ were prepared. These compounds were tested against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli*, *Burkholderia contaminans* and *Pseudomonas aeruginosa* bacteria, to gain insights into the relationship between molecular structure and antimicrobial activity of these compounds. The most effective cyclam derivatives against the bacterial strains tested were the *trans*-disubstituted cyclams displaying fluorinated groups in the *para*-position of the benzyl moieties, for which Minimal Inhibitory Concentration (MIC) values within the range 5 – 18 µg/mL were registered for *E. coli* and S. *aureus*. An inconsistent wider range of MIC values were registered for *P. aeruginosa*, while for *B. contaminans* MIC values for the compounds tested were higher than 512 µg/mL. No significant differences in the antimicrobial activity of the compounds were observed when testing their acetate or chloride salts. For *E. coli*, the highest value estimated for the frequency of spontaneous emergence of resistance to the most effective cyclam derivatives was 6×10^{-8}. This work evidenced that the presence of an increasing number of pendant arms in the cyclam backbone greatly enhances its antimicrobial activity and decrease its solubility in water. The compound bearing 4 pendant arms was not soluble in water and its antimicrobial activity could not be tested.

Keywords: Tetraazamacrocycles, MIC, Drug resistance, Novel drugs, Chemical Synthesis

Resumo

Neste trabalho foram sintetizados derivados de ciclama do tipo $H_4[H_3BnCyclam]Cl_4$, $H_2[H_2Bn_2Cyclam](CH_3COO)_2$, $H_4[H_2Bn_2Cyclam]Cl_4$ e $H_4[H_4Cyclam]Cl_4$. Estes compostos foram testados contra a bactéria Gram-positiva *Staphylococcus aureus* e as bactérias Gram-negativas *Escherichia coli*, *Burkholderia contaminans* e *Pseudomonas aeruginosa*, de forma a tentar relacionar a estrutura molecular com a atividade antimicrobiana destes compostos. Os derivados de ciclama mais ativos contra as estirpes testadas foram as ciclamas *trans*-disubstituídas que apresentavam grupos fluorados na posição *para* dos benzilos, que apresentaram valores de concentrações mínimas inibitórias (CMI) entre os 5 e 18 µg/mL para *E. coli* e S. *aureus*. Para *P. aeruginosa* os valores de CMI tiveram uma variação inconsistente enquanto *B. contaminans* os valores de CMI foram superiores a 512 µg/mL. Não foram observadas diferenças significativas na atividade antimicrobiana quando foram testados os sais de acetato ou de cloreto. Para *E. coli* o valor de frequência de emergência de resistência espontânea mais alto foi 6×10^{-8} para os derivados de ciclama mais ativos. Este trabalho evidencia que a presença de um maior número de braços pendentes na ciclama aumenta significativamente a atividade antimicrobiana e diminui de solubilidade em água. O composto com 4 braços não é solúvel em água pelo que a sua atividade antimicrobiana não possível de ser testada.

Palavras-chave: Tetraazamacrociclos, CMI, Resistência, Novas Drogas, Síntese Química

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Abbreviations

Boc	Tert-butyloxycarbonyl
br	broad
CFU	Colony forming units
COSY	Correlation spectroscopy
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
ESI	Electrospray Ionization
HIV	Human immunodeficiency virus
HSQC	Heteronuclear single quantum coherence spectroscopy
J	Coupling constant
LB	Lennox Broth
m	multiplet
MDR	Multidrug resistance
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
MS	Mass spectroscopy
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PIA	Pseudomonas Isolation Agar
ppm	Parts per million
Py	Pyridine
RNA	Ribonucleic acid
rpm	Rotations per minute
s	singlet
t	triplet
TFA	Trifluoroacetic acid
Ts	Toluenesulfonyl
WHO	World Health Organization
δ	Chemical shift

1. Introduction

Infectious diseases can be defined as transmissible diseases caused by pathogenic microorganisms like viruses, bacteria, fungi, protozoa and helminth worms [1]. As a whole, these diseases are the major cause of mortality throughout the history of humanity. In ancient Greece and Egypt, epidemics of smallpox, leprosy tuberculosis and other infections were already described, which magnitude, morbidity and mortality is difficult to imagine in such period [2]. Between 1348 and 1350, the most devastating pandemics of human history occurred in Europe, the so called Black Death, an epidemic of bubonic plague, that killed between 30% to 60% of Europe's population [3]. This disease was spread by fleas that carried *Yersinia pestis* in the midgut [4]. In 1490, syphilis became epidemic in Spain, Italy and France which spread rapidly through Europe and North America. Only in the 19th century microorganisms were considered to be responsible for several of infectious diseases [5]. Even then in 1914, during the First World War the principal cause of death in the warfield was the infection of wounds and infectious diseases, developed in trenches [6]. It was only with the discovery and development of antimicrobials that the infectious diseases problem was tackled. Prior to the discovery of antibiotics the average life expectancy was 47 years and rose to 78.8 years with the antibiotic era [7].

At the end of the Second World War, in 1962, Sir McFarland Burnett stated that 'By the end of the Second World War it was possible to say that almost all of the major practical problems of dealing with infectious disease had been solved' [8]. At that time the statement seemed logical since there was a continuous discovery and development of new antimicrobials. In addition, the implementation of control measures and prevention protocols decreased the incidence of various infectious diseases. Regardless of the progress achieved, nowadays infectious diseases continue to be a serious problem.

1.1. What are antimicrobials?

The term antimicrobial encompasses numerous pharmaceutical agents that have antibacterial, antifungal, antiviral or anti-parasitic activity [9]. These drugs are used to kill or inhibit the growth of microorganisms, being the antibacterial agents the most commonly used. In Human medicine, antimicrobials are used to treat infectious diseases and for that reason the effect in the host must be null or minimal. This can be achieved if the drug affects the target when used at concentration lower than the concentration that is harmful to the host or if they only harmed the target.

Antibiotics are by definition a class of antimicrobials that are produced exclusively by microorganisms and inhibit or kill other microorganisms. Therefore, antibiotics do not include substances that with antimicrobial activity are synthetic (sulfonamides and quinolones), semi synthetic (methicilin and amoxicillin), or those produced by animals (lysozyme) or plants (quercetin and alkaloids) [9].

1.1.1. Antibacterials Classification

Antibacterials are a subgroup of antimicrobials that specifically target bacteria. These can be classified based on their spectrum of activity effect on bacteria, and mode of action [9]. Depending on the range of bacterial species that the drug affects, antibacterials can be classified as broad-spectrum if they are active against both Gram-positive and Gram-negative organisms or as narrow spectrum if they have limited activity and only affects specific groups of microorganisms.

Regarding their effect, antibacterials may cause inactivation or death [10]. Bactericidal drugs kill the target bacteria, while bacteriostatic drugs inhibit or delay bacterial growth and division [10]. The effects of some antibacterials can be controlled by dosage, duration of exposure, and physiological state of the invading bacteria [9].

Antimicrobials can have various mode of action and this depends on the nature of their structure and affinity to specific target sites within bacteria (**Figure 1**)[9], [11], [12]. Inhibitors of cell wall synthesis disrupt the cell wall synthesis [13], [14]. Animal cells, like human cells, do not have cell walls, so by targeting cell walls it is possible to selectively target bacteria. Inhibitors of cell membrane function disrupt or damage this structure resulting in leakage of important solutes, crucial for cellular functions, leading to cell death [11]. Since both eukaryotic and prokaryotic cells have cell membrane, this type of antibiotics are not very selective and can lead to systemic toxicity when used in humans. Inhibitors of protein synthesis typically bind to the 30S or 50S subunits of the ribosome, leading to the disruption of protein synthesis [11][13]. Proteins are the building blocks of most cellular structures and enzymes, and by disrupting protein synthesis it dysregulates the normal metabolism of bacteria leading to the death or inhibition of growth of the organism. Inhibitors of nucleic acid synthesis bound to components involved in DNA or RNA synthesis. DNA and RNA are the key for all metabolic process and replication of all living forms and the dysregulation of synthesis of these nucleic acids will compromise bacterial multiplication and survival [13], [14]. Antibiotics can also be classified as inhibitors of other metabolic processes and act on selected cellular processes crucial for the survival of the microorganism [13], [14].

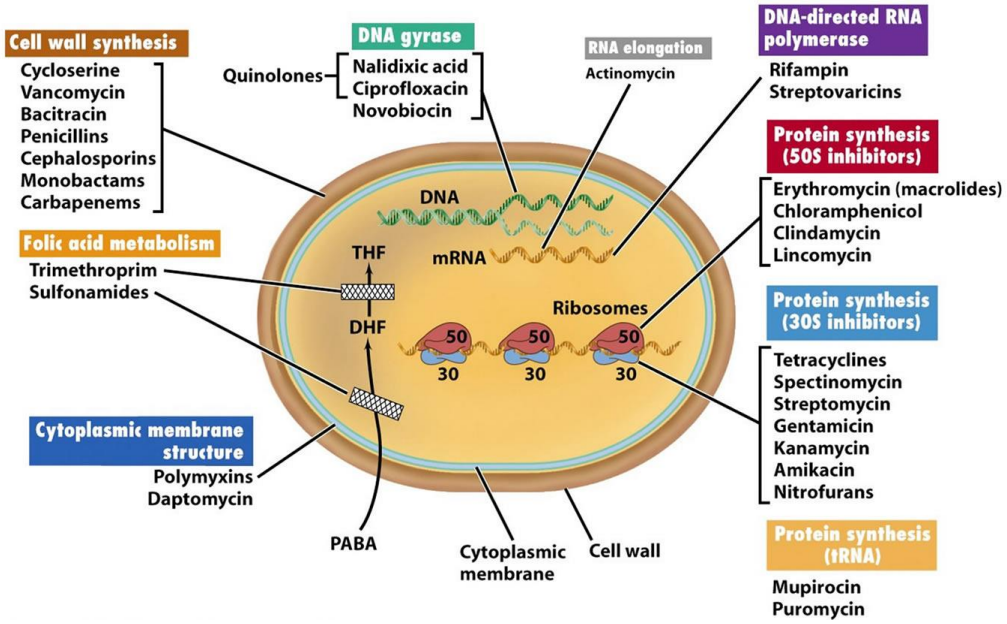


Figure 1- Mode of action of some major antimicrobial agents. Adapted from [11]. Abreviations: PABA- 4-Aminobenzoic acid, DHF- dihydrofolic acid, THF- tetrahydrofolic acid.

1.1.2. Brief history of Antimicrobials

The modern era of antimicrobials started with the discovery of the antibiotic penicillin in 1928 by Sir Alexander Fleming [5], [15]. He found that the growth of *Staphylococus aureus* was inhibited when in contact with blue mold produced by a fungus of the *Penicillium* genus [5]. By 1940 an Oxford team led by Howard Florey and Ernest Chain established a protocol that enabled the purification of penicillin in quantities sufficient for clinical testing [16]. This development led to penicillin mass production and distribution in 1945, saving numerous lives during World War II. Since then, penicillin is considered as an outstanding agent regarding safety and efficacy.

During the subsequent two decades, rapid emergence of new classes of antimicrobial agents led to the so-called golden age of antimicrobial chemotherapy (**Figure 2**). During this time, aminoglycosides, chloramphenicol, tetracycline, macrolides, vancomycin and methicillin were discovered [5]. In the following years, the continuous efforts to improve each class of antimicrobial compounds have led to a broader antimicrobial spectrum and higher antimicrobial activity of many compounds [16].

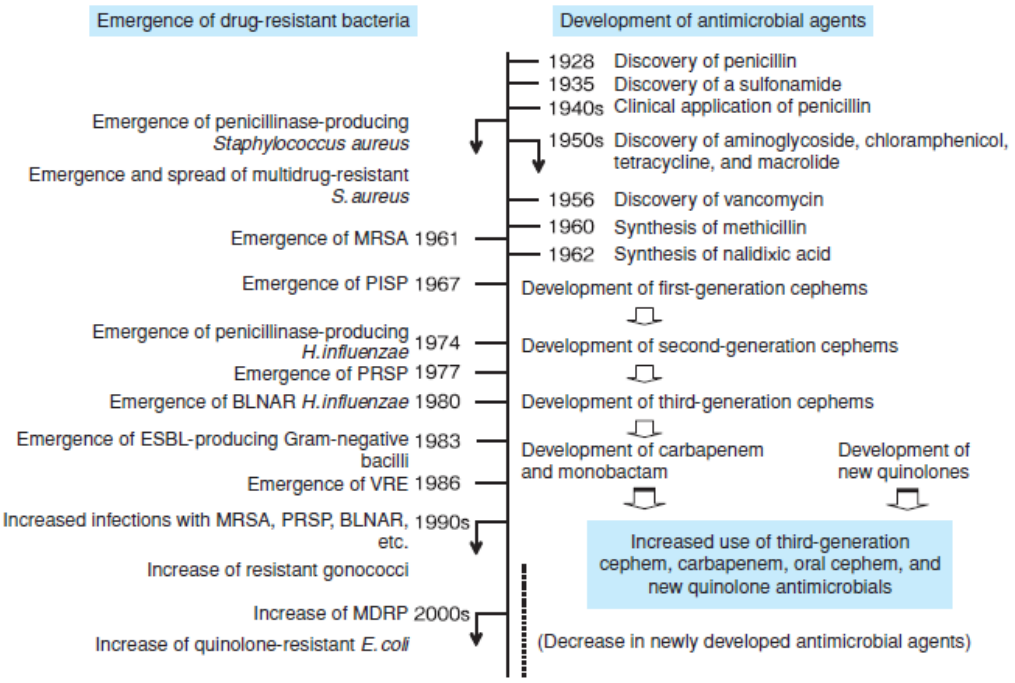


Figure 2- Chronology of the main achievements in antimicrobial agents discovery and resistant bacteria emergence, Adapted from [5]. Abreviations: MRSA- Methicillin-resistant *Staphylococcus aureus*, PISP- penicillin-intermediate *S. pneumoniae,* PRSP- penicillin-resistant *S. pneumoniae,* BLNAR- β-lactamase-negative ampicillin-resistant VRE- vancomycin-resistant enterococci MDRP- multidrug resistant *P. aeruginosa.*

Shortly after the introduction of penicillin, cases of resistance to this antibiotic were reported (**Figure 2**). This appears to be a general phenomenon, as the introduction of a new antibiotic is usually followed by the emergence of resistance to this same antibiotic. Unfortunately, resistance has eventually been reported to virtually all antibiotics, and the overuse and misuse of antimicrobials have been pointed out as the main causes of antibiotic resistance.

1.2 Present Situation

As mentioned previously, infectious diseases constitute a serious threat to Human health despite the development of antimicrobials. Infectious diseases are still a leading cause of death, especially in low-income countries. As **Figure 3** shows, in low-income countries, the majority of people died in 2016 from infectious diseases such as lower respiratory infections, diarrheal diseases among others ones [17]. In countries with a higher income the problem is less evident. Nonetheless, infectious diseases continue to constitute a major threat for public health, in high-income countries, since in 2016 the 6th most common cause of death was lower respiratory infections (**Figure 4**) [17]. Furthermore, hospital-acquired infections are estimated to cause more than ninety thousand deaths per year in USA, being the sixth leading cause of death in the US [18].

The World Health Organization (WHO) appealed in 2007 for an international cooperation to tackle infectious diseases stating that the situation is “anything but stable” [19]. In the same report, it is

stated that these diseases are spreading around the world at a pace faster than ever before[19]. The reasons behind this phenomenon are the increasing easy of international travel, population growth, resistance to drugs, under-resourced healthcare systems, intensive farming practices and degradation of the environment[19].

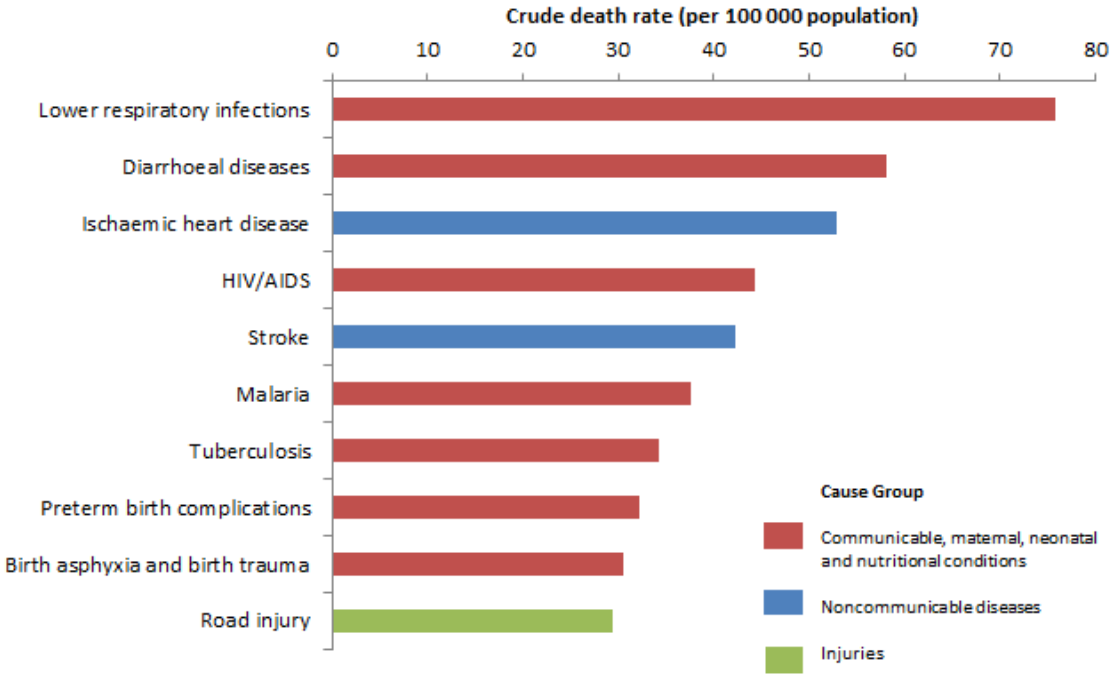


Figure 3- Top 10 causes of deaths in low-income countries in 2016. Adapted from [17].

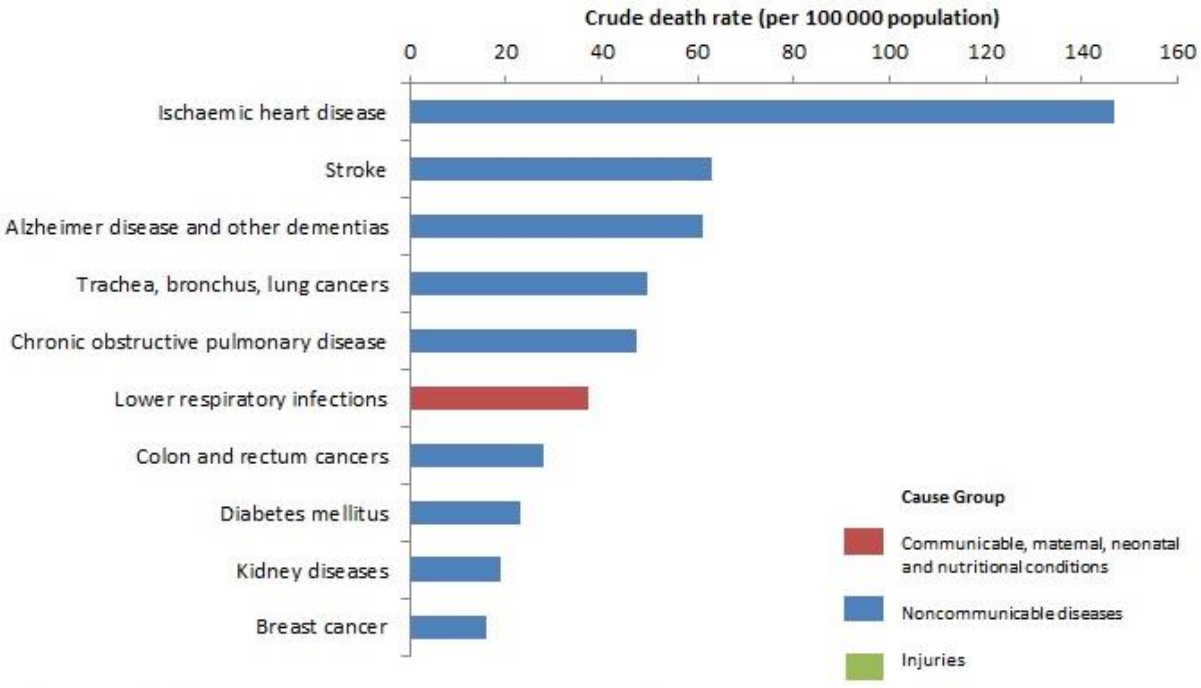


Figure 4- Top 10 causes of death in high-income countries in 2016. Adapted from [17].

The emergence of antimicrobials resistant bacteria is occurring at a rapid pace endangering the efficacy of the existing antibiotics, therefore threatening our ability to treat common infections[20]. Nowadays there is a rise on infections such as pneumonia and tuberculosis, among others, that are becoming harder and sometimes even impossible to treat [20]. WHO states that without a rapid response we are heading to a post-antibiotic era where common infections and minor injuries can become extremely deadly [20]. The antibiotic resistance crisis has been attributed to the overuse and misuse of antimicrobials and lack of new drugs development by the pharmaceutical industry.

1.2.1 Bacterial resistance to antimicrobials

Sir Alexander Fleming, in 1945, raised the alarm regarding antibiotic overuse: “public will demand…then will begin an era of abuses” [21]. Epidemiological studies have demonstrated a direct link between antibiotic consumption and the emergence of bacterial strains resistant to the antibiotics consumed [22]. It is a well known fact that the overuse of antibiotics has a major role on the evolution of resistances. The continued use of antimicrobials led to microorganisms developing mechanisms by which they can resist and survive when in contact to a certain drug or multiple drugs in case of multiresistant organisms.

Bacteria have a genetic plasticity that allows them to respond to a wide array of environmental threats and, by sharing the same ecological niche with antimicrobial-producing organisms, they have evolved mechanisms to withstand the effect of the harmful antibiotic [23]. Bacteria use two major strategies to adapt to the antibiotic contact: mutations in genes, and acquisition of foreign DNA through horizontal gene transfer. Both mechanisms act either by modifying the antibiotic molecule, or by preventing the compound to reaching its target (**Figure 5**) [13], [23]. Several studies estimate that antimicrobial drug-resistant infections increase death, illness and direct costs by 30% to 100% [24].

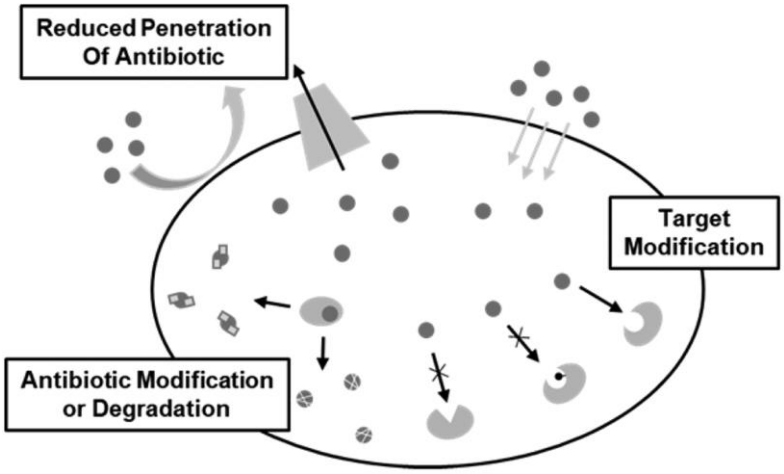


Figure 5- Representation of pathways used by bacteria to resist antimicrobials. Adapted from [13].

1.2.1.1. The ESKAPE pathogens

In recent years, the Infectious Diseases Society of America highlighted the ESKAPE pathogens as common causes of life-threatening nosocomial infections [25]. ESKAPE is an acronym that stands for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species* [26]. These bacterial species are known for being the leading cause of nosocomial infections and for developing resistance to multiple antimicrobials, escaping the biocidal action of these compounds [25]. The understanding of resistance mechanisms, virulence and pathogenicity of ESKAPE pathogens may lead to the development of new drugs that can be used against this group and consequently be active against most bacteria [25].

1.2.1.2. Escherichia coli

E. coli is a Gram-negative, rod-shaped, facultative anaerobic bacterium, that is included in the ESKAPE group [27], [28]. It is a common colonizer of the human gastrointestinal system, but some pathogenic strains can cause a wide variety of diseases like diarrhea, urinary tract infections and neonatal meningitis [29], [30]. Despite that, most of the *E.coli* strains are harmless and can even benefit the host by producing vitamin K_2 and by preventing the colonization of the human gut by harmful pathogenic bacteria [29]. Regardless of their benefits, *E.coli* is a common cause of bacteremia (presence of bacteria in blood) [31] and can have several health consequences since the immune response to the bacteria can cause sepsis and septic shock with a high mortality rate [27]. Nowadays, *E.coli* isolates resistant to various antimicrobials are commonly isolated. This rise makes empirical therapy decisions more difficult since the choice of antibiotic is dependent on the susceptibility of the specific strain.

A survey by Cheong *et al*. compared the survival to bacteremia acquired in community and a bacteremia acquired in a healthcare setting [27]. The results show that survivability decreases when an *E. coli* bacteremia is acquired in healthcare settings (**Figure 6**). In the same study it is stated that Healthcare-associated infections may have a higher mortality because these strains developed multidrug resistance and consequently it is not possible to design an appropriated therapy [27].

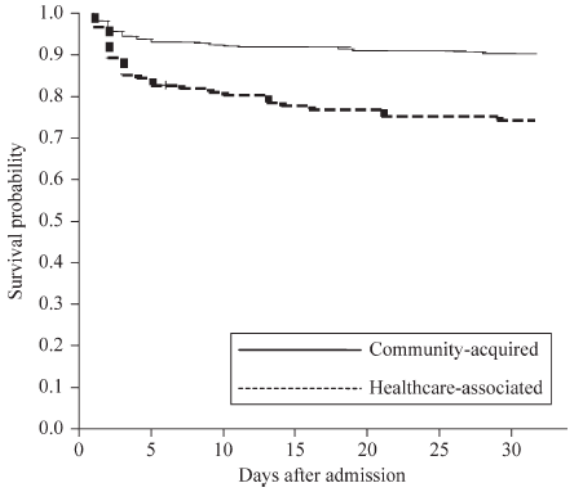


Figure 6- Kaplan-Meler survival curve for community-acquired and healthcare-associated *E. coli* bacteremia (by log-rank method, P<0.001). Adapted from [27].

1.2.1.3. Staphylococcus aureus

S. aureus is a Gram-positive facultative anaerobe, round-shaped bacterium that is both a commensal bacterium and a human pathogen [31], [32]. *S. aureus* is a member of the normal flora of the body, being frequently found in the respiratory tract and on the skin [33].

S. aureus is a major human pathogen causing skin, osteoarticular, pleuropulmonary and device-related infections, being the leading cause for bacteremia and infective endocarditis, worldwide [31]. Pathogenic strains of this species produce virulence factors like toxins and cell-surface proteins that inactivate the host antibodies [31], [34]. *S. aureus* has been recognized as a major human pathogen since the 1880s, and is nowadays the most common cause of infections in hospitalized patients [34].

Nowadays, *S. aureus* has become noticed by its notorious ability to become resistant to antibiotics as it happens for members of the ESKAPE group. Naturally this bacterial species should be susceptible to virtually every antibiotic that has been developed [35]. The acquisition of resistance by *S. aureus* has been demonstrated to occur by horizontal gene transfer and by chromosomal mutations and it was observed to come in waves [35]. The rise of *S. aureus* resistance to antibacterial agents and the increasing prevalence as a nosocomial pathogen constitutes a major concern regarding public health [35], [36].

Hospital-acquired infections with *S. aureus* and especially methicillin-resistant *S. aureus* (MRSA) are a major cause of illness and death [18]. A study done by Klein *et al*, estimated the annual hospitalizations and deaths associated with *S. aureus* and MSRA from 1999 to 2005, in USA [18]. This study showed that the hospitalizations related to *S. aureus* infections increased 62% and the number of MSRA related hospitalization more than doubled (**Figure 7**) [18].

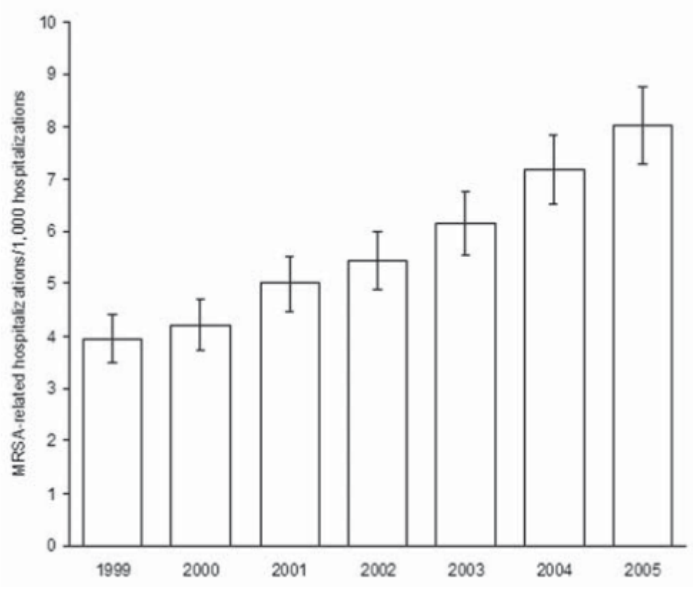


Figure 7- Estimated methicillin-resistant *Staphylococcus aureus* (MRSA)–related hospitalization rates, United States, 1999–2005. Rates are no. MRSA-related discharges/1,000 hospitalizations. Adapted from [18].

However, the authors did not find any trend for *S. aureus*-related deaths, between 1999 and 2005. Despite that, in 2005 a total of 11,406 deaths were related to *S. aureus* 6,639 of which were MSRA-related. Such cases constitute a high number of deaths in the USA [18]. For these reasons, it was suggested by the authors that *S. aureus* and MSRA should be considered a national priority for disease control [18].

1.2.1.4. Pseudomonas aeruginosa

P. aeruginosa is a common Gram-negative rod-shaped bacterium [37]. This bacterium can be found in a wide range of environments, but is considered an opportunistic pathogen, for humans and animals [37], [38]. *P. aeruginosa* is able to survive in a wide range of natural and artificial settings, including surfaces in medical facilities and devices. For this reason, *P. aeruginosa* has been associated with serious illnesses-hospital acquired-infections, especially in patients admitted in critical care units, burn care units and in respiratory infections among cystic fibrosis patients [38]. The ability of *P. aeruginosa* to resist to multiple antimicrobials relies on antibiotic resistance mechanisms including efflux pumps. Therefore, the selection of an antimicrobial agent to treat infections caused by *P. aeruginosa* poses some challenges due to the ability of the species to develop resistance to multiple classes of compounds.

In a study done by Dawra *et al.*, using 938 wound swab isolates from burned patients, the most common isolated organism was *P. aeruginosa*, with a prevalence of 41.79% (392 out of 938 samples)[39]. These 392 isolates were tested for their antimicrobial susceptibility, by the Kirby- Bauer disk diffusion method, which demonstrated that 85% of the isolates were multidrug resitants [39]. The results of this study are not abnormal since other studies showed a prevalence of *P. aeruginosa* among burn patients of 54.9% and 61.9%[40]·[41]. Furthermore a study done by Upadhaya *et al*, 100% of *P. aeruginosa* isolates from burn wounds were multidrug resistant (MDR)[42]. Such high prevalence of MDR *P. aeruginosa* for burn patient's infections poses a serious problem since the therapeutic options are very limited and these infections can lead to death by bacteremia.

1.2.1.5. Burkholderia contaminans

The *Burkholderia cepacia complex* (Bcc) is a major cause of serious respiratory infections in cystic fibrosis patients since they lead to a more rapid decline in lung function [43], [44],[45] . Bcc encompasses more than 20 different bacterial species [46]. However, *B. cenocepacia*, *B. multivorans* and *B. dolosa* are the dominant Bcc species in human infection and have the highest potential for inter–patient transmission [44] [45].

Since the beginning of the millennium *B. contaminans* has been isolated from infected cystic fibrosis patients with a particularly high representation among Portuguese and Argentinean patients [44][47]. More recently, this species was also reported as the Bcc most commonly isolated from

Spanish cystic fibrosis patients. Medina *et al.* reported that 59 out of the 166 Bcc isolates were identified as *B. contaminans*, representing an overall incidence of 36.5% [48]. In 2008 the incidence was 14.3% denoting a significant increase of *B. contaminans* in Spanish cystic fibrosis patients.

In most cases, *B.contaminans* infections are of transient nature. However some patients developed chronic infection[44]. This bacterium does not belong to the ESKAPE group, but due to its clinical relevance and multidrug resistant nature of most clinical isolates in the context of cystic fibrosis, was included in this work to attest the antimicrobial activities of the compounds synthesized [49].

1.2.2. Investment decline on the research for new drugs

After the year 2000, pharmaceutical companies practically abandoned the research and development of novel antimicrobials [21]. This is apparent considering the number of new drug approvals after 2000 (**Figure 8**). The development of new antibiotics by pharmaceutical industries has been hampered mainly by economic and regulatory obstacles [21], [50]. The apparent lack of return on investment has led to reduction in the research and development of new antimicrobial drugs. This return on investment is low, when compared to other drugs, because antimicrobials are usually used for short-term courses of treatment (5-7 days) and are only used for long-term therapies in case of chronic infections [50]. In fact, estimates of the antimicrobials financial return are now lower than for any other drug or vaccine.

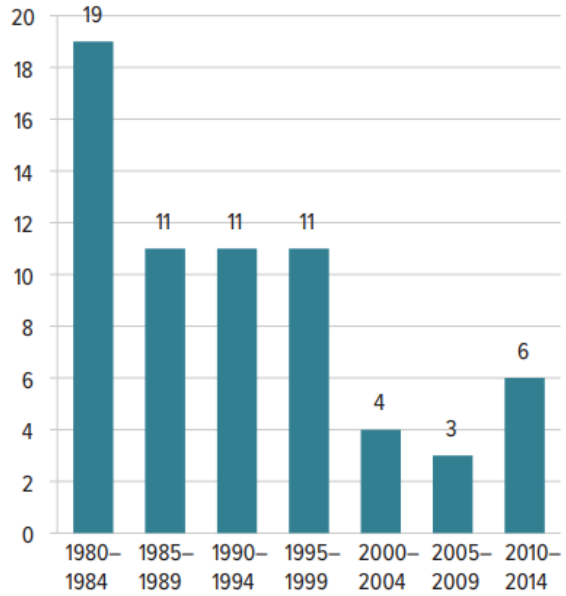


Figure 8- Number of new antibacterial drug approvals *versus* year intervals. Adapted from [21],

To further aggravate the lack of investment in antimicrobials, the complexity and high costs of phase 3 clinical trials is beyond the financial means of smaller companies that try to occupy the space left by large pharmaceutical companies. Therefore, many promising new compounds are not being

tested. Despite that, meaningful incentives have been suggested to encourage pharmaceutical companies to re-enter this area of drug development. All initiatives that try to improve the antibiotic pipeline conclude that antibiotic resistance and consequently the need for new antibiotics are a global problem needing multiple stakeholders and multifaceted actions.

1.3. What is being done?

As said before, considering the diversity of resistance mechanisms exhibited by bacteria against antimicrobials and the emergence of resistant strains, it is necessary to organize a multifaceted response to contain these resistant microorganisms with effective treatments. To address this issue, two possible approaches can be undertaken, the development of entirely new compounds or modify compounds already used in treatments [13].

1.3.1. Modification of existing antimicrobials

Modifying already existing compounds seems to be the best approach when it comes for short-term solutions to the present shortage of antimicrobials. It is possible to generate new lead molecules based on combinatory chemistry or by modifications to compounds previously characterized as antimicrobials, know as scaffold diversification (semisynthetic) (**Figure 9**) [13]. Combinatory chemistry has not been quite successful regarding the development of new drugs when compared to the scaffold diversification approach, which has extensively been applied in developing compounds through relatively few chemical transformations. These two approaches have the potential to develop compounds which expand beyond the known antibacterial motifs [13].

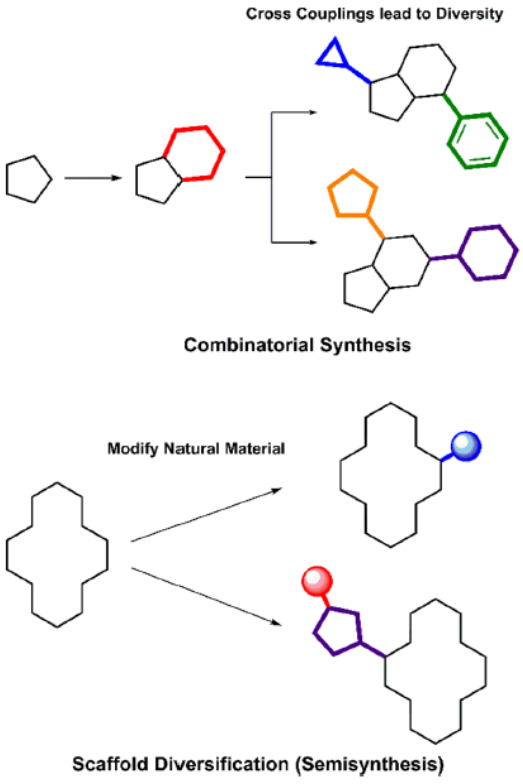


Figure 9- Schematic representation of the combinatorial synthesis and scaffold diversification approaches for new antimicrobials development. Adapted from [13].

Cationic microbial peptides are antibiotics that are considered promising for treatment against multi-resistant bacteria. In a study done by Thamri *et al*. the functionalization of a small synthetic cationic peptide with a cysteine residue led to spontaneous formation of a dimer with a 30 to 60-fold enhancement of its antimicrobial activity when compared to the precursor [51]. This study demonstrates the potential in modifying existing antimicrobials to achieve novel and better performing antimicrobials. It is possible that this kind of approach enhances the activity of a compound and possibly enhance its selectivity, resulting in less systemic toxicity for the patients [51].

1.3.2. Development of new antimicrobial molecules

The development of entirely new compounds for treatment of infectious diseases is a time consuming and risky approach. Since there is no guarantee that the compound synthesized possesses antimicrobial activity, most of the research falls short when it comes to develop a new antimicrobial, resulting in a low incentive to this approach and low return on investments. The discovery of natural compounds with antimicrobial activity also seems to have reached a bottleneck where the amount of effort needed largely exceeds the results achieved.

Taking into account the complexity and time consumption of this approach, the production of entirely new compounds has been left aside by pharmaceutical companies and most of the effort to find new compounds comes from academia. As shown in a study by Theuretzbacher *et al.*, the majority of drugs in development, in 2009, do not target novel molecules (**Figure 10**) [52]. Nevertheless, new compounds can trigger new ways to kill microorganisms that have not yet been used and possibly resulting in a more efficient and selective treatment.

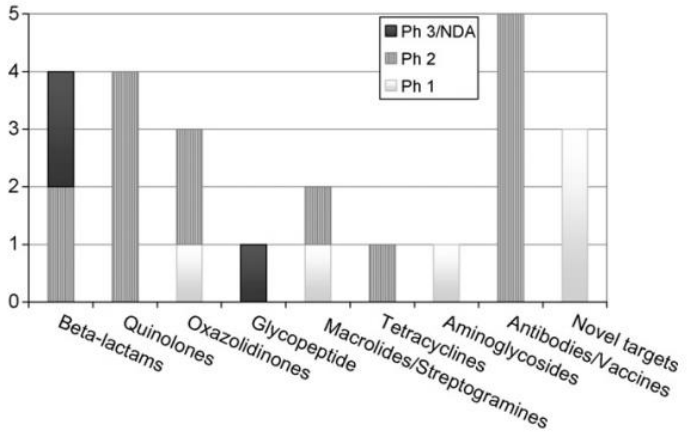


Figure 10- Number of antibiotics in clinical development (Phases 1, 2 and 3 or filed new drug application (NDA)) **according to antibacterial group**. Adapted from [52]**.**

Teixobactin (**Figure 11)** is an example of a recently new antimicrobial which was discovered in 2015. This antibiotic was discovered by mining “unculturable” bacteria and it is a promising therapeutic candidate since it is effective against drug-resistant pathogens. It is prospected that it will take longer

for the emergence of resistance to this compound and therefore Teixobactin turns into an even more interesting drug. Giving that this compound exists in nature, it is likely that additional natural compounds with similar characteristics can also be found in nature [53].

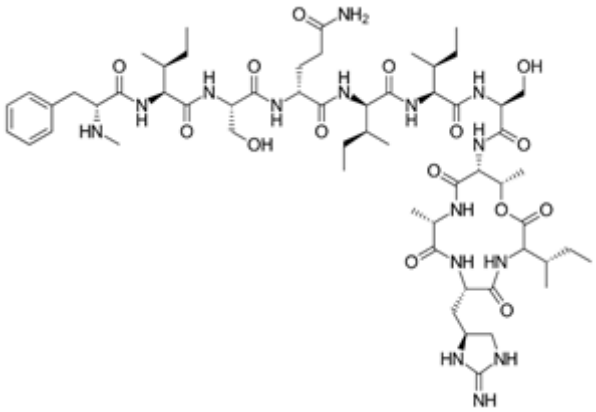


Figure 11- Molecular structure of Teixobactin. Adapted from [54]

1.4. Tetraazamacrocycles as potential new drugs

Macrocyclic polyamines have generated a great interest over the last years because of their chemical and biological properties. These compounds have properties that go beyond those anticipated from the mere assembly of amines, like the ability to bind to a wide variety of metals and to undergo conformational changes during binding. Nowadays, a vast number of azamacrocycles with ring size ranging from 12 to 16 atoms are known [55]. Examples of applications include their use in studies of selective uptake and transport of oxygen and metal ions in organisms, as agents which cleave phosphate ester bonds like those existing in DNA and RNA, as contrasting agents in Magnetic Resonance Imaging (MRI), and as anti-HIV agents [55].

Cyclam ($C_{10}H_{24}N_4$) is a white solid soluble in water and is one of the most studied tetraazamacrocycles (**Figure 12**), having demonstrated antiviral activity, especially against human immunodeficiency virus (HIV), and also antimicrobial activity [55]–[58].

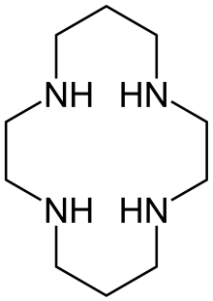


Figure 12- Cyclam structure

The bicyclam derivatives represent a new class of anti-HIV agents. Studies done by Clercq *et al*., placed them as attractive candidates for further development of anti-HIV drugs [57]. Unlike other drugs that are HIV-1 specific, as is the case of the HIV-1 reverse transcriptase inhibitors that do not affect HIV-2 reverse transcriptase, bicyclams have shown to be active against both HIV-1 and HIV-2. One of the derivatives was even capable of inhibiting virus at a concentration of 1 to 10 ng/mL, while not being toxic to the host at concentrations up to 500 µg/mL (**Figure 13**). These derivatives do not show to act as other drugs act. They do not inhibit HIV reverse transcriptase, HIV protease, virus binding to cells and they do not directly inactivate the virus. Bicyclams inhibit a specific event that is involved in viral uncoating, representing the first compound to act in such a manner against retroviruses [57].The compound in **Figure 13** inhibits syncytium formation, which is regarded as essential for virus-cell fusion [57]. Also, it was found that when in contact with the compound RNA degradation occurs suggesting that bicyclams interact with capsid proteins from the virus [57]. The authors suggest that future work should address the investigation of which of the viral capsid proteins the bicyclams interact with and where exactly the interaction takes place [57].

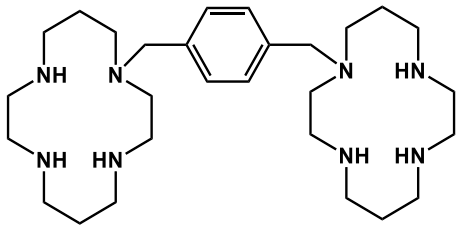


Figure 13- Structure of the Cyclam derivative with anti-HIV activity synthesized by Clercq *et al.*[57].

Alves *et al.* synthesized a series of cyclam derivatives and screened their antibacterial activities[56]. The compound with the highest antimicrobial activity was identified, the $H_2(^{4\text{-}CF3}PhCH_2)_2$Cyclam acetate salt (**Figure 14**). MIC values found for this compound against *S. aureus*, *P. aeruginosa* and *E. coli* of 15, 261 and 9 µg/mL respectively, were reported [56]. The compound was found to be bactericidal against all the three bacterial strains. These authors also used the nematode *C. elegans* as a muticellular animal model to test the compound toxicity, concluding that concentrations up to 32 µg/mL did not affected the survival of the worms[56].

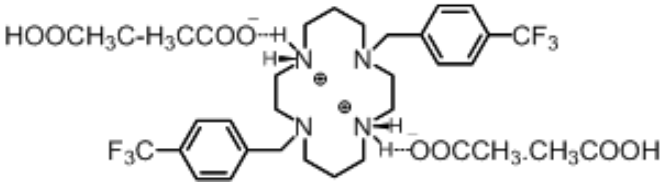


Figure 14- Structure of the compound synthesized by Alves *et al*. with highest antimicrobial activity[56]**.**

Compound 4,11-Bis((1-(2-ethyl-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl)-1H-1,2,3-triazol4-yl)methyl)-4,11-diaza-1,8-diazoniacyclotetradecane-1,8-diium 2,2,2-trifluoroacetate (**Figure 15**) revealed to be highly active against several *Mycobacterium* species such as *M. avium*, *M. bovis* and *M. tuberculosis* in a study carried out by Yu *et al.* [59]. These compounds inhibit intracellular growth of *M. tuberculosis* being nontoxic to human cell lines. Furthermore, the authors state that the compound is active against multidrug-resistant *M. tuberculosis*, and this may indicate a distinct mode of action.

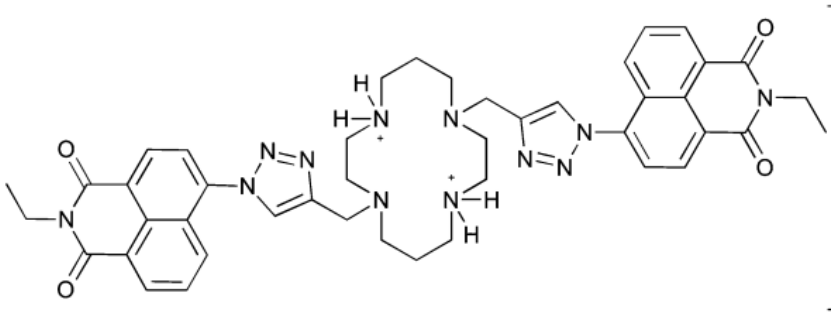


Figure 15- Structure of the compound synthesized by Yu *et al*. with activity against Mycobacterium species. Adapted from [59]

Cyclens (1,4,7,10-tetraazacyclododecane) (**Figure 16**) are another class of macrocyclic tetraamines with applications in organometalic chemistry revealing a high interest as MRI contrast agents[60]. Looking at the structure of cyclens, it is possible to see that they resemble cyclams as both possess four secondary amines that might be functionalized with pendant groups. Therefore, it is possible to obtain compounds similar to that of **Figure 14**, with a cyclen backbone instead of a cyclam. To the best of my knowledge, cyclens were never studied as antimicrobials. Thus, the use of this type of compounds may open a window of opportunity for the development of novel drugs.

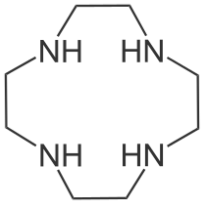


Figure 16- Cyclen structure.

1.5. Thesis Objectives

The work developed in the master project had the objective of contributing to the establishment of tetraazamacrocycles as a new family of compounds with antimicrobial activity with potential use as antimicrobials. Therefore, an array of tetraazamacrocycles derivatives were synthesized and their activity against selected bacteria was tested. The bacteria selected were *E.coli, P.aeruginosa*, *S. aureus* and *B. contaminans*. These bacteria were chosen because they have clinical significance and constitute a serious risk in human health. The main motivation for the present work come from the fact that the antimicrobials discovered to date are not sufficient to treat infectious diseases, in particular those caused by multidrug resistant bacteria.

The antimicrobial activity of the tetraazamacrocycles derivatives synthesized was assessed based on the determination of MIC values. The bacteriostatic/bactericidal activity of the compouds was also assessed. The MIC values allowed to determine the most effective cyclam derivatives for the bacteria tested. Results gathered are expected to shed light on the structure/ function relationships in cyclam derivatives, and to guide the work towards the identification of chemical modifications that can be introduced in the cyclam backbone to obtain derivatives with higher antimicrobial activity.

Preliminary experiments envisaging the quantification of the frequency of spontaneous resistance to the compounds studied were performed. Spontaneous resistant mutants are important biological tools to gain clues on the mode of action of these compounds.

2. Materials and Methods

2.1. General considerations

Compound **16d** was prepared as previously described **[56]**. All other reagents were of commercial grade and used without further purification.

NMR spectra were recorded in Bruker AVANCE II 300 or 400 MHz spectrometers referenced internally to residual proton-solvent (^{1}H) or solvent (^{13}C) resonances, and reported relative to tetramethylsilane (0 ppm). ^{19}F NMR was referenced to external CF_3COOH (-76.55 ppm). ^{1}H-$^{13}C\{^{1}H\}$ HSQC and ^{1}H-^{1}H COSY NMR experiments were used to assign all proton and carbon resonances.

Elemental analyses were performed by the Laboratório de Análises of IST.

Suitable crystals for single crystal X-ray diffraction of compounds **12** and **16b** were coated and selected with Fomblin® oil. Data were collected using graphite monochromated Mo-Kα radiation (λ = 0.71073 Å) on a Bruker AXS-KAPPA APEX II diffractometer. Cell parameters were retrieved using Bruker SMART software and refined using Bruker SAINT on all observed reflections [61]. Absorption corrections were applied using SADABS [62]. The structure was solved by direct methods using SIR97[63]. Structure refinement was done using SHELXL-97 [64]. These programs are part of the WinGX software package version 1.80.05 [65]. The hydrogen atoms of the NH and COOH groups were located in the electron density maps. The other hydrogen atoms were inserted in calculated positions and allowed to refine in the parent atoms. Torsion angles, mean square planes and other geometrical parameters were calculated using SHELX [64]. Illustrations of the molecular structures were made with Mercury CSD 3.9 for Windows [66].

2.1.1. Synthesis of compounds

Cyclam (1):

The compound was prepared according to an established procedure previously developed by Barefield[67]. $Ni(ClO_4)_2.6H_2O$ (55.08 g, 0.15 mol) was dissolved in 400 mL of distilled water and 28 mL of tetraazadodecane (0.15 mol) was added. The orange solution formed was cooled to around 5 ºC and 22 mL of glyoxal at 40% was added (0.48 mol). The reaction mixture was stirred overnight at room temperature. The solution was cooled, once again, to 5 ºC and two equivalents of $NaBH_4$ (11.12 g, 0.29 mol) were slowly added. The solution was stirred until the foam disappeared and refluxed for 1h. After cooling to room temperature, NaOH (15.65 g, 0.39 mol) were added and the reaction mixture was stirred for 2 days. The solvent was evaporated, and the product was extracted with several portions of chloroform. The organic phases were combined and dried with $MgSO_4$ anhydrous. The

solution was filtered, and the solvent was evaporated under reduced pressure, affording the compound as a white solid with a 47% yield (13.50 g, 0.07 mol).

^{1}H NMR ($CDCl_3$, 300.1 MHz, 298 K): δ (ppm) 2.71-2.64 (overlapping, 16H total, 8H, [C3]C*H*$_2$N and 8H, [C2]C*H*$_2$N), 2.48 (br, 4H, N*H*), 1.69 (m, 4H, $CH_2CH_2CH_2$).

tri-*tert*-butyl 1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate (2):

The procedure to prepare this compound was based on a previously described method[68]. Cyclam (1.00 g, 4.99 mmol) was dissolved in 200 mL of dichloromethane and 4.0 mL of triethylamine (29 mmol) was added under an inert atmosphere of nitrogen. A solution of di-*tert*-butyl-dicarbonate (1.96 g, 8.98 mmol) in 60 mL of dichloromethane was added dropwise. After the addition was completed the reaction mixture was cooled to -15 ºC and another portion of di-*tert*-butyl-dicarbonate (1.39 g, 6.36 mmol) was added. The reaction mixture was left stirring overnight. A saturated aqueous solution of Na_2CO_3 was added and the organic phase separated and dried with anhydrous $MgSO_4$. Compound **2** was obtained as a white powder with a 68 % yield (1.69 g, 3.37 mmol).

MS (CH_3CN/MeOH (1%H_2O), ESI): *m/z* 501.6 $[M+H]^+$

tri-*tert*-butyl11-(4-(trifluoromethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate (3):

Compound **2** (1.02 g, 2.04 mmol) was dissolved in a minimum volume of dimethylformamide. K_2CO_3 (0.70 g, 5.06 mmol) and 4-(trifluoromethyl)benzyl bromide (0.49 g, 2.05 mmol) was added. The reaction mixture was left stirring overnight. A saturated aqueous solution of $KHCO_3$ and brine were added and the product extracted with small portions of chloroform. The organic phases were combined and dried with anhydrous $MgSO_4$. After filtration, the solvent was evaporated under reduced pressure and the product was obtained with a 98 % yield (1.32 g, 2.00 mmol).

^{1}H NMR ($CDCl_3$, 300.1 MHz, 296 K): δ (ppm) 7.50 (d, $^{3}J_{H\text{-}H}$ = 8 Hz, 2H, *o-Ph* or *m-Ph*), 7.34 (d, $^{3}J_{H\text{-}H}$ = 8 Hz, 2H, *o-Ph* or *m-Ph*), 3.53 (s, 4H, PhC*H*$_2$N), 3.30 (overlapping, 12H total, 6H, [C3]*C*H$_2$N and 6H, [C2]*C*H$_2$N), 2.56 (m, 2H [C2]*C*H$_2$N), 2.34 (m, 2H [C3]*C*H$_2$N), 1.84 (m, 2H, $CH_2CH_2CH_2$), 1.64 (m, 2H, $CH_2CH_2CH_2$), 1.41-1.22 (br, 27H, C*H*$_3$).

^{13}C {^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 155.8 (*C*O), 155.6 (overlapping, *C*O), 143.4 (*i-Ph*), 129.2 (*o-Ph* or *m-Ph*), 128.0 (t, $^{2}J_{C\text{-}F}$ = 93 Hz, *p-Ph*), 124.6 (q, $^{1}J_{C\text{-}F}$ = 273 Hz, *C*F$_3$), 122.5 (*o-Ph* or *m-Ph*), 79.7 (*C*(CH$_3$)$_3$), 79.6 (*C*(CH$_3$)$_3$), 79.5 (*C*(CH$_3$)$_3$), 59.5 (Ph*C*H$_2$N), 53.5 ([C2]*C*H$_2$N), 51.9 ([C3]*C*H$_2$N), 47.9 - 46.3 (overlapping, [C2]*C*H$_2$N and [C3]*C*H$_2$N), 28.5 - 28.4 (overlapping ($CH_2CH_2CH_2$) and (*C*H$_3$).

^{19}F NMR (D_2O/$(CD_3)_2CO$, 282.4 MHz, 296K): δ (ppm) -62.4 (s, C*F*$_3$).

MS (CH_3CN, ESI): *m/z* 501.20 $[M-^{CF3}PHCH_2+H]^+$, 659.21 $[M+H]^+$

11-(4-(trifluoromethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-triium trifluoracetate (4):

Compound **3** (0.66g, 1.00 mmol) was dissolved in 20mL of dichloromethane and 10mL of trifluoracetic acid (0.13 mol) were added. The reaction mixture was refluxed overnight. The solvent was evaporated and the product was obtained as brown oil with a 67% yield (0.24g, 0.67mmol).

^{1}H NMR (D_2O, 300.1 MHz, 296 K): δ (ppm) 7.80 (d, $^{3}J_{H-H}$ = 9 Hz, 2H, *o-Ph* or *m-Ph*), 7.61 (d, $^{3}J_{H-H}$ = 9 Hz, 2H, *o-Ph* or *m-Ph*), 4.31 (s, 2H, PhC*H*$_2$N), 3.49-3.14 (overlapping, 16H total, 8H, [C3]*C*H$_2$N and 8H, [C2]*C*H$_2$N), 2.08 (m, 4H, CH$_2$C*H*$_2$CH$_2$).

^{19}F NMR (D_2O, 282.4 MHz, 296K): δ (ppm) -75.7 (s, C*F*$_3$).

1-(4-(trifluoromethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane (5):

Compound **4** (0.20 g, 0.27 mmol) was dissolved in water and KOH was added until the reaction mixture reached pH ≈ 13. The product was extracted with dichloromethane, the organic phase was washed with brine and dried with anhydrous $MgSO_4$. After filtration the solvent was evaporated to dryness affording the product as a white solid with a 87% yield (0.11 g, 0.31 mmol).

^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 7.57 (d, $^{3}J_{H-H}$ = 8 Hz, 2H, *o-Ph* or *m-Ph*), 7.48 (d, $^{3}J_{H-H}$ = 8 Hz, 2H, *o-Ph* or *m-Ph*), 4.39 (br, 3H, N*H*) 3.65 (s, 2H, PhC*H*$_2$N), 2.90 (m, 2H, [C2]*C*H$_2$N), 2.85 (m, 2H [C3]*C*H$_2$N), 2.79 (overlapping, 4H total, 2H, [C3]*C*H$_2$N and 2H, [C2]*C*H$_2$N), 2.71(overlapping, 4H total, 2H, [C3]*C*H$_2$N and 2H, [C2]*C*H$_2$N), 2.60 (overlapping, 4H total, 2H, [C3]*C*H$_2$N and 2H, [C2]*C*H$_2$N), 1.89 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.81 (m, 2H, CH$_2$C*H*$_2$CH$_2$),

^{13}C {^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 142.4 (*i-Ph)*, 129.7 (*p-Ph*), 129.3 (q, $^{1}J_{C-F}$ = 233 Hz, *C*F$_3$), 129.5 (*o-Ph*), 125.3 (q, $^{3}J_{C-F}$ = 4 Hz, *m-Ph*), 57.8 (Ph*C*H$_2$N), 53.5 ([C2]*C*H$_2$N or [C3]*C*H$_2$N), 53.1 [C2]*C*H$_2$N or [C3]*C*H$_2$N), 50.5 ([C2]*C*H$_2$N or [C3]*C*H$_2$N), 49.2 ([C3]*C*H$_2$N), 48.8 ([C2]*C*H$_2$N or [C3]*C*H$_2$N), 48.6 ([C2]*C*H$_2$N or [C3]*C*H$_2$N), 47.2 ([C2]*C*H$_2$N or [C3]*C*H$_2$N), 47.1 ([C2]*C*H$_2$N), 27.0 (CH$_2$*C*H$_2$CH$_2$), 25.5 (CH$_2$*C*H$_2$CH$_2$).

^{19}F NMR ($CDCl_3$), 376.5 MHz, 296K): δ (ppm) -62.4 (s, C*F*$_3$).

1-(4-(trifluoromethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraiumchloride (6):

Two distinct approaches were used to synthesize the compound, as follows:

Method A: Compound **3** (0.66 g, 1.00 mmol) was dissolved in dichloromethane and HCl at 37% was added until the pH ≈ 1. The solution was left stirring overnight at room temperature. The solvent was evaporated under reduced pressure affording the product as white solid with a 47% yield (0.29 g, 0.47 mmol).

Method B: Compound **5** (0.11 g, 0.31 mmol) was dissolved in ethanol and HCl at 37% was added. The product precipitated out of solution. After filtration the product was dried in vacuum affording the compound was a white solid with a 52 % yield (0.08 g, 0.16 mmol).

^{1}H NMR (D_2O/$(CD_3)_2CO$, 300.1 MHz, 296 K): δ (ppm) 7.87 (d, $^{3}J_{H\text{-}H}$ = 9 Hz, 2H, *o-Ph* or *m-Ph*), 7.78 (d, $^{3}J_{H\text{-}H}$ = 9 Hz, 2H, *o-Ph* or *m-Ph*), 4.48 (s, 2H, PhCH_2N), 3.72-3.68 (overlapping, 6H total, [C2]CH_2N), 3.60 (m, 2H [C2]CH_2N), 3.52-3.47 (overlapping, 6H total, [C3]CH_2N), 3.36 (m, 2H [C3]CH_2N), 2.33 (m, 4H, $CH_2CH_2CH_2$),

^{13}C {^{1}H} NMR (D_2O/$(CD_3)_2CO$, 75.5 MHz, 296 K): δ (ppm) 135.1 (*i-Ph)* , 131.7 (*o-Ph*), 131.2 (q, $^{2}J_{C\text{-}F}$ = 32 Hz, *p-Ph*), 126.4 (d, $^{3}J_{C\text{-}F}$ = 3 Hz, *m-Ph*), 124.2 (q, $^{1}J_{C\text{-}F}$ = 272 Hz, CF_3), 57.4 (PhCH_2N), 48.7 ([C3]CH_2N), 46.3 ([C2]CH_2N), 43.0 ([C3]CH_2N), 42.4 (overlapping, [C3]CH_2N), 39.6 (overlapping, [C2]CH_2N), 39.1 ([C2]CH_2N), 20.0 ($CH_2CH_2CH_2$), 19.7 ($CH_2CH_2CH_2$).

^{19}F NMR (D_2O/$(CD_3)_2CO$, 282.4 MHz, 296K): δ (ppm) -62.6 (s, CF_3).

Anal. Calcd for $C_{18}H_{33}Cl_4F_3N_4.H_2O$: C, 41.39; H, 6.75; N, 10.73. Found: C, 41.29; H, 6.60; N, 10.68.

1,4,8,11-tetraazatricyclo[9.3.1.1^{4,8}]hexadecane (7):

Compound **7** was synthesized according to the method previously described by Royal *et al.*[69]. Cyclam (6.0 g, 30.0 mmol) was dissolved in water and 4.9 mL of formaldehyde (37 % in water) (0.13 mol) was added. After a while the formation of a white precipitate was observed. The mixture was stirred for 2h at 0 ºC and for an additional period of 1h at room temperature. The precipitate was filtered and dried under reduced pressure giving the compound as a white solid with a 58% yield (3.9 g, 17.4 mmol).

^{1}H NMR ($CDCl_3$, 300.1 MHz, 298 K): δ (ppm) 5.40 (d, 2H, NCH_2N), 3.11 (m, 4H, [C2]CH_2N), 2.86 (d, 2H, NCH_2N), 2.70 (m, 4H, [C3]CH_2N), 2.59 (m, 4H, [C3]CH_2N), 2.34 (m, 4H, [C2]CH_2N), 2.20 (m, 2H, $CH_2CH_2CH_2$), 1.14 (m, 2H, $CH_2CH_2CH_2$).

1,8-bis(4-methylbenzyl)-1l^{4},4,8l^{4},11-tetraazatricyclo[9.3.1.14,8]hexadecane-1,8-diiumbromide (8):

Compound **7** (5.0 g, 22.3 mmol) was dissolved in a minimum volume of acetonitrile and two equivalents of 4-(methyl)benzyl bromide (9.1 g, 49.2 mmol) were added. The solution was stirred overnight at room temperature, resulting in a white precipitate that was filtered and dried under reduced pressure. The product was obtained as a white solid with a 47% yield (6.2 g, 10.4 mmol).

^{1}H NMR (D_2O/$(CD_3)_2CO$, 300.1 MHz, 296 K): δ (ppm) 7.81 (d, $^{3}J_{H\text{-}H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 7.62 (d, $^{3}J_{H\text{-}H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 5.94 (d, $^{3}J_{H\text{-}H}$ = 9 Hz, 2H, NCH_2N), 5.12 (d, $^{3}J_{H\text{-}H}$ = 15Hz, 2H, PhCH_2N), 4.94-4.86 (overlapping, 4H total, 2H, [C2]CH_2N and 2H, PhCH_2N), 4.05-3.97 (overlapping,

4H total, 2H, [C2]C*H*$_2$N and 2H, NC*H*$_2$N), 3.78 (m, 2H, [C3]C*H*$_2$N), 3.65-3.56 (overlapping, 4H total, 2H, [C3]C*H*$_2$N and 2H, [C3]C*H*$_2$N), 3.34 (m, 2H, [C2]C*H*$_2$N), 3.24 (m, 2H, [C2]C*H*$_2$N), 2.96-2.79 (overlapping, 4H total, 2H, [C3]C*H*$_2$N and 2H, $CH_2CH_2CH_2$), 2.65 (s, 6H, C*H*$_3$), 2.21 (m, 2H, $CH_2CH_2CH_2$).

^{13}C {^{1}H} NMR (D_2O/$(CD_3)_2CO$, 75.5 MHz, 296 K): δ (ppm) 141.4 (*i-Ph* or *p-Ph*), 133.4 (*o-Ph* or *m-Ph*), 130.3 (*o-Ph* or *m-Ph*), 123.6 (*i-Ph* or *p-Ph*), 77.1 (N*C*H$_2$N), 63.0 (Ph*C*H$_2$N), 59.9 ([C3]*C*H$_2$N), 51.7 ([C3]*C*H$_2$N), 48.0 (overlapping, [C2]*C*H$_2$N and [C2]*C*H$_2$N), 20.9 ($CH_2CH_2CH_2$), 19.9 (*C*H$_3$).

Anal. Calcd for $C_{28}H_{42}Br_2N_4$: C, 56.57; H, 7.12; N, 9.42. Found: C, 56.51; H, 6.85; N, 9.50.

1,8-bis(4-(2,2,2-trifluoroethyl)benzyl)-1l^4,4,8l^4,11-tetraazatricyclo[9.3.1.1^{4,8}]hexadecane-1,9diiumbromide (9):

Compound **7** (0.42 g, 1.89 mmol) was dissolved in a minimum volume of acetonitrile and two equivalents of 1-(bromomethyl)-4-(2,2,2-trifluoroethyl)benzene (1.00 g, 3.97 mmol) were rapidly added. The mixture was stirred overnight at temperature room. The white precipitate formed was filtered and dried under reduced pressure affording **9** with a 52% yield (0.72 g, 0.98 mmol).

^{19}F NMR (D_2O/$(CD_3)_2CO$, 282.4 MHz, 296K): δ (ppm) -65.8 (s, CH_2CF_3).

Anal. Calcd for $C_{30}H_{40}Br_2F_6N_4O_4.(H_2O)$: C, 48.14; H, 5.66; N, 7.49. Found: C, 47.64; H, 5.38; N, 7.32

1,8-bis(4-methylbenzyl)-1,4,8,11-tetraazacyclotetradecane (12):

Compound **8** (6.0 g, 10.1 mmol) was hydrolyzed in an aqueous NaOH solution (3M) for 4h under stirring at room temperature. The product was extracted with small portions of chloroform that were combined and dried with anhydrous $MgSO_4$. Evaporation of the solvent to dryness gave an oil that was converted into a white solid after successive freeze-trituration-pump-thaw cycles. Compound **12** was obtained with a 92% yield (3.8 g, 9.3 mmol).

^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 7.19 (d, $^3J_{H\text{-}H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.10 (d, $^3J_{H\text{-}H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 3.69 (s, 4H, Ph*C*H$_2$N), 2.80 (s, 2H, N*H*), 2.73 (m, 4H, [C3]C*H*$_2$N), 2.68 (m, 4H, [C2]C*H*$_2$N), 2.55 (m, 4H, [C2]C*H*$_2$N), 2.51 (m, 4H, [C3]C*H*$_2$N), 2.29 (s, 6H, C*H*$_3$), 1.83 (m, 4H, $CH_2CH_2CH_2$).

^{13}C {^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 136.6 (*i-Ph* or *p-Ph*), 134.2 (*i-Ph* or *p-Ph*), 129.7 (*o-Ph* or *m-Ph*), 128.9 (*o-Ph* or *m-Ph*), 57.5 (Ph*C*H$_2$N), 54.2 ([C2]*C*H$_2$N), 51.8 ([C3]*C*H$_2$N), 50.5 ([C3]*C*H$_2$N), 47.9 ([C2]*C*H$_2$N), 26.1 ($CH_2CH_2CH_2$), 21.8 (*C*H$_3$).

Anal. Calcd for $C_{26}H_{40}N_4$: C, 76.42; H, 9,87; N, 13.71. Found: C, 76.06; H, 9.91; N, 13.69.

1,8-bis(4-(2,2,2-trifluoroethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane (13):

Compound **13** was prepared by the same procedure described for **12,** using **9**, being obtained as a white solid with a 52% yield (0.28 g, 0.51 mmol).

^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 7.29 (d, $^{3}J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.21 (d, $^{3}J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 3.71 (s, 4H, PhCH_2N), 3.31 (q, 4H, $^{3}J_{H-F}$ = 11 Hz, CH_2CF_3), 2.71-2.52 (overlapping, 18H total, 2H, NH, 8H, [C3]CH_2N and 8H, [C2]CH_2N), 1.84 (m, 4H, $CH_2CH_2CH_2$).

^{13}C {^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 137.6 (*i-Ph* or *p-Ph*), 130.1 (*o-Ph* or *m-Ph*), 129.9 (*o-Ph* or *m-Ph*), 129.0 (*i-Ph* or *p-Ph*), 125.9 (q, $^{1}J_{H-F}$ = 279 Hz, CH_2CF_3), 57.6 (PhCH_2N), 54.3 ([C2]CH_2N), 51.7 ([C3]CH_2N), 50.1 ([C2]CH_2N or [C3]CH_2N), 47.9 ([C2]CH_2N or [C3]CH_2N), 40.0 (q, $^{2}J_{H-F}$ = 29 Hz, CH_2CF_3), 26.1 ($CH_2CH_2CH_2$).

^{19}F NMR ($CDCl_3$), 376.5 MHz, 296K): δ (ppm) -65.9 (s, CF_3).

Anal. Calcd for $C_{28}H_{38}F_6N_4$: C, 61.75; H, 7.03; N, 10.29. Found: C, 60.82; H, 6.75; N, 10.00.

4,11-bis(4-methylbenzyl)-1,4,8,11-tetraazacyclotetradecane-1,8-diium acetate (16a):

Compound **12** (1.00 g, 2.45 mmol) was dissolved in a small volume acetonitrile and 1 mL of glacial acetic acid was added to the solution. This mixture was refluxed for 1h and the solvent was evaporated under reduced pressure resulting in **16a** with a 98% yield (1.27 g, 2.40 mmol).

^{1}H NMR (D_2O, 300.1 MHz, 296 K): δ (ppm) 7.31 (d, $^{3}J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 7.23 (d, $^{3}J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 3.48 (s, 4H, PhCH_2N), 3.31 (m, 4H, [C3]CH_2N or [C2]CH_2N), 3.23 (m, 4H, [C3]CH_2N or [C2]CH_2N), 2.80 (m, 4H, [C3]CH_2N or [C2]CH_2N), 2.76 (m, 4H, [C3]CH_2N or [C2]CH_2N), 2.25 (s, 6H, CH_3), 1.99 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO^- and 6H, CH_3COOH).

^{1}H NMR ($CDCl_3$, 300.1 MHz, 296 K): δ (ppm) 10.74 (overlapping, 6H total, 4H, NH_2^+ and 2H, CH_3COOH), 7.12 (d, $^{3}J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 7.02 (d, $^{3}J_{H-H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 3.79 (s, 4H, PhCH_2N), 3.16-3.14 (overlapping, 8H total, 4H, [C3]CH_2N and 4H, [C2]CH_2N), 2.75 (m, 4H, [C2]CH_2N), 2.66 (m, 4H, [C3]CH_2N), 2.32 (s, 6H, CH_3), 1.99 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO^- and 6H, CH_3COOH).

^{13}C{^{1}H} NMR ($CDCl_3$, 75.5 MHz, 296 K): δ (ppm) 176.6 (*C*OO), 137.3 (*i-Ph*), 130.9 (*p-Ph*), 130.8 (*o-Ph* or *m-Ph*), 129.0 (*o-Ph* or *m-Ph*), 52.7 (PhCH_2N), 51.3 ([C3]CH_2N), 48.7 ([C2]CH_2N), 48.3 ([C3]CH_2N), 45.6 ([C2]CH_2N), 22.8 ($CH_2CH_2CH_2$), 22.6 (CH_3COO), 21.2 (CH_3).

Anal. Calcd for $C_{30}H_{48}N_4O_4.(CH_3COOH)_2$: C, 62.94; H,8.70; N,8.64. Found: C, 62.26; H, 9.17; N, 8.46.

4,11-bis(4-(2,2,2-trifluoroethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane-1,8-diiumacetate (16b):

This compound was prepared using a similar protocol described for **16a** using **13** instead of **11**. Compound **16b** was obtained as a white solid with a 84% yield (0.29 g, 0.43 mmol).

^{1}H NMR (D_2O, 400.1 MHz, 296 K): δ (ppm) 7.46 (d, $^{3}J_{H\text{-}H}$ = 8Hz, 4H, *o-Ph* or *m-Ph*), 7.36 (d, $^{3}J_{H\text{-}H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 3.60 (s, 4H, PhCH_2N), 3.48 (q, 4H, $^{3}J_{H\text{-}F}$ = 11 Hz, CH_2CF_3), 3.34 ([C3]CH_2N or [C2]CH_2N), 3.29 (m, 4H, [C3]CH_2N or [C2]CH_2N), 2.83 (m, 4H, [C3]CH_2N or [C2]CH_2N), 2.73 (m, 4H, [C3]CH_2N or [C2]CH_2N), 2.00 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO^- and 6H, CH_3COOH).

^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 10.28 (overlapping, 6H total, 4H, NH_2^+ and 2H, COO*H*), 7.25 (d, $^{3}J_{H\text{-}H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.16 (d, $^{3}J_{H\text{-}H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 3.83 (s, 4H, PhCH_2N), 3.34 (m, $^{3}J_{H\text{-}F}$ = 11 Hz, 4H, CH_2CF_3), 3.10 (overlapping, 8H total, 4H, [C2]CH_2N and 4H, [C3]CH_2N), 2.76 (m, 4H, [C2]CH_2N), 2.68 (m, 4H, [C3]CH_2N), 2.00-1.96 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO^- and 6H, CH_3COOH).

^{13}C{^{1}H} NMR ($CDCl_3$,100.6 MHz, 296 K): δ (ppm) 176.7 (*C*OO), 134.6 (*i-Ph*), 131.0 (*o-Ph* or *m-Ph*), 130.2 (*o-Ph* or *m-Ph*), 129.6 (m, $^{3}J_{H\text{-}F}$ = 3 Hz, *p-Ph*), 125.8 (q, $^{1}J_{H\text{-}F}$ = 277 Hz, CH_2CF_3), 53.3 (Ph*C*H_2N), 51.1 ([C3]*C*H_2N), 49.1 ([C2]*C*H_2N), 48.2 ([C3]*C*H_2N or [C2]*C*H_2N), 45.9 ([C3]*C*H_2N or [C2]*C*H_2N), 40.0 (q, $^{2}J_{H\text{-}F}$ = 29 Hz, *C*H_2CF_3), 23.2 ($CH_2CH_2CH_2$), 22.6 (*C*H_3COO).

^{19}F NMR (D_2O/$(CD_3)_2CO$, 376.5 MHz, 296 K): δ (ppm) -65.8 (s, CH_2CF_3)

^{19}F NMR ($CDCl_3$, 376.5 MHz, 296 K): δ (ppm) -65.9 (s, CH_2CF_3)

Anal. Calcd for $C_{32}H_{46}F_6N_4O_4.(CH_3COOH)_2$: C, 55.09; H, 6.94; N, 7.14. Found: C, 55.00; H, 6.86; N, 7.01.

4,11-dibenzyl-1,4,8,11-tetraazacyclotetradecane-1,8-diiumacetate (16c):

4,11-bis(4-(2,2,2-trifluoroethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane (0.35 g, 0.92 mmol) was dissolved in a small volume acetonitrile and 1 mL of glacial acetic acid was added to the solution. This mixture was refluxed for 1h and the solvent was evaporated under reduced pressure resulting in **16c** with a 84% yield (0.48 g, 0.77mmol).

^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 10.82 (overlapping, 6H total, 4H, NH_2^+ and 2H, CH_3COO*H*), 7.31-7.29 (overlapping, 6H total, 2H, *p-Ph* and 4H, *m-Ph*), 7.21 (d, $^{3}J_{H\text{-}H}$ = 8 Hz, 4H, *o-Ph*), 3.97 (s, 4H, PhCH_2N), 3.25 (m, 4H, [C3]CH_2N), 3.20 (m, 4H, [C2]CH_2N), 2.78 (m, 4H, [C2]CH_2N), 2.74 (m, 4H, [C3]CH_2N), 2.00-1.96 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO^- and 6H, CH_3COOH).

$^{13}C\{^{1}H\}$ NMR ($CDCl_3$,100.6 MHz, 296 K): δ (ppm) 176.5 (*C*OO), 134.0 (*i-Ph*), 130.8 (*o-Ph*), 128.4 (*m-Ph*), 127.7 (*p-Ph*), 53.9 (Ph*C*H_2N), 51.9 ([C3]*C*H_2N), 48.5 (overlapping, [C2]*C*H_2N and [C3]*C*H_2N), 45.5 ([C2]*C*H_2N), 22.8 (CH_2*C*H_2CH_2), 22.5 (*C*H_3COO).

Anal. Calcd for $C_{28}H_{44}N_4O_4.(CH_3COOH)_2$: C, 61.91; N, 9.03; H, 8.44. Found: C, 61.85; N, 9.81; H, 8.40.

4,11-bis(4-methylbenzyl)-1,4,8,11-tetraazacyclotetradecane-1,8-tetraiumchloride (17a):

Compound **12**(0.80g, 1.96 mmol) was dissolved in a minimum volume of ethanol and HCl at 37% was added until the solution reached pH≈1. The white precipitated formed was filtered, washed with ethanol and dried under reduced pressure affording the product with a 91% yield (0,99 g, 1.79 mmol).

^{1}H NMR (D_2O/C_6D_5N, 300.1 MHz, 296 K): δ (ppm) 6.51(d, $^{3}J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 6.51 (d, $^{3}J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 2.92 (m, 4H, [C3]C*H*$_2$N), 2.83 (overlapping, 8H total, 4H, [C2]C*H*$_2$N and 4H, PhC*H*$_2$N), 2.09 (m, 4H, [C2]C*H*$_2$N), 1.96 (m, 4H, [C3]C*H*$_2$N), 1.42 (m, 4H, CH_2C*H*$_2CH_2$) 1.37 (s, 6H, C*H*$_3$).

$^{13}C\{^{1}H\}$ NMR (D_2O/C_6D_5N, 75.5 MHz, 296 K): δ (ppm) 139.9 (*i-Ph* or *p-Ph*), 133.2 (*i-Ph* or *p-Ph*), 132.7 (*o-Ph* or *m-Ph*), 131.6 (*o-Ph* or *m-Ph*), 57.3 ([C3]*C*H_2N), 53.9 ([C3]*C*H_2N), 53.0 ([C2]*C*H_2N), 50.9 (Ph*C*H_2N), 47.5 ([C2]*C*H_2N), 24.2 (CH_2*C*H_2CH_2), 22.7 (*C*H_3).

Anal. Calcd for $C_{26}H_{44}Cl_4N_4.H_2O$: C, 54.55; H, 8.10; N, 9.79. Found: C, 54.41; H, 8.30; N, 9.57.

4,11-bis(4-(2,2,2-trifluoroethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane-1,8-tetraiumchloride (17b):

Compound **17b** was obtained, through **13,** quantitatively as a white solid using the procedure described for the preparation of **17a**.

^{1}H NMR ($D_2O/(CD_3)_2CO$, 300.1 MHz, 296 K): δ (ppm) 7.61 (d, $^{3}J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.54 (d, $^{3}J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 4.45 (s, 4H, PhC*H*$_2$N), 3.72 (overlapping, 8H total, [C2]C*H*$_2$N), 3.61 (q, $^{3}J_{H-F}$ = 11 Hz, 4H, C*H*$_2$CF$_3$), 3.51-3.46 (overlapping, 8H total, [C3]C*H*$_2$N), 2.31 (m, 4H, CH_2C*H*$_2CH_2$).

$^{13}C\{^{1}H\}$ NMR ($D_2O/(CD_3)_2CO$, 75.5 MHz, 296 K): δ (ppm) 133.1 (*i-Ph*), 131.8 (*o-Ph* or *m-Ph*), 131.7 (*o-Ph* or *m-Ph*), 129.5 (*p-Ph*), 126.4 (q, $^{1}J_{H-F}$ = 277 Hz, CH_2*C*F$_3$), 58.5 (Ph*C*H_2N), 48.2 ([C3]*C*H_2N), 45.2 ([C2]*C*H_2N), 42.4 ([C3]*C*H_2N), 39.0 (q, $^{2}J_{H-F}$ = 29 Hz, *C*H_2CF$_3$), 38.2 ([C2]*C*H_2N), 18.7 (CH_2*C*H_2CH_2).

^{19}F NMR ($D_2O/(CD_3)_2CO$, 282.4 MHz, 296 K): δ (ppm) -65.7 (s, CH_2C*F*$_3$).

Anal. Calcd for $C_{28}H_{42}Cl_4F_6N_4.(H_2O)_2$: C, 46.29; N, 7.71; H, 6.38. Found: C, 46.29; N, 747; H, 6.24.

4,11-dibenzyl-1,4,8,11-tetraazacyclotetradecane-1,8-tetraiumchloride (17c):

4,11-bis(4-(2,2,2-trifluoroethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane (1.00 g, 2.63 mmol) was dissolved in a minimum volume of ethanol and HCl at 37% was added until the solution reached pH≈1. The white precipitated formed was filtered, washed with ethanol and dried under reduced pressure affording the product in 53% yield (0.73 g, 1.39 mmol). Suitable crystals for single crystal X-ray diffraction were obtained from an aqueous hydrochloric acid solution.

^{1}H NMR (D_2O/$(CD_3)_2CO$, 300.1 MHz, 296 K): δ (ppm) 7.53 (overlapping, 10H total, *Ph*), 4.46 (m, 4H, [C2]C*H*$_2$N), 3.65-3.40 (overlapping, 16H total, 8H, [C3]C*H*$_2$N, 4H, [C2]C*H*$_2$N and 4H, PhC*H*$_2$N), 1.14 (m, 4H, $CH_2CH_2CH_2$).

^{13}C{^{1}H} NMR (D_2O/$(CD_3)_2CO$, 75.5 MHz, 296 K): δ (ppm) 131.3 (*o-Ph* or *m-Ph*), 129.6 (*o-Ph* or *m-Ph*), 57.4 (Ph*C*H$_2$N), 57.0 ([C2]*C*H$_2$N), 49.2 ([C2]*C*H$_2$N), 44.3 ([C3]*C*H$_2$N), 40.6 ([C3]*C*H$_2$N), 17.6 ($CH_2CH_2CH_2$).

Anal. calcd for $C_{24}H_{40}Cl_4N_4.H_2O$: C, 52.95; H, 7.78; N, 10.29. Found: C, 52.78; H, 7.64; N, 10.25.

1,8-bis(4-(trifluoromethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane-1,8-tetraiumchloride (17d):

1,8-bis(4-(trifluoromethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane (1.00 g, 1.94 mmol) was dissolved in a minimum volume of ethanol and HCl at 37% was added until the solution reached pH≈1. The white precipitated formed was filtered, washed with ethanol and dried under reduced pressure affording the product in 68% yield (0.88 g, 1.33 mmol).

^{1}H NMR (D_2O/$(CD_3)_2CO$, 400.1 MHz, 296 K): δ (ppm) 7.85 (d, $^{3}J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.75 (d, $^{3}J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 4.24 (s, 4H, PhC*H*$_2$N), 3.65 (m, 4H, [C2]C*H*$_2$N), 3.51 (m, 4H, [C3]C*H*$_2$N), 3.42 (m, 4H, [C2]C*H*$_2$N), 3.21 (m, 4H, [C3]C*H*$_2$N), 2.28 (m, 4H, $CH_2CH_2CH_2$).

^{13}C{^{1}H} NMR (D_2O/$(CD_3)_2CO$, 100.6 MHz, 296 K): δ (ppm) 136.3 (*i-Ph*), 131.7 (*o-Ph*), 130.8 (q, $^{2}J_{C-F}$ = 24 Hz, *p*-Ph), 126.3 (d, $^{3}J_{C-F}$ = 3 Hz, *m*-Ph), 124.3 (q, $^{1}J_{C-F}$ = 204 Hz, *C*F$_3$), 57.1 (Ph*C*H$_2$N), 49.8 ([C3]*C*H$_2$N), 47.7 ([C2]*C*H$_2$N), 44.8 ([C3]*C*H$_2$N), 41.3 ([C2]*C*H$_2$N), 20.4 ($CH_2CH_2CH_2$).

^{19}F NMR ($CDCl_3$, 376.5 MHz, 296 K): δ (ppm) -62.6 (s, C*F*$_3$).

Anal. calcd for $C_{26}H_{38}Cl_4F_6N_4.H_2O$: C, 45.90; H, 5.93; N, 8.23. Found: C, 46.01; H, 5.80; N, 8.23.

1,4,8,11-tetrakis(4-(trifluoromethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane (21):

Cyclam (0.20g, 1.00mmol) was dissolved in 10 mL of a NaOH solution (1M). An acetonitrile solution of 4-(trifluoromethyl)benzyl bromide (0.98g, 4.10mmol) was added and the reaction mixture was left stirring for 2 hours. The white precipitate formed was filtered off and dried under reduced pressure. Compound **21** was obtained with a 84% yield (0.70 g, 0,84mmol).

^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 7.50 (m, 8H, *o-Ph* or *m-Ph*), 7.39 (m, 8H, *o-Ph* or *m-Ph*), 3.48 (m, 8H, PhC*H*$_2$N), 2.62 (m, 8H, [C2]C*H*$_2$N), 2.54 (m, 8H, [C3]C*H*$_2$N), 1.79 (m, 4H, $CH_2CH_2CH_2$).

^{13}C{^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 129.2 (*o-Ph* or *m-Ph*), 125.2 (*o-Ph* or *m-Ph*), 58.9 (Ph*C*H$_2$N), 51.7 ([C2]*C*H$_2$N or [C3]*C*H$_2$N), 50.7 ([C2]*C*H$_2$N or [C3]*C*H$_2$N), 24.5 ($CH_2CH_2CH_2$).

^{19}F NMR ($CDCl_3$, 282.4 MHz, 296 K): δ (ppm) -62.4 (s, C*F*$_3$)

Anal. Calcd for $C_{42}H_{44}F_{12}N_4$: C, 60.57; N, 6.73; H, 5.33. Found: C, 59.03; N, 6.72; H, 5.34.

1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraiumchloride (23): The procedure to obtain this compound was similar to those described for **17a** and **17b** using cyclam as starting reagent. Compound **23** was obtained quantitatively as a white solid.

^{1}H NMR (D_2O/$(CD_3)_2CO$, 400.1 MHz, 296 K): δ (ppm) 3.59 (m, 8H, [C2]C*H*$_2$N), 3.44 (m, 8H, [C3]C*H*$_2$N), 2.25 (m, 4H, $CH_2CH_2CH_2$).

^{13}C {^{1}H} NMR (D_2O/$(CD_3)_2CO$, 100.6 MHz, 296 K): δ (ppm) 40.4 ([C3]*C*H$_2$N), 37.3 ([C2]*C*H$_2$N), 17.5 ($CH_2CH_2CH_2$).

Anal. Calcd for $C_{10}H_{28}Cl_4N_4.(H_2O)_2$: C, 31.43; H, 8.44; N, 14.66. Found: C, 31.34; H, 7.59; N, 14.56.

Cyclen (24):

Cyclen was prepared according to a previously published procedure[70]. Triethylenetetramine (10.0 g, 68.4 mmol) was dissolved in 400 mL of water and K_2CO_3 (14.6 g, 308.0 mmol) was added. The mixture was warmed to 80 ºC and stirred vigorously. *p*-toluenesulfonyl chloride (57.5 g, 302.0 mmol) was added during a period of 3h. The reaction mixture was heated and stirred overnight. After cooling to room temperature, the precipitate formed was filtered and subsequently washed with water, methanol and diethyl ether. The solid obtained (33.8 g, 44.4 mmol) was dissolved in 520 mL of dried DMF and K_2CO_3 (12.5 g, .90.mmol) was added. A solution of 1,2-bis(p-toluenesulfonato)ethane (16.3 g, 44.1 mmol) in 75 mL of anhydrous DMF was added through an addition funnel over a period of 3-4 h. The reaction mixture was heated to 70 ºC for 3h. The residue obtained after removal of the solvent under reduced pressure was taken into 100 mL of dichloromethane and 100 mL of water. The aqueous layer was separated and washed with dichloromethane. The organic phases were combined

and dried with $MgSO_4$ anhydrous. The solution was filtered, and the solvent dried under reduced pressure to yield a solid. This compound was dissolved in hot toluene and the solution was allowed to cool to room temperature. The white precipitate formed was collected and washed with cold toluene to yield a cyclic tetratosylamide (14.4 g, 18.2 mmol). This solid was charged into an Erlenmeyer flask and 25 mL of concentrated sulfuric acid was added. The mixture was heated under nitrogen atmosphere for a period of 40 h at 110 ºC. The reaction mixture was poured into 20 mL of water cooled in an ice bath. KOH pellets were added until pH>13. Upon addition of 150 mL of ethanol a white precipitate was formed. The solid was filtered off and washed with ethanol. The ethanol extracts were dried on a rotating evaporator yielding a yellow residue that was dissolved in a minimum volume of HCl (0.1 M). Dichloromethane was added to the solution and the aqueous layer was separated and washed with dichloromethane. KOH pellets were added to the aqueous layer until pH≈13 and the product extracted with chloroform. The organic phases of the extractions were combined and dried with anhydrous $MgSO_4$. After filtration the solvent was removed under reduced vacuum achieving the product as a white solid with a yield of 12% (1.4g, 8.1mmol).

H NMR (d_8-toluene, 300.1 MHz, 296 K): δ (ppm) 2.66 (m, 16H, C*H*$_2$N), 2.05 (br, 4H, N*H*)

1,7-ditosyl-1,4,7,10-tetraazacyclododecane (25):

Tosyl chloride (0,76g, 4.00 mmol) was dissolved in 10mL of dried pyridine and charged to a round-bottomed flask attached to a pressure equalizing addition funnel, with a nitrogen inlet adaptor. The apparatus was flushed with dry nitrogen gas. A solution of **24** (0.34g, 2.00 mmol) in 3 mL of dry pyridine was added with the aid of an addition funnel. The reaction flask was cooled to 0ºC and the contents of the addition funnel were added, under nitrogen atmosphere, over a period of 15 min. Afterwards the mixture was allowed to warm to room temperature and it was left stirring for 3h. Once completed the reaction, the pyridine was removed under reduced pressure yielding a yellow solid that was washed with water. This mixture was filtered and the precipitate was washed with water, saturated potassium carbonate solution, water and methanol. Finally it was dried in an electric oven (120ºC) and product was achieved as a yellow solid with a yield of 63% (0,60g, 1.25 mmol)

^{1}H NMR ($CDCl_3$, 300.1 MHz, 296 K): δ (ppm) 7.70 (d, $^3J_{H\text{-}H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 7.37 (d, $^3J_{H\text{-}H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 3.41 (m, 8H, [C2]C*H*$_2$N), 3.16 (m, 8H, [C2]C*H*$_2$N), 2.45 (s, 6H, C*H*$_3$).

2.2. Biological assays

2.2.1. Bacterial strains and media

The bacterial strains used were *Escherichia coli* ATCC 2592, *Burkholderia contaminans* IST408, *Pseudomonas aeruginosa* 477 and *Staphylococcus aureus* Newman. All these strains are human clinical isolates. When in use, *E. coli* ATCC 2592 and *S. aureus* Newman were maintained in

Lennox Broth (LB) solid medium, while *B. contaminans* IST408 and *P. aeruginosa* 477 were maintained in *Pseudomonas* Isolation Agar (PIA) plates.

2.2.2. Minimum inhibitory Concentration Assays

Minimal inhibitory concentration (MIC) assays were performed in Mueller Hinton Broth (Sigma) (MHB) medium using a microdilution assay, based on previously described methods[71][72]. Optical densities (OD) were determined at 640nm using a U-2000 Spectrophotometer (HITACHI) and the absorbance of microbial cultures in microtiter plates were measured at 600 nm in a SPECTROstar^{Nano} microplate reader (BMG LABTECH).

Briefly, bacteria were grown in MHB liquid medium overnight with orbital agitation (250 rpm) at 37 ºC. The cultures were then diluted in fresh MHB to an OD_{640} of 0.05 and incubated for 5h under the same conditions. Cultures were then diluted to an OD_{640} of 0.02 and 100 µL aliquots of these cell suspensions were inoculated in 96-well polystyrene microtiter plate containing 100 µL of MHB supplemented with different concentrations of each compound under study, achieved by serial 1:2 dilutions starting at 512 µg/mL to 0.5 µg/mL. Compounds were dissolved in distilled water and filtered using a 0.22 µm sterile filter. As positive control an aliquot of 100 µL of 1x concentrated MHB and 100 µL of the bacteria inocula (OD_{640nm}= 0.02) were used, while for negative controls aliquots of 200 µL of sterile MHB 1x concentrated was used. The compounds solutions sterility were also tested. After the inoculation, the microtiter plates were incubated at 37 ºC for 20h and bacterial growth was assessed by determining the OD of cultures at 600nm using a SPECTROstar^{Nano} microplate reader. Experiments were carried out at least four times.

2.2.3. Bacteriostatic /Bactericidal tests

To assess the bacteriostatic or bactericidal activity of the compounds, 20 µL aliquots the 96-well polystyrene microtiter plates used in the MIC assays were collected from the wells exhibiting the highest concentration of compound with bacterial growth and the two subsequent wells with no evident growth. These aliquots were serially diluted 1:10 until 10^{-7}. 10 µL of each serial dilution were spot inoculated onto to the surface of LB solid medium. The spot inoculated plaques were incubated at 37ºC for 18h. After incubation, the plaques were photographed using a Biorad Imaging System and the CFUs were enumerated.

2.2.4. Spontaneous emergence of resistance assays

The assays to assess the frequency of emergence of spontaneous resistance to the compounds under study were done based on published procedures [73]. Since the published procedure used larger plates, the conditions needed to be adjust to the ones used in this study. Plates were prepared with 50 mL of solid MHB and contained the compounds at concentrations four times higher than the MIC calculated for each strain. For this purpose, the number of bacteria inoculated was adjusted to 1-9 x 10^9.

MHB was inoculated with an isolated colony of each bacterial strain and incubated overnight at 37°C with orbital agitation (250 rpm). Afterwards aliquots of the cultures were collected and diluted

in 20mL of MHB to obtain an OD_{640} of 0.05. These cultures were further incubated at 37°C with orbital agitation (250 rpm) for five hours. After 5 hours, the OD_{640} was measured in order to determine the volume of culture correspondent to the CFUs desired. These volumes were centrifuged in microtubes using a microcentrifuge (8000 rpm; 5min). The supernatant was discarded and the pellets were ressuspended in 300 µL 0.9% NaCl solution and spread onto the surface of square plates, followed by incubation at 37°C for 5 days.

To assess the precise number of initial CFUs, separate assays were done. For this purpose aliquots of the cultures used to inoculate the plates with the compounds, at the same optical density, were used. These aliquots were serially diluted 1:10 until concentration $1x10^{-7}$ was reached. Aliquots of 10 µL of each dilution were spot inoculated in MHB solid medium and incubated overnight at 37ºC. The colonies formed were counted after 24h of incubation.

3. Results and Discussion

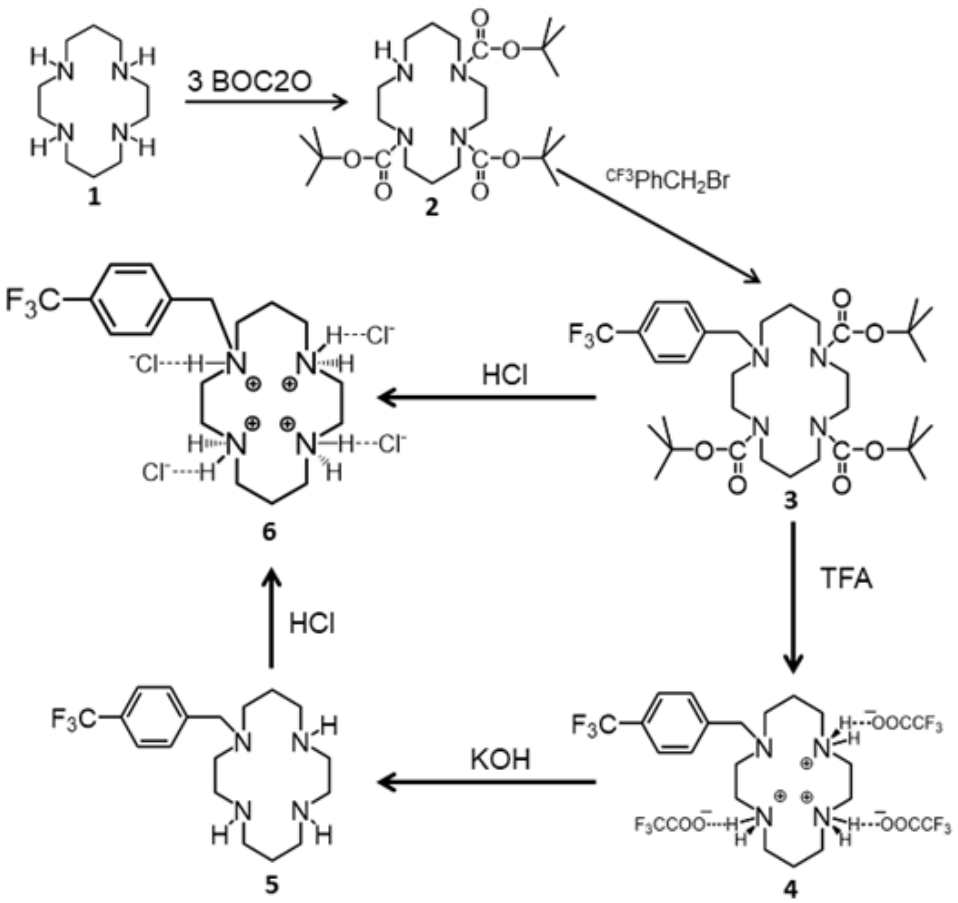
3.1 Chemical Synthesis

Cyclam derivatives with a varying number of pendant arms on the macrocyclic ring were prepared in order to test if the number and chemical nature of the pendant arms had any effect on the antimicrobial activity of these compounds. Since the compounds must be soluble in water for the MIC assays, their neutral forms were converted into the corresponding acetate and/or chloride salts in order to increase their solubility.

For clear and better understanding of the various chemical structures mentioned in this thesis, a detachable with the depiction of their molecular structure is presented, as a separate sheet.

3.1.1 Synthesis of mono-substituted Cyclam Derivatives

To synthesize the mono-substituted cyclam salt (**6**) it was necessary to protect three of the four nitrogen atoms of the cyclam ring in order to selectively functionalize only one of them. As so, protection was done using *tert*-butylcarbamate groups (Boc) by reaction of cyclam with di-*tert*-butyldicarbonate (Boc_2O) leading to the formation of $H(Boc)_3$Cyclam (**2**) (see **Scheme 1**). The subsequent alkylation reaction with 4-(trifluoromethyl)benzyl bromide led to the formation of ($^{4\text{-}CF3}PhCH_2$)$(Boc)_3$Cyclam (**3**). Removal of the Boc protecting groups was done by two methods. In ***Method A*** compound **3** and concentrated HCl (37%) were used, which gave the chloride salt $H_4[H_3(^{4\text{-}CF3}PhCH_2)Cyclam]Cl_4$ (**6**) with a 47% yield (see **Scheme 1**). In ***Method B***, trifluoracetic acid was used to remove the Boc protecting groups, affording the corresponding trifluoracetate salt (**4**). This species was subsequently neutralized with KOH (**5**), which was reconverted into the corresponding chloride salt by reaction with HCl (**6**) (see **Scheme 1**). Both methods are convenient procedures for the preparation of **6** but since the latter one involves more steps, it seems obvious that the direct reaction with HCl is a more efficient method.



Scheme 1

The ^{1}H NMR spectrum of **6** reveals ten multiplets corresponding to the methylene protons of the macrocycle backbone integrating to two protons each and one singlet that correspond to the two benzylic protons of the pendant arm due to the C_1 symmetry of the compound (**Figure 17**). In addition, two doublets integrating to two protons each appear in the aromatic region of the spectra. The NH_2^+ protons are absent because the spectrum was recorded in D_2O, revealing proton exchange. The ^{13}C $\{^{1}H\}$ NMR spectrum of **6** displays ten different resonances for the macrocycle carbons and one for the benzylic carbon of the pedant arm, as expected. Additional resonances due to aromatic rings and the CF_3 group are also present. Despite the multiplicity observed in the ^{1}H and ^{13}C $\{^{1}H\}$ NMR spectra did not correspond to a C_1 symmetry due to overlapping of the resonances, its confirmation was based on 2D NMR experiments (COSY and HSQC). The ^{19}F NMR spectrum shows a singlet due to the CF_3 group at -62.6 ppm.

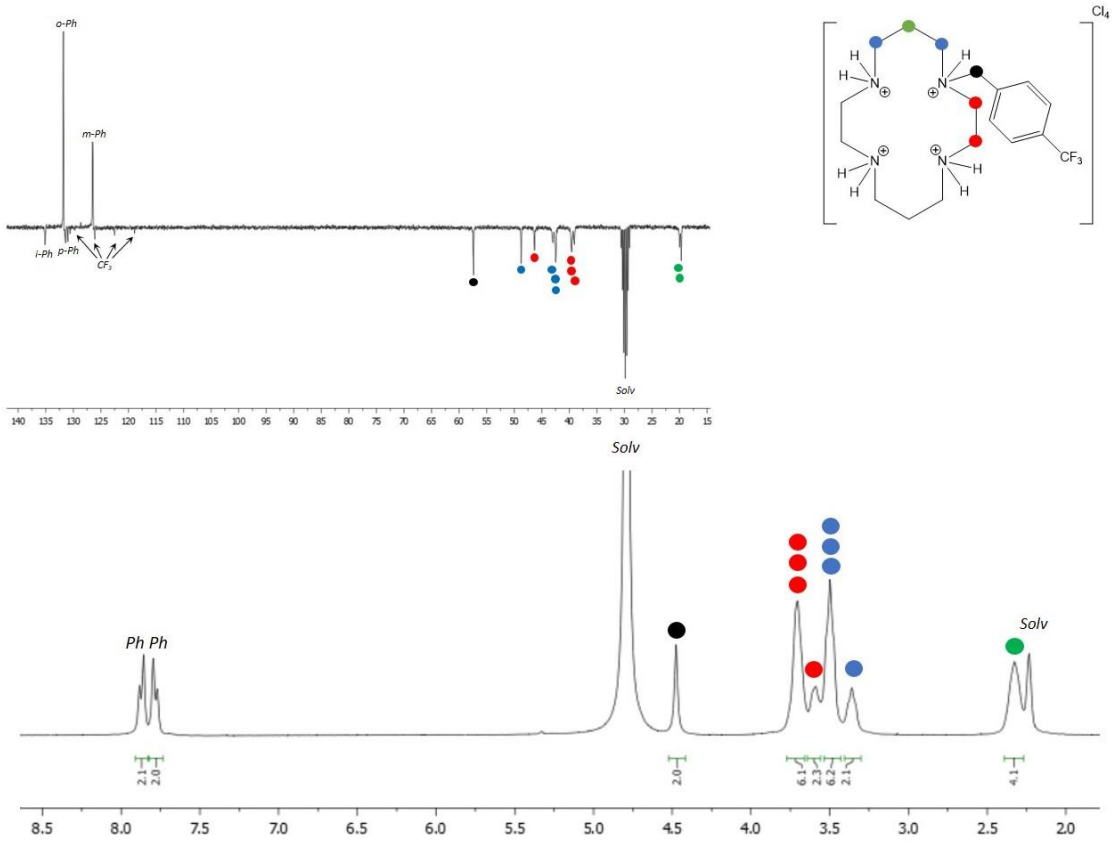
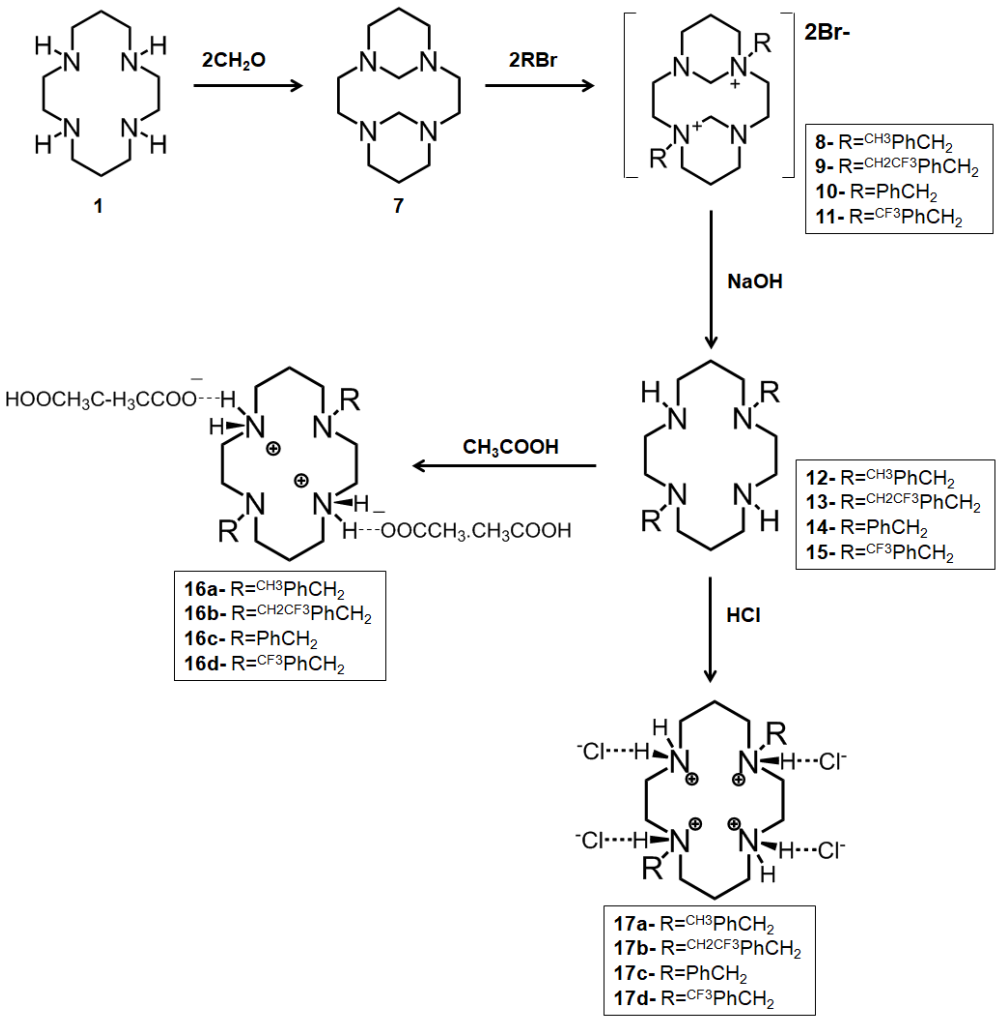


Figure 17- ^{1}H and ^{13}C {^{1}H} NMR spectra of compound 6, in $D_2O/(CD_3)_2CO$, at 296K. Red, green and black dots indicate the position of carbon or hydrogen atoms in the molecule structure and the respective spectra

3.1.2 Synthesis of *trans*-disubstituted Cyclam Derivatives

trans-disubstituted cyclams of formula H_2Bn_2Cyclam (Bn = $^{4\text{-}CH3}PhCH_2$, **12**, $^{4\text{-}CH2CF3}PhCH_2$, **13**, $PhCH_2$, **14** and $^{4\text{-}CF3}PhCH_2$, **15**) were prepared as shown in **Scheme 2**. Conversion of cyclam (**1**) into 1,4,8,11-tetraazatricyclo[9.3.1.1^{4,8}]hexadecane (**7**) was achieved by reaction with two equivalents of formaldehyde. Compound **7** displays two methylene cross-bridges between adjacent nitrogen atoms that point to opposite faces of the macrocycle. In this species, due to the stereochemical conformation imposed by the methylene cross-bridge, the deepest negative potential of the cyclam ring corresponds only to one pair of *trans* nitrogen atoms. These atoms are not only the less sterically hindered but they also have the electron lone pair pointing out of the macrocycle backbone; this structure is crucial for the second step of the reaction as it directs the nucleophilic attack and controls the selectivity[74]. In this way, the formation of **8**-**11** was attained by reaction of **7** with two equivalents of the appropriate benzyl bromide. All compounds are slightly hygroscopic solids that can hydrolyze in air. This behavior is more pronounced in **9** which hampered its characterization by NMR. Full hydrolysis of **8**-**11** in basic conditions affords the correspondent neutral compounds **12**-**15**.



Scheme 2

In the ^{1}H NMR spectra of compounds **12**-**15**, the macrocycle geminal protons are equivalent, giving rise to the emergence of only five signals integrating to four protons each in agreement with a C_2 symmetry in solution. The methylene protons of the pendant arms show up as singlets in accordance with allowed nitrogen inversion. The $^{13}C\{^{1}H\}$ NMR spectra display five different resonances for the macrocycle backbone and one set of resonances that corresponds to the benzyl moieties. The proton and carbon NMR spectra of **12**-**15** are similar to those obtained for other *trans*-disubstituted cyclams already reported and do not deserve further details[74].

Crystals of **12** suitable for single crystal X-ray diffraction were obtained from slow evaporation of a chloroform solution. Crystallographic and experimental details of data collection and crystal structure determination are presented in **Table 1**. A Mercury depiction of the molecular structure of **12** is shown in **Figure 18**.

Table 1- Crystallographic data of compounds 12 and 16b

Compound	12	16b
Empirical formula	C_{26} H_{40} N_4	C_{36} H_{54} F_6 N_4 O_8
Formula weight	408.62	784.83
Temperature (K)	150(2)	150(2)
Wavelength (Å)	0.71073	0.71073
Crystal system	Monoclinic	Triclinic
Space group	C2/c	P-1
Unit Cell Dimensions:		
***a* (Å)**	20.719(2)	9.578(1)
***b* (Å)**	9.2432(8)	10.528(2)
***c* (Å)**	14.962(1)	20.376(2)
α(º)	90	81.323(5)
β(º)	121.894(4)	82.311(6)
γ(º)	90	79.096(5)
Volume (Å^3)	2432.8(4)	1982.7(3)
Z	4	2
Calculated density (g m^{-3})	1.116	1.315
Absorption coefficient (mm^{-1})	0.066	0.110
***F* (000)**	896	832
Crystal size (mm)	0.16 x 0.16 x 0.18	0.12 x 0.14 x 0.14
Theta range for data collection (º)	3.207 – 27.182	2.346 – 26.534
Limiting indices	-26≤*h*≤26, -11≤*k*≤8, -14≤*l*≤19	-11≤*h*≤12, -13≤*k*≤13, -25≤*l*≤25
Reflections collected/unique [R_{int}]	8410/2683 [0.0489]	17572/8095 [0.0578]
Completeness to θ (%)	99.5 (θ = 25.242)	99.1 (θ = 25.242)
Refinement method	Full-matrix least squares on F^2	Full-matrix least squares on F^2
Data/restraints/parameters	2683/0/141	8095/0/515
Goodness-of-fit on F^2	1.003	0.939
Final *R* indices [$I>2\sigma(I)$][a]	R_1 = 0.0528, wR_2 = 0.1154	R_1 = 0.0649, wR_2 = 0.1543
***R* indices (all data)[a]**	R_1 = 0.1066, wR_2 = 0.1293	R_1 = 0.1438, wR_2 = 0.1798
Absorption correction	Multi-scan	Multi-scan
Largest diff. peak/hole (e Å^{-3})	0.213 and -0.189	0.774 and -0.303

[a] $R_1 = \Sigma \, ||F_0| - |F_c|| \, / \, \Sigma \, |F_0|$; $wR_2 = \{\Sigma[w(F^2_0 - F^2_c)^2] / \Sigma[w(F^2_0)^2]\}^{1/2}$

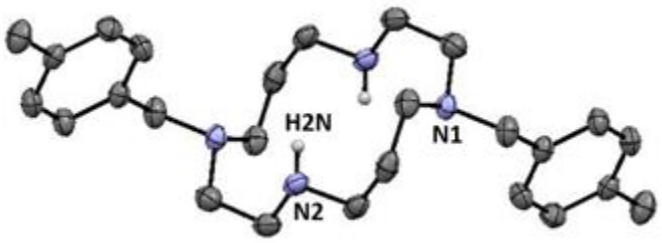


Figure 18- Mercury diagram of 12. Selected hydrogen atoms are omitted for clarity.

The solid state molecular structure of **12** shows the two benzyl pendant arms located at opposite sides of the macrocyclic ring. Despite the structural arrangement of the cyclam ring, one cannot consider intramolecular hydrogen bonds between N(2)-H(2N) and N(1) as the corresponding angles are narrower than 110º. These features were also observed in the previously reported solid state molecular structure of $H_2(^{4\text{-}CN}PhCH_2)_2$Cyclam (**14**) [74].

In acetic acid medium, compounds **12**-**15** were converted into the corresponding acetate salts **16** that were obtained in high yields after taking the solution to dryness. On the other hand, the chloride salts were obtained by addition of HCl to a solution of the compounds in ethanol that cause them to precipitate (see **Scheme 2**). Therefore salts **17** were obtained in very high yields due to the nature of the reaction.

The ^{1}H NMR spectra of compounds **16** and **17** are similar to the ones described for **12**-**15**, showing five multiplets corresponding to the methylene protons of the macrocycle backbone integrating to four protons each and one singlet that corresponds to the four benzylic protons of the pendant arms. In addition, one set of resonances appear in the aromatic region of the spectra. In compounds **16a** and **17a** the protons of the CH_3 groups show up as singlets at 2.25 and 1.32ppm, respectively. In **16b** and **17b** the CH_2CF_3 groups appear as a quartet with $^3J_{H\text{-}F}$=11Hz at 3.34 and 3.32ppm, respectively. The NH_2^+ and COOH protons overlap at 10.74 and 10.28 ppm in the spectra of **16a** and **16b** respectively. These protons are absent if the spectra are performed in D_2O, revealing proton exchange. The ^{13}C {^{1}H} NMR spectra of **16** and **17** are also similar to the ones described for **12**-**15** displaying five different resonances for the macrocycle and one for the benzylic carbon of the pedant arm as expected. Additional resonances due to aromatic rings are also presented. **Figure 19** depicts the ^{1}H and ^{13}C{^{1}H} NMR spectra of compound **16b** as an example.

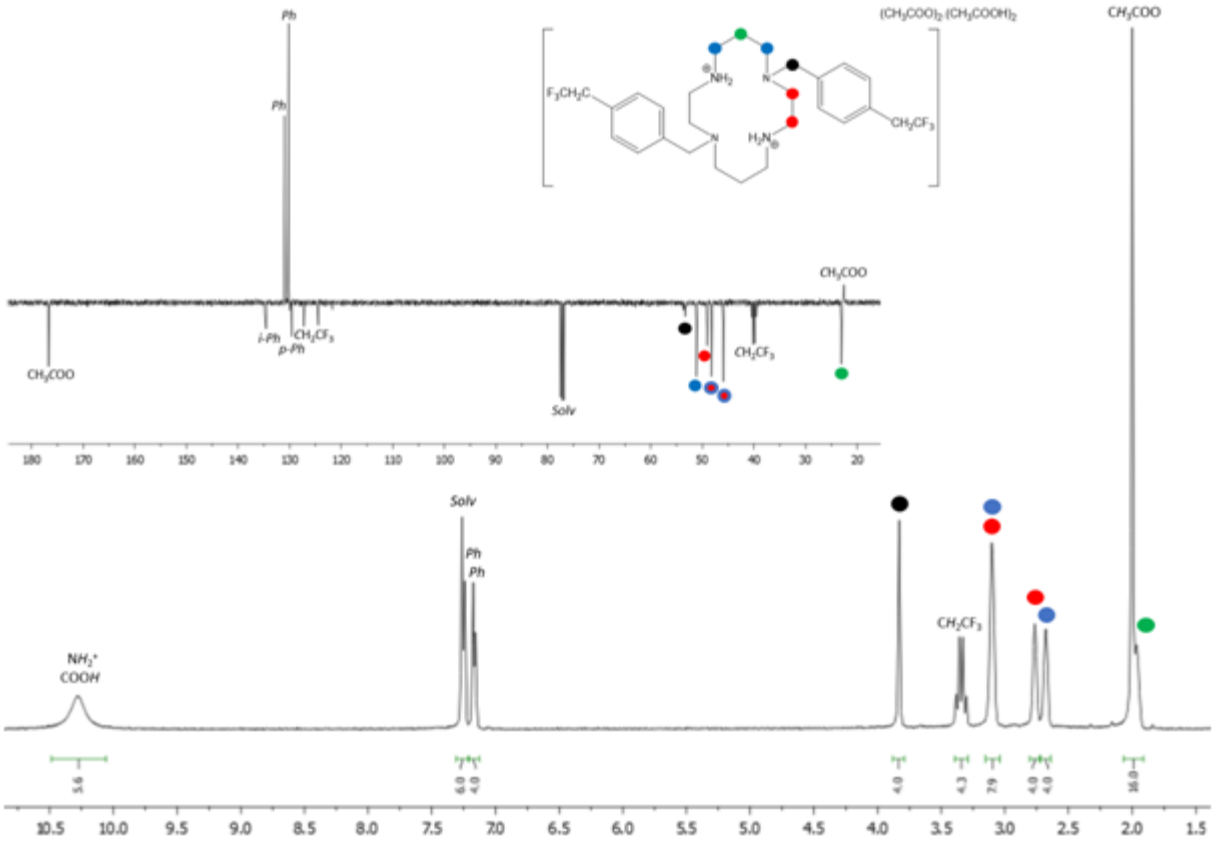


Figure 19-^{1}H and ^{13}C {^{1}H} NMR spectra of compound 16b, in $CDCl_3$, at 296K. Red, green and black dots indicate the position of carbon or hydrogen atoms in the molecule structure and the respective spectra.

Crystals of **16b** suitable for single crystal X-ray diffraction were obtained by concentration of an aqueous acetic acid solution (**Table 1**). A Mercury depiction of **16b** is presented in **Figure 20**, showing the two benzyl pendant arms in mutually *trans*-positions pointing to opposite sides of the macrocyclic ring. The structural arrangement of **16b** reveals the establishment of hydrogen bonds between the acetate anions, the cyclam framework and acetic acid molecules of co-crystallization. Each $[CH_3COO\cdots HOOCCH_3]^-$ pair is located at opposite sides of the plane that contains the four nitrogen atoms of the cyclam ring. Such interactions are not present in solution as revealed by the C_2 symmetry observed in the NMR spectra. The solid state molecular structure of **16b** reveals similar features to those observed for $[H_2\{H_2(^{4\text{-}CF3}PhCH_2)_2Cyclam\}](CH_3COO)_2\cdot(CH_3COOH)_2$, previously reported[56].

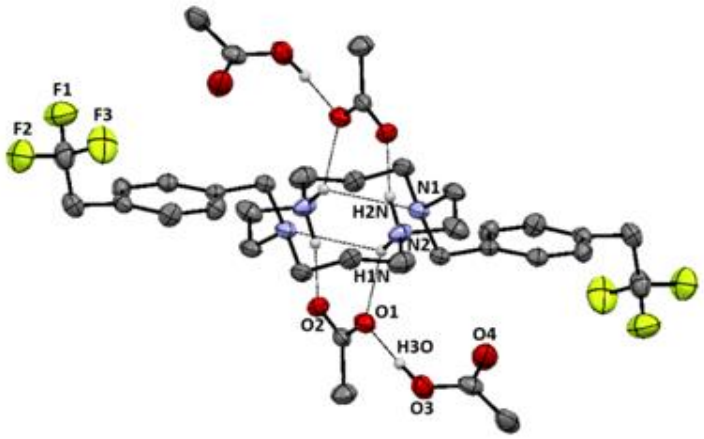
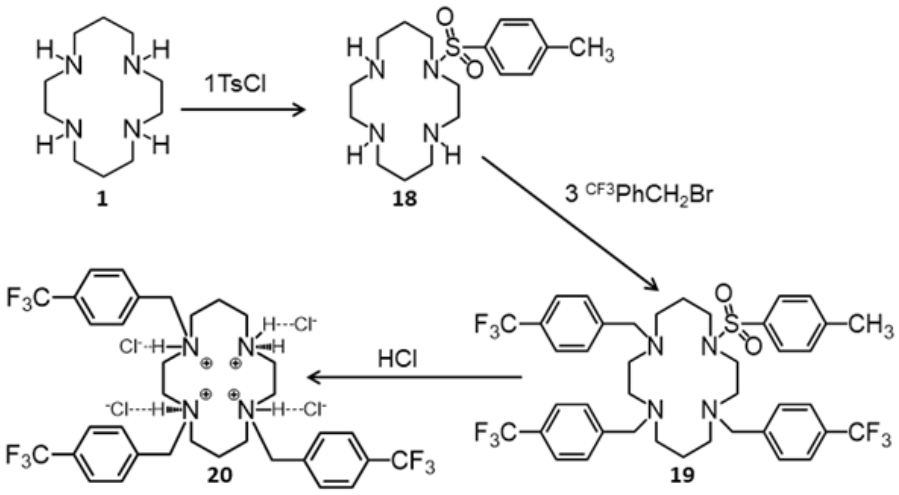


Figure 20 Mercury diagram of 16b. Selected hydrogen atoms are omitted for clarity. The dashed lines represent hydrogen bonds.

3.1.3 Synthesis of trisubstituted Cyclam Derivatives

The synthesis of a cyclam derivative with three pendant (**19**) arms was also attempted but it was not possible to develop a convenient method to its preparation.

The synthetic route that was tried to be implemented is represented in **Scheme 3**. The tosyl group should work as a protecting group for one nitrogen atom of the cyclam ring, so then it would be possible to direct the alkylation to the remaining three secondary amine groups. After alkylation, deprotection with HCl like it was done for Boc groups in compound **3** would give the desired compound **20**. Despite the design of the route, the production of the compound proved to be more difficult since the protection of the amine was not achievable. The reaction of cyclam with 1 eq of TsCl yielded a racial mixture. Therefore, it was not possible to proceed to the next steps precluding the acquisition of the desired compound. I have also tried to use Boc as a protecting group but similar results were obtained.

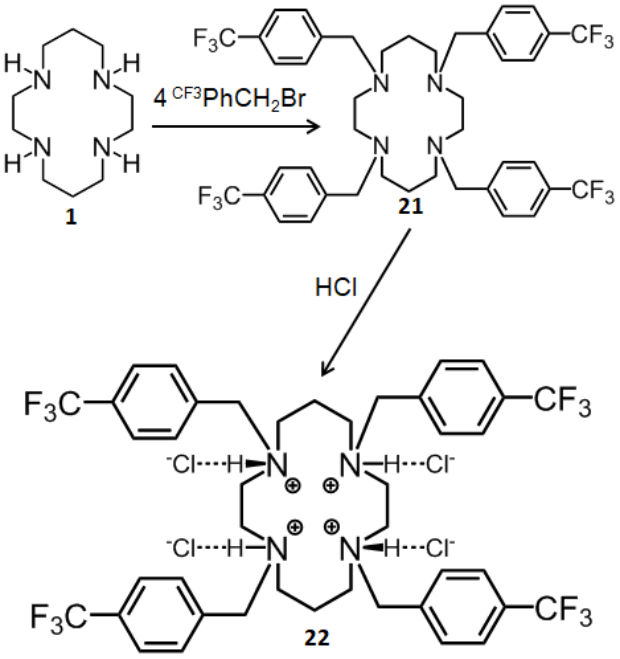


Scheme 3

3.1.4 Synthesis of tetrasubstituted Cyclam Derivatives

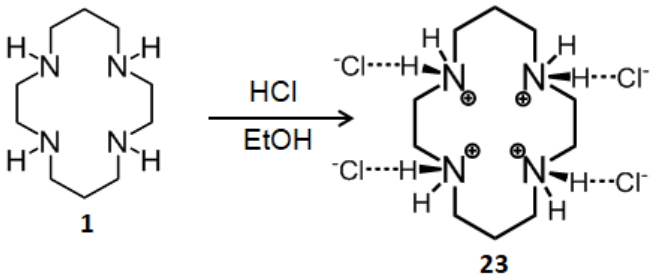
Contrasting to the reactions that produce mono- and di-substituted cyclams**,** the production of the tetra-substituted derivative (**21**) revealed to be challenging. To obtain this compound several reactions were performed, with various conditions (*e.g.* different stoichiometries, reaction time, temperature and base) that are classically described in literature for the desired transformation. Surprisingly, none of them was able to give the final product affording a racial mixture of products.

To obtain the compound **21**, cyclam was reacted with 4.2 equivalents of 4-(trifluoromethyl)benzyl bromide in a mixture of acetonitrile and an aqueous solution of NaOH (1M) in a 1:1 ratio as shown in **Scheme 4**. The product precipitated out of solution and with a very high yield. In order to obtain the corresponding chloride salt (**22**), compound **21** was reacted with concentrated HCl. The product formed is not soluble in the most common solvents which hampered its characterization.



Scheme 4

The convertion of the cyclam in its chloride salt form **23** was really straightforward using the same method used to obtain the chloride salts (see **Scheme 5**). This reaction had a very high yield as expected.



Scheme 5

The NMR spectra of **23** is consistent with a tetraprotonated cyclam salt revealing only three resonances in both proton and carbon spectra. These signals correspond to the CH2 groups of the [C2] and [C3] chains of the cyclam ring as depicted in **Figure 21**.

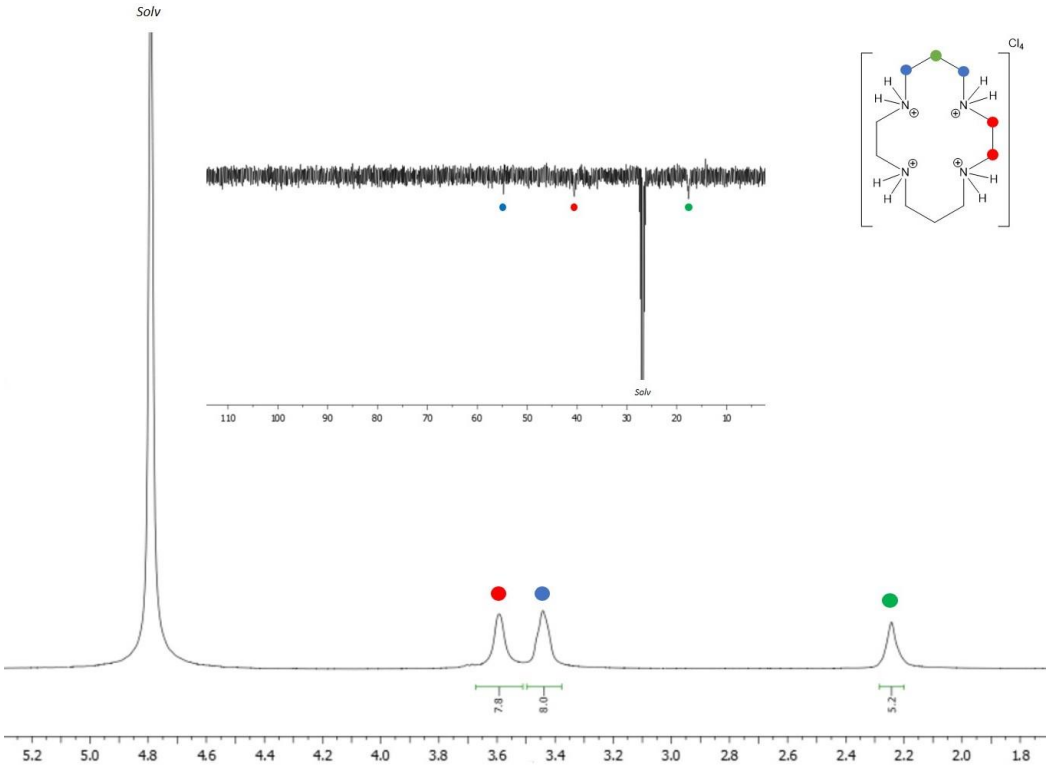


Figure 21-^{1}H and ^{13}C {^{1}H} NMR spectra of compound 23, in $CDCl_3$, at 296K. Red dots, green dots and black dots indicate the position of carbon or hydrogen atoms in the molecule structure and the respective spectra

3.1.5. Synthesis of cyclen derivatives

In order to assess if the size of the macrocycle had any influence on the activity of this family of compounds, cyclen derivatives were also synthesized. The classical procedures for the synthesis of both cyclen and cyclam do not lead to very high yields but the preparation of cyclen requires more demanding steps because this synthesis involves the protection of amine groups and removal of the same protecting groups, which are usually steps with low efficiency [70]. To overcome this issue, a different methodology was tried [60]. Despite the literature description points to the synthesis of cyclen in high yield, the compound obtained did not display the purity level required for the microbiological assays. Therefore, a method was chosen that leads to lower yields but higher purity.

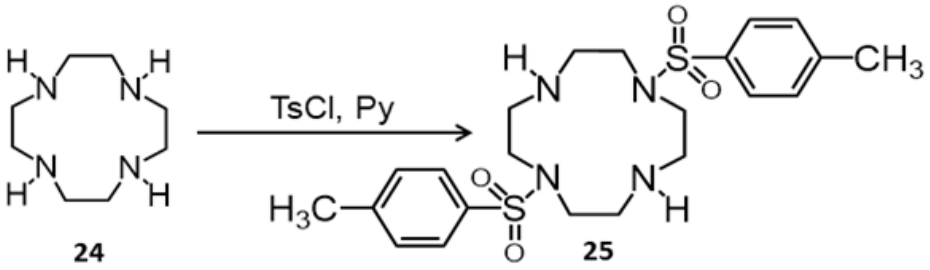
It was reported that to achieve the *trans*-disubstituted cyclen it is not necessary to protect the amine groups to direct the alkylation [70]. Therefore, the reactions to obtain the *trans*-disubstituted cyclens were done without the use of a protecting group. Different reaction conditions to obtain the desired product were attempted as follow:

- Cyclen, 5 equivalents of K_2CO_3 and 2.1 equivalents of $^{CF3}PhCH_2Br$ were dissolved in DMF at room temperature and the reaction mixture stirred overnight.
- Cyclen and 2.1 equivalents of $^{CF3}PhCH_2Br$ were dissolved in pyridine at room temperature and the reaction mixture stirred overnight.
- Cyclen was dissolved in an aqueous solution of NaOH 1M and an equal volume of an acetonitrile solution containing 2.1 equivalents of $^{CF3}PhCH_2Br$ was added and stirred for 2h.

All these conditions resulted in a racial mixture of compounds. Interestingly the third condition yielded tetrasubstituted cyclen. Other attempts to achieve *trans*-disubstituted cyclen were also tried using the mono-substituted derivative in order to reach the intended product with the addition of another equivalent of $^{CF3}PhCH_2Br$. To obtain the mono-substituted species the following reactions were done:

- Cyclen, 1.5 equivalents of triethylamine and 1 equivalent of $^{CF3}PhCH_2Br$ were dissolved in DMF, react at 85 ºC and stirred for 3h.
- Cyclen, 1 equivalent of K_2CO_3 and 1 equivalent of $^{CF3}PhCH_2Br$ were dissolved in DMF at room temperature and stirred over weekend

Surprisingly these reactions also gave a racial mixture of mono and di-substituted cyclen as well as reagents. Giving the difficulty of attaching the pendant arm desired in cyclen an already described protocol was followed to protect two amine groups in *trans* positions, to further drive the reaction with more extreme conditions. The protection of the cyclen was fairly easy to achieve but a low yield, was obtained (see **Scheme 6**).

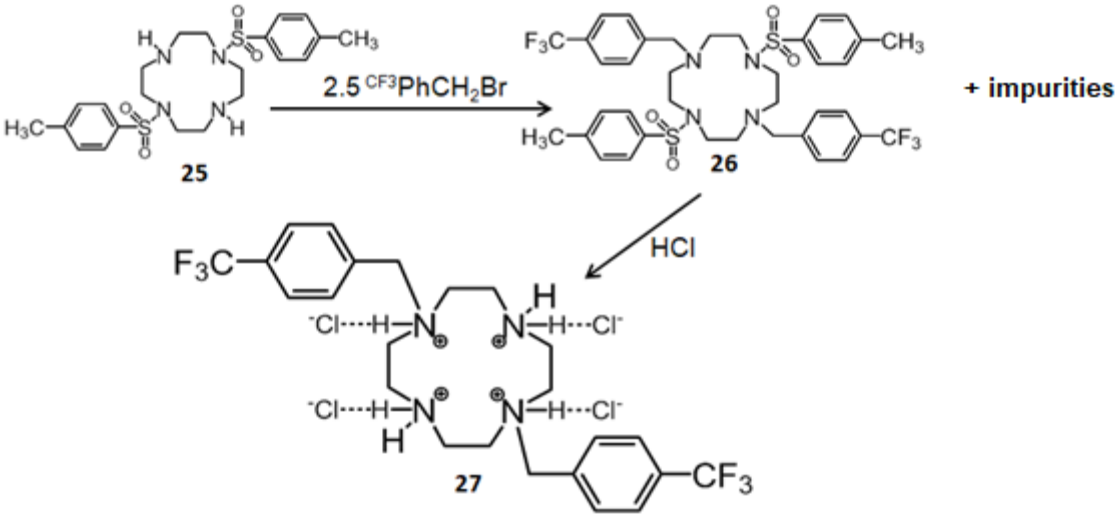


Scheme 6

Using **25** as starting material, two reactions were performed in order to achieve a *trans*,*trans*-tetrasubstituted cyclen (**26**). The reactions conditions were as follow:

- Compound **25** was dissolved in an aqueous solution of NaOH 1M and an equal volume of an acetonitrile solution containing 2.5 equivalents of 4-(trifluoromethyl)benzyl bromide was added with the help of an addition funnel.
- Compound **25** was dissolved in a minimum of DMF and 2.5 equivalents of 4-(trifluoromethyl)benzyl bromide plus 5 equivalents of K_2CO_3 were added. The reaction mixture was left stirring over the weekend.

The first reaction would mimic the formation of the tetrasubstituted cyclam that precipitates out of solution in the conditions described. However, this procedure did not result for cyclen may be due to the high solubility of the product in water caused by the presence of the tosyl groups. The analysis of the bulk revealed a mixture of species. The second reaction also gave a mixture of compounds. Upon dissolution in ethanol, the desired product precipitated out of solution by addition of HCl to the crude (see **Scheme 7**). Nevertheless, the global yield of the reaction was so low after purification that it was not possible to fully characterize the product or even to proceed to biologic assays.



Scheme 7

3.2 Biologic assays

The MICs estimative for the cyclam derivatives were calculated by measuring the OD at 640nm of cultures in microtiter plates, after 20h incubation at 37ºC in the presence of the compounds at concentrations up to 512 µg/mL, obtained upon 1:2 serial dilutions. The data was later adjusted using the Gompertz model in GraphPad Prism software[71].

3.2.1 MIC assays

As discussed above, *trans*-disubstituted cyclams with different pendant arms and different salt forms were used to assess the effects of the nature and number of pendant arms on the compounds antimicrobial activity. The results of the biologic assays for the acetate and chloride salts of the *trans*-disubstituted cyclams synthesized are represented in **Figure 22** and summarized in **Table 2**. Tables with the software outputs (best fitted values and 95% confidence intervals) are presented as annexes (**Annexes – Table A1-A8**).

The assays did not allow us to determine a MIC value for *B. contaminans* IST 408 for any compound synthesized indicating that the MIC values of the compounds for this strain are higher than 512 µg/mL (**Annexes - Figure A1**). These results led us to conclude that the compounds synthesized in this work are not candidates to treat infectious diseases caused by this pathogen.

Results presented on **Table 2** and **Figure 22** show that, the two most active *trans*-disubstituted derivatives were those containing the CF_3 group variations. In fact, the MIC values estimated for *E. coli ATCC* 25922 and *S. aureus* Newman were respectively 10 µg/mL and 10 µg/mL for **16b**, 16 µg/mL and 18 µg/mL for **17b**, 7 µg/mL and 8 µg/mL for **16d** and 5 µg/mL and 5 µg/mL for **17d** . Since the similarities of these four compounds are the presence of a CF_3 group attached to the phenyl ring, in the case of the **16b** and **17b** compounds with a CH_2 linker between, our results indicate that the CF_3 is a group that greatly enhances the antimicrobial activity for these strains. In addition, since the MIC values of **16b, 17b, 16d** and **17d** are very similar, our results suggest that the CH_2 linker group in **16b** and **17b** does not significantly affect the activity. These results also point to the importance of the fluorine atoms in the pendant arms of the compounds. However, in a previous study, the presence of perfluorinated phenyl groups in the pendant arms of the cyclam ring did not increase the antimicrobial activity of the compound[56]. Therefore the group CF_3 must be required, most probably in that position of the phenyl ring for the enhanced antimicrobial activity observed in this study.

Table 2- MIC values obtained from the Gompertz model for the compounds **16** and **17**. Towards *E. coli*, *S. aureus* and *P. aeruginosa.*

	***E. coli* ATCC 25922**	***S. aureus* Newman**	***P. aeruginosa* 477**
	MIC for Acetate salt (µg/mL)		
16a	55	49	9
16b	10	10	164
16c	80	206	19
16d	7	8	15
	MIC for Chloride salt (µg/mL)		
17a	43	119	25
17b	16	18	20
17c	124	80	26
17d	5	5	70

For the compounds **16a**, **16c**, **17a** and **17c** a higher MIC values for *E. coli* and *S. aureus* were obtained, compared to **16b**, **16d**, **17b** and **17d**. It is also possible to observe the importance of a group attached to the carbon in para-position of the phenyl ring since compounds **16a**, **16b** and **16d** have lower MIC values than **16c** and this behavior is also possible to observe in compounds **17**. When analyzing the MIC values for **17a** and **17c** for *S. aureus* it is possible to see that the pattern does not occur. This may indicate a true result or possibly an erroneous calculation of one of the compounds MIC, since what was expected was a decreased in activity in **17c** since there is no group attached to the phenyl group. Furthermore, the curve in **Figure 22-D** indicates that the decay of OD is less pronounced in **17c** when compared to the curves obtained for the other compounds, indicating that it is less active.

For *Pseudomonas aeruginosa* 477 the results obtained are not consistent. As shown in **Table 2**, the MIC values estimated for this strain were lower than it was expected. When observing the microtiter plates no growth could be detected only in the wells containing concentrations of 512 µg/mL of **16b**, **16d**, **17b** and **17d**. This indicates that the MIC values for these compounds should be between 512µg/mL and 256µg/mL. For the other compounds, growth was detected in all wells and therefore it should not be possible to calculate the MIC value. As observed in **Figure 22,** OD around 0 was only obtained for **16b**, **16d**, **17b** and **17d** while OD of at least 0.15 was obtained for the other compounds. Results in **Figure 22** also show that *P. aeruginosa* final biomass decreases gradually with rise of the concentration of the compounds instead of a more pronounced reduction like it happens for *E. coli* and *S. aureus*. These results suggest that the method used is not the most effective to determined MIC values for *P. aeruginosa*.

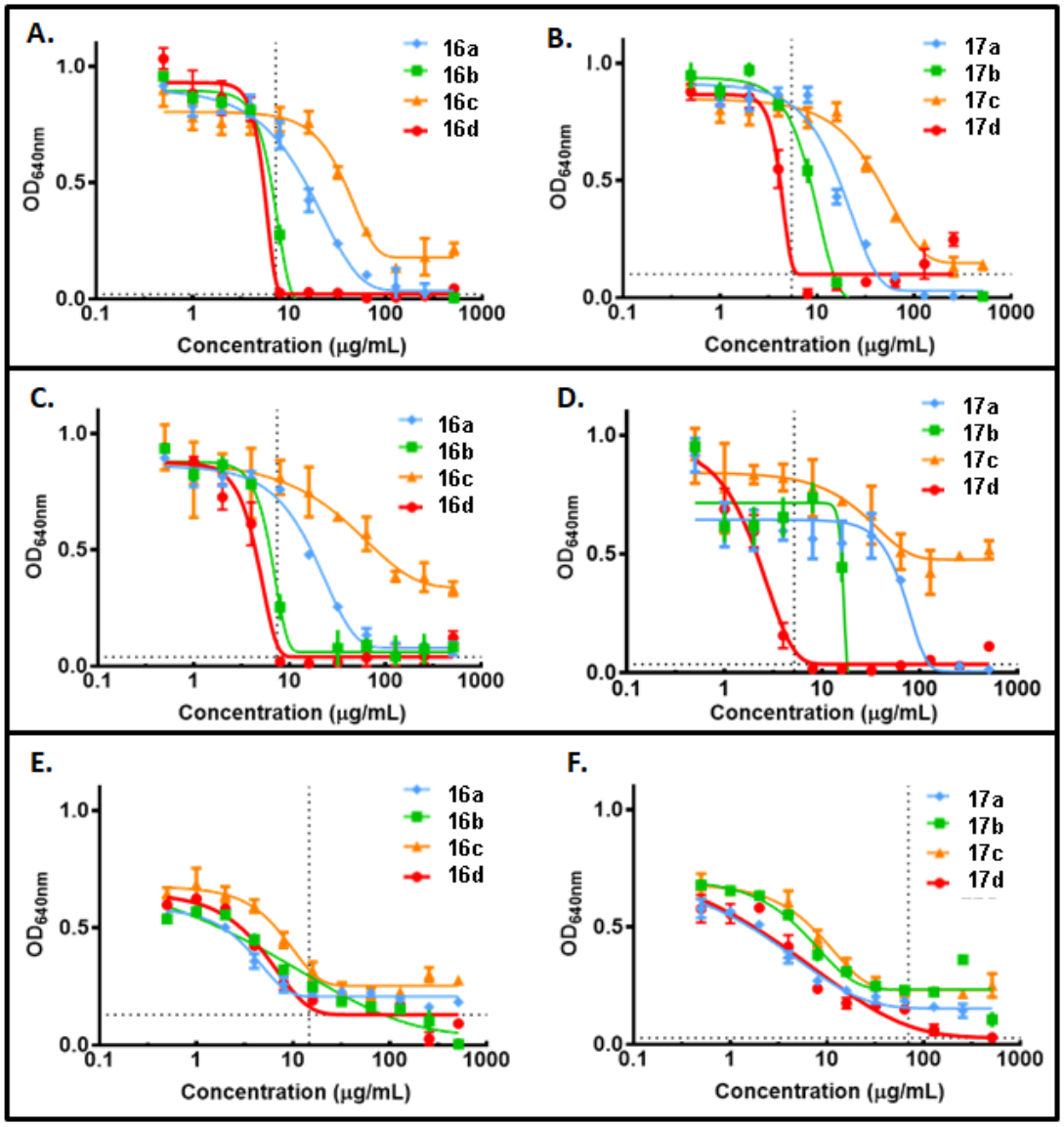


Figure 22- OD $_{640nm}$ adjusted to Gompertz model to determine MIC values for compounds 16 (A. C. and E.) and 17 (B., D, and E) *for E. coli* (A. and B.), *S. aureus* (C. and D.) *and P. aeruginosa* (E. and F.).

In conclusion, the results obtained with *trans*-disubstituted cyclams show that the compounds with the highest antimicrobial activity were those with a CF_3 group, most probably attached at *para*-position since the non-inclusion of a methyl or trifluormethyl group at that position dramatically reduces the compound activity. In addition, results presented in **Table 2** also indicate that the change from acetate to chloride salts did not significantly affect the activity of the compounds. The minimal difference that it is possible to observe in the MIC values of the compounds is probably due to the fact that the acetate salt has a higher molecular mass than the chloride salt and therefore for the same mass concentration there will be more molecules of **16** than **17**. Furthermore **16** has, also acetic acid co-crystallized as shown above.

Cyclam derivatives with varying number of pendant arms were synthesized to assess the effect of the number of pendant arms. For this purpose, the pendant arm used in **16d** and **17d** was selected since it led to be the most active compounds. Besides that, since acetic acid is not strong enough to create acetate salts for tetra-substituted cyclams, chloride salts were used for the purpose of testing the effect of the number of pendant arms.

As discussed, mono, *trans*-di, and tetra-substituted cyclams and their chloride salts were synthesized. To test the cyclam with no substituent, a chloride salt of cyclam (**23**), was prepared. Since the tetra-substituted cyclam was not soluble in water its antimicrobial activity was not possible to be assessed.

For the remainder compounds tested, results show that the reduction of the number of pendant arms significantly reduced the antimicrobial activity. Furthermore, **23** had no antimicrobial activity within the range of concentrations towards the strains tested (**Annexes - Figure A2**). The MIC values determined for compound **6** were 235 µg/mL and 261 µg/mL (**Annexes - Table A9**) for *E. coli* and *P. aeruginosa*, respectively, and >512 µg/mL for *S. aureus* and *B. contaminans* (**Annexes - Figure A3**). **Figure 23** shows a decline in bacterial growth with the increasing of compound concentration *E.coli*. **Figure 23** also depicts the difference in activity between the compounds with none, one and two pendant arms. The results, obtained for *E.coli* and *P. aeruginosa* with compounds **6**, **17d** and **23,** show us that the number of pendant arms is important for the activity of the compound.

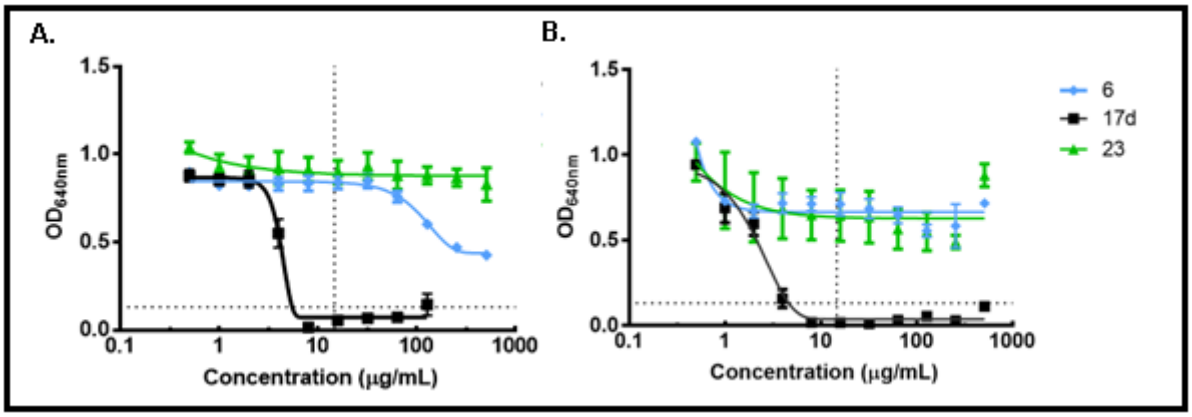


Figure 23- **Optical density of *E. coli* (A.) and *S. aureus* (B.) after incubation for 24h at 37ºC in the presence of the indicated concentrations** of 6, 17d or 23.

3.2.2 Bactericidal / Bacteriostatic assays for the *trans*-disubstituted cyclams

As described in the Material and Methods section, aliquots of the cultures in the microtiter plates, after 24h of incubation were taken from the wells, containing compounds at concentrations equal to the respective MIC value and from the wells with concentrations immediately below and higher than the MIC. These samples were serially diluted 1:10 until 10^{-7} and spot inoculated on LB solid medium followed by incubation for 18h. After incubation the CFUs were enumerated.

In this study only results from *E. coli* and *S. aureus* were presented since *P. aeruginosa* and *B. contaminans* seemed to be insensitive to the compounds. Also, the compounds with higher antimicrobial activity and probable candidates for the development of antimicrobial drug were selected. Therefore, the compounds **16c** and **17c** were not tested concerning their bactericidal or bacteriostatic activity. The results for these assays are presented in **Figures 24** and **25** for *E. coli* and *S. aureus*, respectively. The results suggest that these compounds have bactericidal activity since there was no growth at concentrations equal or higher than the MIC. The exceptions were the results obtained with compounds **16a** for *S. aureus* (**Figure 25-A.**) and **17c** for *E. coli* (**Figure 24-F.**), suggesting that they present a bacteriostatic activity. Giving that the other compounds behave as bactericidal, it is possible that some problems occurred during experiments. Further experiments are required to better characterize the antimicrobial activity of the compounds.

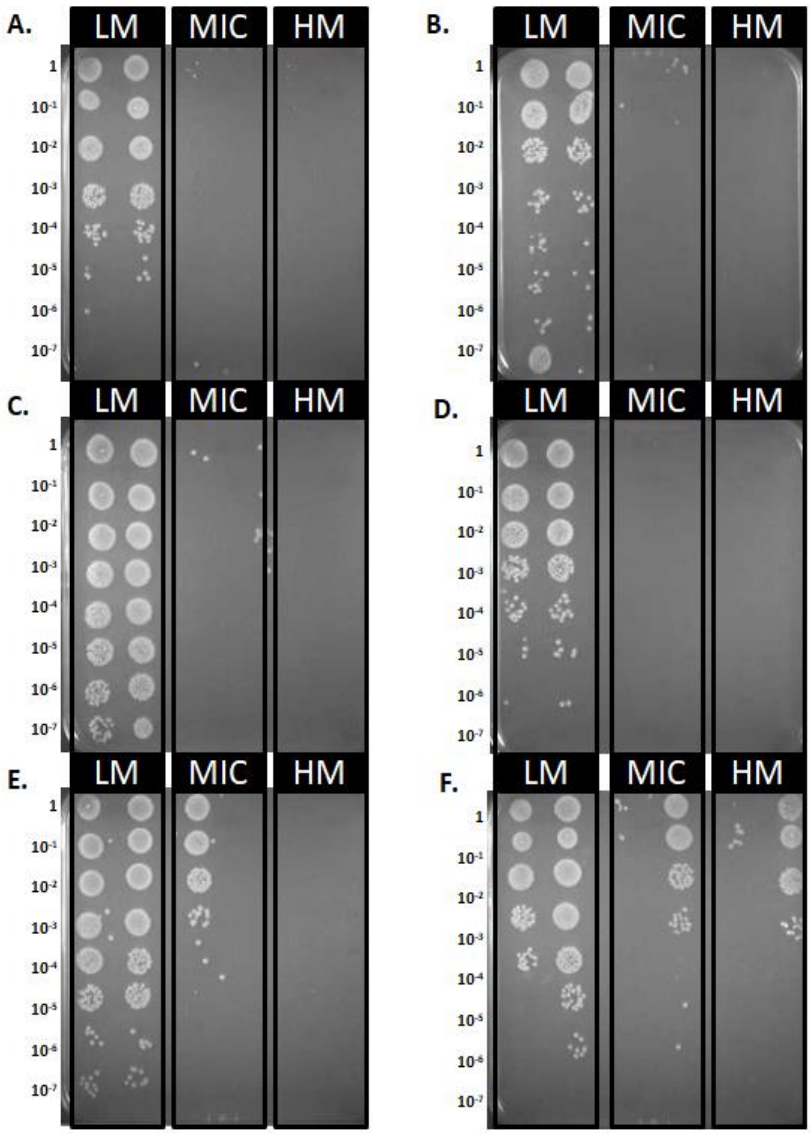


Figure 24- Photographs of spot inoculated plates to assess the bactericidal / bacteriostatic activity of compounds 16a (A.) 16b (C.) 16d (E.) and 17a (B.) 17b (D.) 17d (F.) towards ***E.coli*** for concentrations equal to the MIC, lower (LM) and higher (HM) than the MIC. Numbers indicate 1:10 serial dilutions.

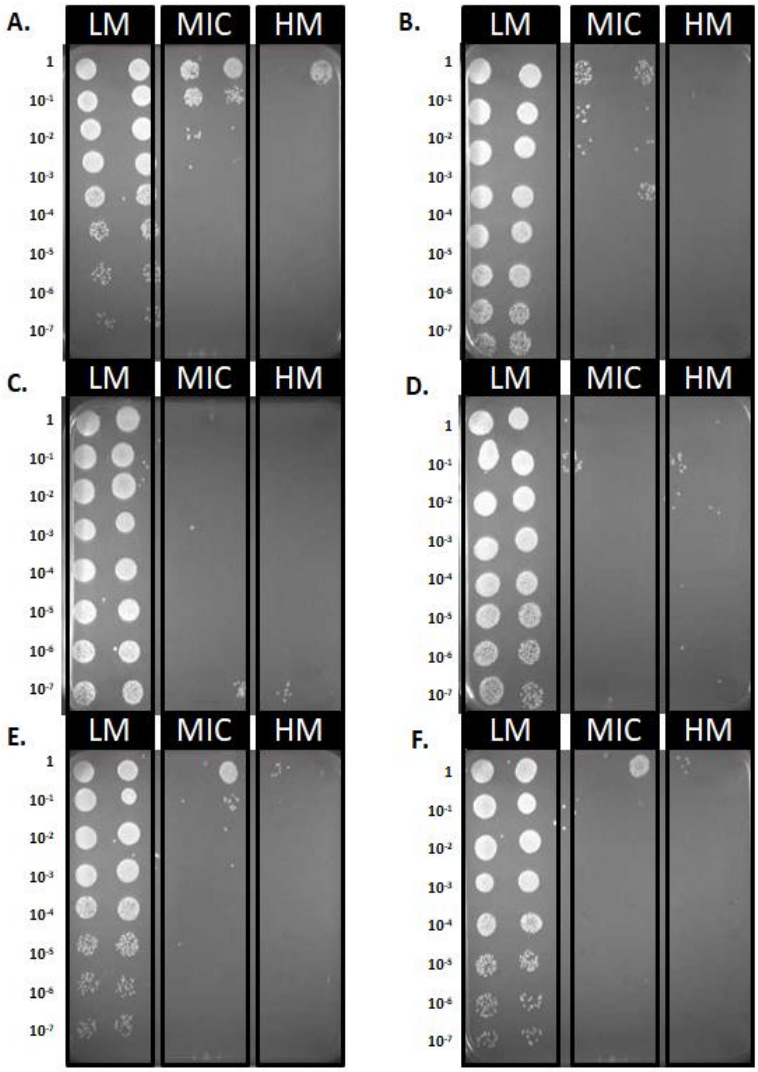


Figure 25- Photographs of spot inoculated plates to assess the bactericidal / bacteriostatic activity of compounds 16a (A.) 16b (C.) 16d (E.) and 17a (B.) 17b (D.) 17d (F.) towards ***S. aureus*** for concentrations equal to the MIC, lower (LM) and higher (HM) than the MIC. Numbers indicate 1:10 serial dilutions.

3.2.3 Spontaneous emergence of resistance to cyclam derivatives

Experiments were conducted to determine the frequency of spontaneous emergence of resistance, for the compounds **16b**, **16d**, **17b** and **17d**, which were found as the most active towards *E. coli* and *S. aureus*. These assays were made only for *E. coli* and *S. aureus* due to the lack of confidence in the MIC values determined for *P. aeruginosa*. As explained above for these assays, cells were incubated at 37ºC for a week in the presence of a concentrations of compounds equal to 4x the MICs determined.

In the case of experiments performed for *S. aureus* with compounds **16b**, **16d**, **17b** and **17d,** the initial CFUs used were too high, and a lane of bacteria capable of growing on the surface of the plates appeared after 24h of incubation. No individual colonies were observed. Further experiments are required with a lower number of initial cells.

In the case of experiments carried out for *E. coli* it was possible to use adequate number of initial CFUs. No colonies were obtained for compounds **16d** and **17d**. Since the plates were inoculated with a number of CFUs of 2.2 x10^{8} results obtained indicate a frequency of emergence of spontaneous resistance lower than 4.5 x10^{-9}. For compound **16b** some colonies were observed which indicate the growth of spontaneous resistant colonies. The frequency of resistance emergence for this compound and this strain was $\frac{13}{2.2 \text{ x} 10^{8}}$= 5.9 x$10^{-8}$.

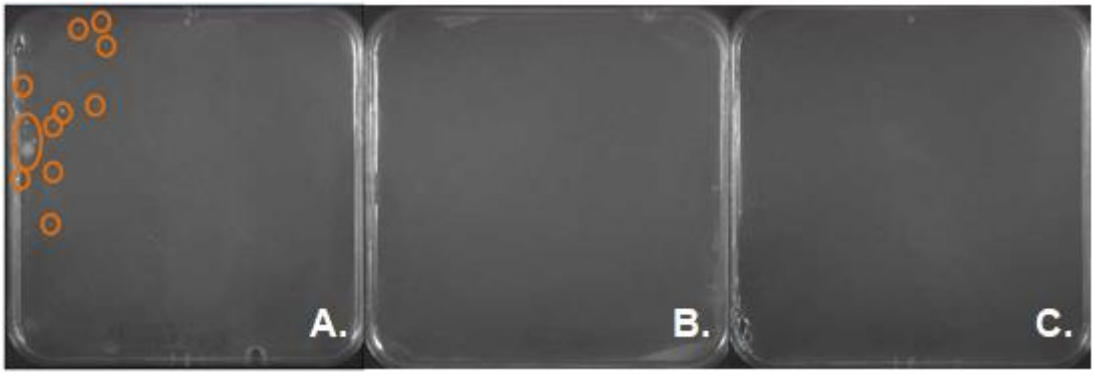


Figure 26- *E. coli* exposed to a concentration 4x MIC of 16b (A.), 16d (B.) and 17d (C.). 2.2 x10^{8} CFU were plated and incubated for 37ºC for a week.

Locke *et al*. in a study done in 2009, with *S. aureus* against compounds Linezolid (LZD) and Torezolid (TR-700) obtained a frequency of spontaneous resistance in the order of 10^{-9} and10^{-10}, respectively [73]. Despite the strain not being the same, the frequency of resistance obtained for *E. coli* to the compounds **16d** and **17d** are in the same order of values to the ones obtained by Locke *et al*. which suggests that the compounds are effective and should not lead to the development of resistance strains easily. As for compound **16b**, despite the value of frequency being a little higher it should not be excluded as a good candidate for drug development. This type of assays are a critical component for drug development since a compound for which bacteria exhibit a high frequency of spontaneous emergence of resistance does not constitute an appropriate therapeutic since bacteria can create resistance easily, requiring other therapeutics.

4. Conclusion and Future Perspectives

Cyclam derivatives were recently shown by Alves *et al.* as a new family of compounds with antimicrobial activity[56]. In particular, these authors showed that a cyclam derivative with a CF_3 group bound to a benzyl moiety presented MIC values of 9, 261 and 15µg/mL towards *E. coli*, *P. aeruginosa* and *S. aureus*, respectively.

In this work, cyclam derivatives with the CF_3 group bound to a benzyl moiety and replaced by CH_3 (compound **16a** and **17a**), by CH_2CF_3 (compound **16b** and **17b**), or substituted with only one benzyl group (compound **16c** and **17c**) were synthesized. These cyclam derivatives were conceived to gain knowledge on the role played by the fluorine presence and position on the antimicrobial activity of the molecule. In addition, compounds were prepared as acetate salts (**16**) or chloride salts (**17**) to investigate any possible role of the counter ion in the compound's activity.

The studies revealed in first hand that the anion do not change the activity of the compounds as minor differences were observed between an acetate (**16**) and chloride salts (**17**). These minor differences can be explained by the different molecular mass of the compounds and also by the error window of the assays. Since the synthesis of chloride salts were simpler and led to higher yields, these salts are better candidates for mass production. In addition, acetate salts are hygroscopic turning the quantification of cyclam derivatives acetate salts more difficult.

The work developed showed the importance of fluorine atoms in the pendant group since the compounds without fluorine had lower antimicrobial activity, as indicated by the higher MIC values for *E. coli* and *S. aureus*. The CF_3 group alone (**16b** and **17b**) or linked by CH_2 to the same carbon (**16d** and **17d**) on the antimicrobial activity of compounds were also investigated. The differences in MIC values obtained for **16d**, **17d** and **16b**, **17b** suggest that the space between the CF_3 moiety and the remaining of the molecule did not affect activity, at least when the space corresponds to 1 carbon atom. To further study the effect of spacing between CF_3 moiety and the phenyl ring it would be interesting to synthesize a compound with a larger spacer. As for the other two *trans*-substituted cyclams, the results show the importance of a methyl moiety attached to the phenyl ring since **16c** and **17c** had the lower activity towards both *E. coli* and *S. aureus*.

During this work, the determination of MIC values for *P. aeruginosa* was more difficult to achieve than for the other bacterial strains. Furthermore, it should be stated that there is a lack of confidence about the results presented, since variations in results were obtained. The observation of the microtitter plates showed that *P. aeruginosa* did not growth in concentrations higher than 256 µg/mL for **16d**, **17d** and **16b**, **17b** while growth were observed for all the other compounds, at all concentrations tested. This might be the result of the expression of several efflux pumps encoded in the organism's genome, or due to subpopulations with different levels of resistance to the compounds tested. It is also possible that the Gompertz model is not the best to determine MIC values for *P. aeruginosa* since the organism presents a slow decrease of OD with increasing concentrations of compounds.

The present work also shows that the number of pendant arms impacts the antimicrobial activity of the cyclam derivatives. The results obtained thus far allow to conclude that the activity decreases with the decrease of pendant arms and vice-versa. To further complete this study, the synthesis of a tri-substituted cyclam could give interesting results and further confirm the hypothesis made. In that scope, the tetra-substituted compound was synthesized. However the compound was not soluble and could not be tested. It is possible that the complexation of the tetra-substituted cyclam with a metal ion could turn the compound more soluble in water and make possible the characterization of its antimicrobial activity.

During this work attempts to synthesize a *trans*-disubstituted cyclen were carried out, but that was not possible. The synthesis of the *trans*-substituted cyclen with the particular pendant arm studied in this work seems to be complicated. Therefore *trans*-substituted cyclens are probably more easily synthesized with other pendant arms with a more efficient reaction. This is shown by the formation of **25** that was achieved fairly easy. Since compound **25** resemble a well known class of antimicrobials, the sulfonamides, in future studies its antimicrobial activity should be studied. Furthermore, the synthesis of the non-substituted cyclen was achieved with a very low yield turning it in an expensive antimicrobial for mass production. Despite that, future work should encompass the synthesis of cyclen derivatives and the characterization of their antimicrobial activity. Such results are expected to further the knowledge on the relationship between the size of the macrocycle and its antimicrobial activity. This discovery could potentially give rise to novel families of compounds with enhanced antimicrobial activities.

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Annexes

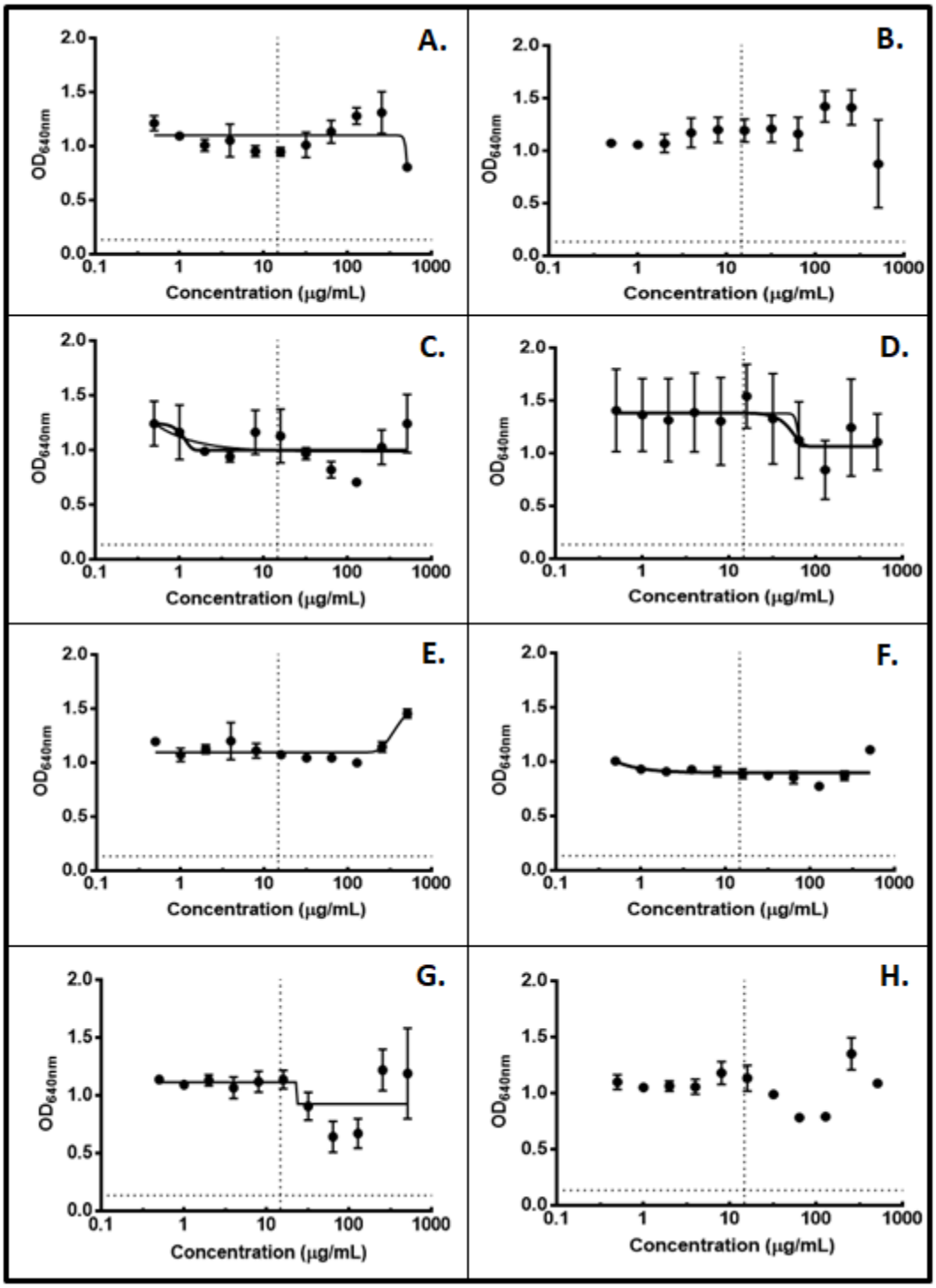


Figure A 1- Optical densities 640nm adjusted to Gompertz model to determine MIC values for *B. contaminans*: **A.16a**, **B.17a**, **C.16b**, **D.17b**, **E.16c**, **F.17c**, **G.16d** and **H.17d**.

Table A 1- Table of results from Gompertz model adjust for compound **16a** for *E. coli*, *S. aureus*, and *P. aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**55 µg/mL**	**49 µg/mL**	**9 µg/mL**
	Slope	2.583	3,205	3,559
	Bottom	0,03552	0,07873	0,2067
	Span	0,8672	0,7823	0,3779
95%Confidence Intervals	**MIC**	36,35 to 83,04 µg/mL	32,87 to 72,70 µg/mL	5,133 to 15,19 µg/mL
	Slope	1,626 to 3,541	1,872 to 4,538	0,8201 to 6,298
	Bottom	-0,01836 to 0,08939	0,02594 to 0,1315	0,1777 to 0,2357
	Span	0,7653 to 0,9692	0,6933 to 0,8713	0,2845 to 0,4713
Goodness of fit	**R^2**	0,9918	0,9903	0,9738

Table A 2- Table of results from Gompertz model adjust for compound **16b** for *E. coli*, *S. aureus*, and *P. aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**10 µg/mL**	**10 µg/mL**	**164 µg/mL**
	Slope	7,956	8,025	0,8965
	Bottom	-0,01253	0,06089	0,04203
	Span	0,9072	0,8179	0,7304
95%Confidence Intervals	**MIC**	8,995 to 11,74 µg/mL	8,189 to 11,23 µg/mL	12,67 to 2118 µg/mL
	Slope	4,549 to 11,36	3,770 to 12,28	-0,3786 to 2,172
	Bottom	-0,04245 to 0,01740	0,02068 to 0,1011	-0,1227 to 0,2068
	Span	0,8526 to 0,9619	0,7445 to 0,8913	-0,02598 to 1,487
Goodness of fit	**R^2**	0,9964	0,9921	0,9560

Table A 3- Table of results from Gompertz model adjust for compound **16c** for *E. coli*, *S. aureus*, and *P. aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**80 µg/mL**	**206 µg/mL**	**19 µg/mL**
	Slope	4,296	1,983	3,572
	Bottom	0,1782	0,3378	0,2534
	Span	0,6254	0,5408	0,4219
95%Confidence Intervals	**MIC**	50,08 to 128,0 µg/mL	67,45 to 630,6 µg/mL	11,85 to 30,60 µg/mL
	Slope	1,698 to 6,893	0,6615 to 3,304	1,458 to 5,685
	Bottom	0,1073 to 0,2490	0,2361 to 0,4395	0,2212 to 0,2856
	Span	0,5307 to 0,7202	0,3918 to 0,6899	0,3530 to 0,4909
Goodness of fit	**R^2**	0,9781	0,9676	0,9823

Table A 4- Table of results from Gompertz model adjust for compound **16d** for *E. coli*, *S. aureus*, and P. *aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**7 µg/mL**	**8 µg/mL**	**15 µg/mL**
	Slope	11,37	6,764	3,572
	Bottom	0,02018	0,04032	0,2534
	Span	0,9094	0,8346	0,4219
95%Confidence Intervals	**MIC**	4,729 to 11,22 µg/mL	4,635 to 11,81 µg/mL	11,85 to 30,60 µg/mL
	Slope	2,248 to 20,50	1,254 to 12,27	1,458 to 5,685
	Bottom	-0,02862 to 0,06899	-0,01836 to 0,09899	0,2212 to 0,2856
	Span	0,8227 to 0,9962	0,7147 to 0,9544	0,3530 to 0,4909
Goodness of fit	**R^2**	0,9910	0,9824	0,9823

Table A 5- Table of results from Gompertz model adjust for compound **17a** for *E. coli*, *S. aureus*, and *P. aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**43 µg/mL**	**119 µg/mL**	**25 µg/mL**
	Slope	3,590	5,809	1,407
	Bottom	0,02996	0,007286	0,1525
	Span	0,8806	0,6364	0,5773
95%Confidence Intervals	**MIC**	25,55 to 73,36 µg/mL	34,74 to 409,2 µg/mL	11,80 to 52,48 µg/mL
	Slope	1,396 to 5,784	-4,781 to 16,40	0,2484 to 2,565
	Bottom	-0,05518 to 0,1151	-0,1800 to 0,1946	0,1144 to 0,1905
	Span	0,7415 to 1,020	0,4075 to 0,8653	0,2573 to 0,8973
Goodness of fit	**R^2**	0,9796	0,8954	0,9786

Table A 6- Table of results from Gompertz model adjust for compound **17b** for *E. coli*, *S. aureus*, and *P. aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**16 µg/mL**	**~ 18 µg/mL**	**20 µg/mL**
	Slope	5,203	~ 31,73	2,640
	Bottom	-0,005767	-0,01363	0,2343
	Span	0,9433	0,6648	0,4684
95%Confidence Intervals	**MIC**	13,67 to 19,40 µg/mL	(Very wide)	5,475 to 70,85 µg/mL
	Slope	3,814 to 6,591	(Very wide)	-1,049 to 6,329
	Bottom	-0,03725 to 0,02571	-0,1473 to 0,1201	0,1573 to 0,3112
	Span	0,8874 to 0,9993	0,4643 to 0,8654	0,2376 to 0,6993
Goodness of fit	**R^2**	0,9968	0,9091	0,9134

Table A 7- Table of results from Gompertz model adjust for compound **17c** for *E. coli*, *S. aureus*, and *P. aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**124 µg/mL**	**80 µg/mL**	**26 µg/mL**
	Slope	2,982	2,918	2,841
	Bottom	0,1481	0,4765	0,2313
	Span	0,6984	0,3657	0,4526
95%Confidence Intervals	**MIC**	69,07 to 225,7 µg/mL	28,50 to 226,1 µg/mL	19,06 to 36,77 µg/mL
	Slope	1,413 to 4,551	0,1252 to 5,710	1,855 to 3,828
	Bottom	0,06641 to 0,2298	0,4093 to 0,5438	0,2104 to 0,2522
	Span	0,5865 to 0,8103	0,2623 to 0,4692	0,4036 to 0,5017
Goodness of fit	**R^2**	0,9808	0,9433	0,9938

Table A 8- Table of results from Gompertz model adjust for compound **17d** for *E. coli*, *S. aureus*, and *P. aeruginosa*.

		E. coli	***S. aureus***	***P. aeruginosa***
Best-fit Values	**MIC**	**5 µg/mL**	**5 µg/mL**	**70 µg/mL**
	Slope	13,66	3,587	1,036
	Bottom	0,1004	0,03613	0,02769
	Span	0,7656	0,9200	0,7935
95%Confidence Intervals	**MIC**	1,707 to 13,90 µg/mL	3,355 to 7,964 µg/mL	13,04 to 373,8 µg/mL
	Slope	-59,75 to 87,06	0,9360 to 6,239	-0,3643 to 2,437
	Bottom	0,02384 to 0,1769	-0,01664 to 0,08889	-0,08557 to 0,1409
	Span	0,6105 to 0,9207	3,355 to 7,964	0,005020 to 1,582
Goodness of fit	**R^2**	0,9707	0,9789	0,9499

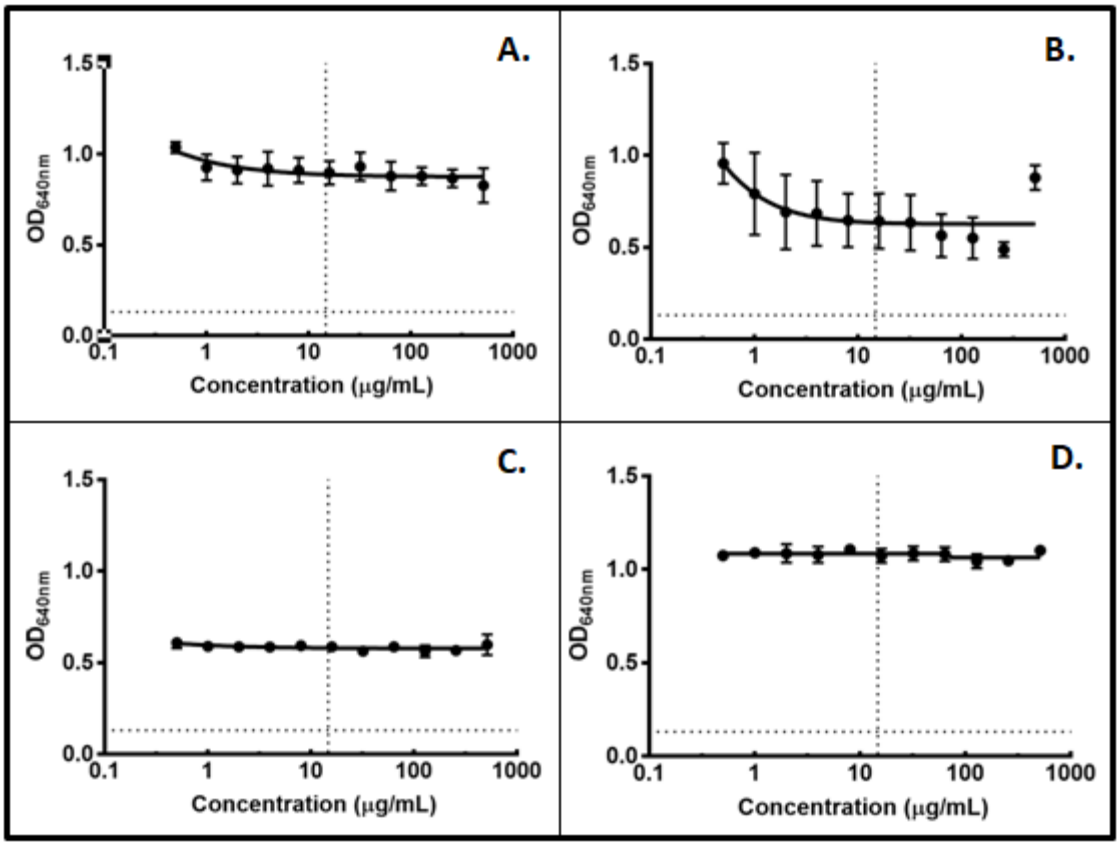


Figure A 2- Optical densities 640nm adjusted to Gompertz model to determine MIC values for compound **23** for the strains: *E. coli* **(A.)**, *S. aureus* **(B.)**, *P. aeruginosa* **(C.)** and *B. contaminans* **(D.)**

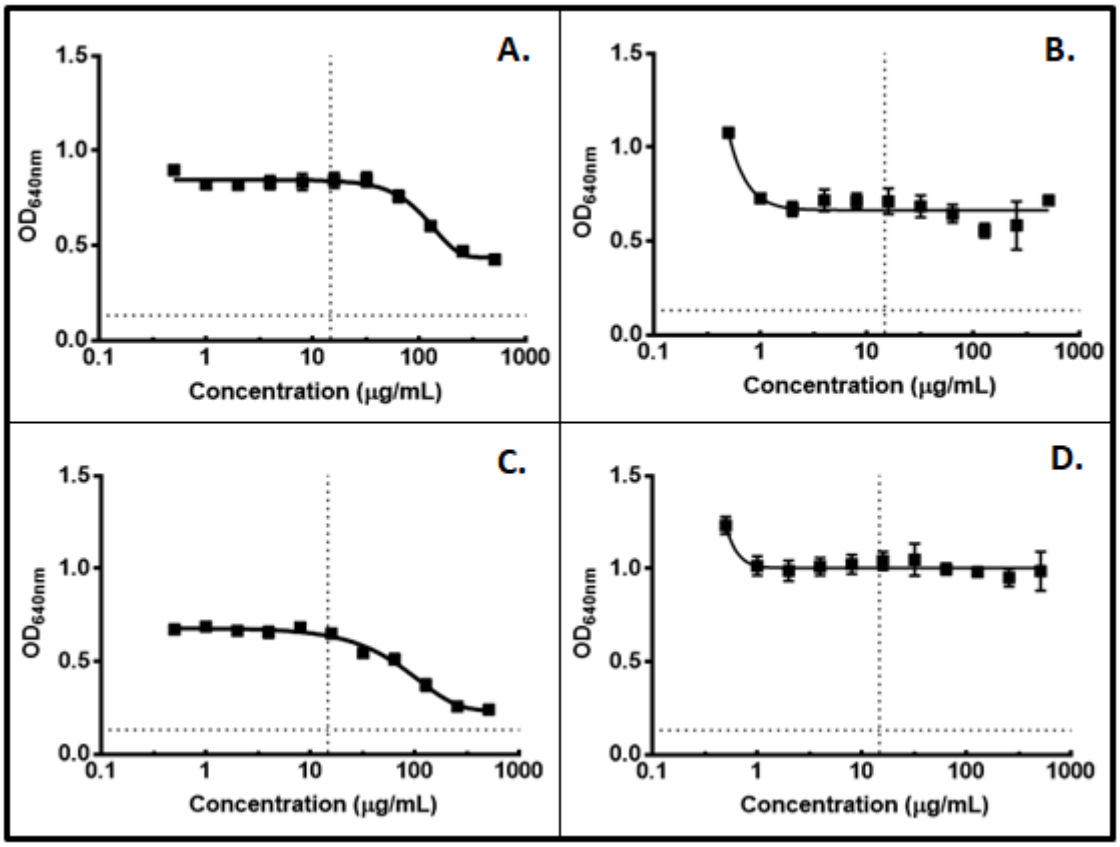
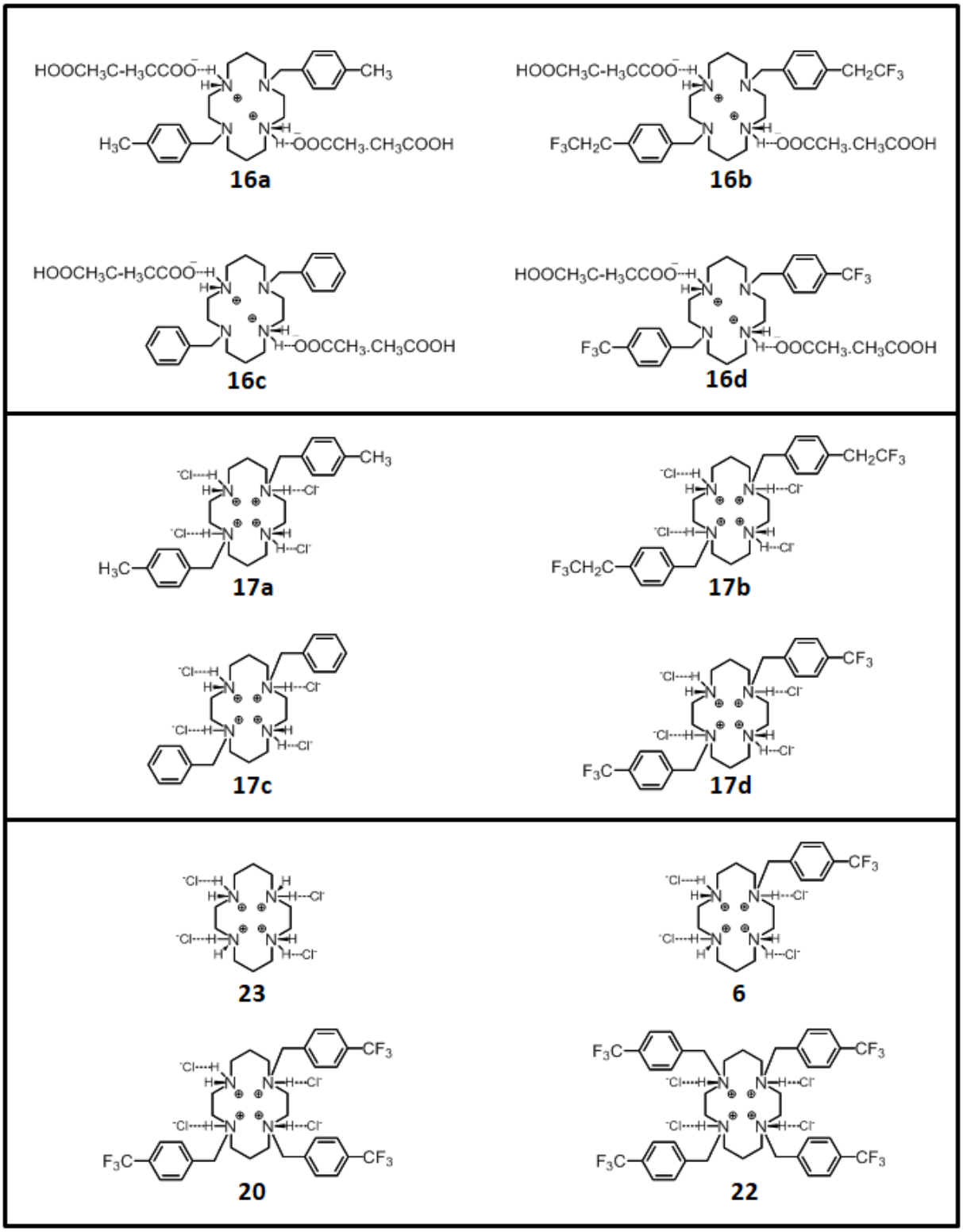


Figure A 3- Optical densities 640nm adjusted to Gompertz model to determine MIC values for compound **6** for the strains: *E. coli* **(A.)**, *S. aureus* **(B.)**, *P. aeruginosa* **(C.)** and *B. contaminans* **(D.)**

Table A 9- Table of results from Gompertz model adjust for compound **6** for *E. coli*, *and P. aeruginosa*.

		E. coli	***P. aeruginosa***
Best-fit Values	**MIC**	**235 µg/mL**	**261 µg/mL**
	Slope	4,378	2,657
	Bottom	0,4368	0,2345
	Span	0,4088	0,4440
95%Confidence Intervals	**MIC**	141,5 to 388,6 µg/mL	157,2 to 431,7 µg/mL
	Slope	1,820 to 6,937	1,705 to 3,609
	Bottom	0,3787 to 0,4949	0,1837 to 0,2853
	Span	0,3416 to 0,4759	0,3821 to 0,5059
Goodness of fit	**R^2**	0,9801	0,9891

Detachable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16c
Cl
17a
17b
17c
17d
23
6
20
22