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- CICLO XXXI -

POLYALKYLGUANIDINES: NEW WEAPONS TO TACKLE BACTERIAL RESISTANCE

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To Antonietta and Pasquale
Miles away but always close to me

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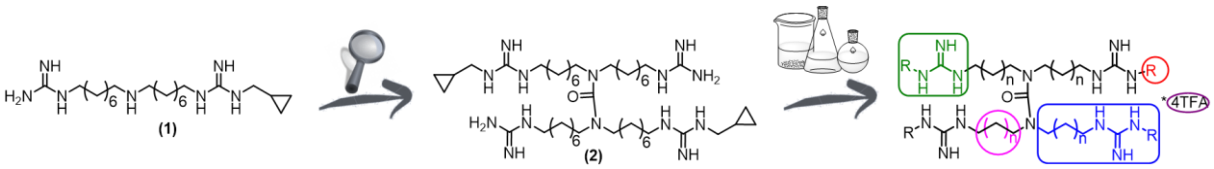
Abstract

In this historical period known as the antibiotic crisis era, the ever faster rise of bacterial strains resistant to the clinically used antibiotics along with the scientific research silent gap in the antibacterial field is treating seriously to the worldwide public health. Hence, we urgently need to develop new antibacterials agents with an innovative mode of action, able to trick the mechanisms of the pathogen resistance.
In this alarming frame, aware of the antibacterial properties of guanidine moieties, Prof. M. Botta and his research group have evaluated the biological activity of a linear polyalkylguanidino series, synthesized for different medicinal purpose, toward a panel of bacterial microorganisms. Only one compound (**1**) emerged to have an interesting broad-spectrum antibacterial activity. Later, the serendipitous discovery that the test batch of compound **1** was actually a mixture of oligomers led us to identify the chemical structure of the main component, dimer **2**, which was the responsible for the activity. From its scaffold, we designed and synthesized a small library of analogs to make some preliminary consideration on the pharmacophores with the aim of improving the selectivity index and studying the mode(s) of action.

Riassunto

In questo periodo storico, noto come era della crisi antibiotica, la sempre più rapida insorgenza di ceppi batterici resistenti agli antibiotici usati in clinica e la silenziosa stasi della ricerca scientifica nel campo antibatterico stanno seriamente minacciando la salute pubblica mondiale. Diventa, quindi, necessario sviluppare nuovi antibatterici con meccanismo d'azione innovativo, che possano raggirare i meccanismi di resistenza creati dal patogeno.
In questo scenario allarmante, consapevoli dell'attività antibatterica delle guanidine, il Prof. M. Botta e il suo gruppo di ricerca hanno valutato l'attività biologica di una serie di composti lineari a struttura poliachilguanidinica, sintetizzati con altri propositi farmaceutici, su un pannello di batteri. Solamente il composto **1** è risultato avere un interessante profilo antibatterico ad ampio spettro d'azione. Successivamente, la scoperta casuale che il campione analizzato era in realtà una miscela di oligomeri ci ha portato ad identificare la strttura chimica del componente principale, il dimero **2**, che era responsabile dell'attività. Partendo dal suo scaffold, abbiamo disegnato e sintetizzato una piccola libreria di composi per poter fare delle considerazioni preliminari sui farmacofori, con lo scopo di migliorare l'indice di selettività e studiare i meccanismi d'azione.



Graphical Abstract Content.

Abbreviations

ADME, Absorption, Distribution, Metabolism, Excretion; **AGP**, α1-acid glycoprotein; **AIFA**, Italian Italian Agency of Drugs; **AMP**, antimicrobial peptide; **ATCC**, American Type Culture Collection; **BCRP**, Breast Cancer Resistance Protein; **BCS**, Biopharmaceutics Classification System; $\mathbf{B_{max}}$, maximum number of binding sites; **Boc**, *tert*-butyloxycarbonyl; **BSA**, Bovine Serum Albumin; **Cbz**, carboxybenzyl; **CCUG**, Culture Collection of University of Gothenburg; **CFU**, Colony Forming Unit; **CLSI**, Clinical Laboratory Standards Institute; **CV**, Crystal Violet; **DCC**, *N,N'*-dicyclohexylcarbodiimide; **DCM**, dichloromethane; **DCU**, dicyclohexylurea; **DIBAL(H)**, diisobutylaluminium hydride; **DIPEA**, *N,N*-Diisopropylethylamine; $\mathbf{DISC_3}$**-5**, 3,3'-dipropylthiacarbocyanine; **DMAP**, 4-dimethylaminopyridine; **DMF**, *N,N*-Dimethylformamide; **DMSO**, *N,N*-Dimethylsulfoxide; **DMS**, dimethyl sulfate; **DMSO**, *N,N*-dimethylsulfoxide; **DNP**, dinitrophenol; **ESBL**, extended-spectrum beta-lactamase; **ESKAPE**, *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species; **FBS**, fetal bovine serum; **FDA**, Food and Drug Administration; **HPLC**, high performance liquid chromatography; **HS**, Human Serum; **HSA**, Human Serum Albumin; **HeLa** cells, cells of Henriette Lacks; $\mathbf{K_d}$, ligand concentration that binds to half the receptor sites at equilibrium; **LDH**, lactate dehydrogenase; **LC**, Liquid Chromatography; **LAH**, lithium aluminum hydride; **logD**, distribution constant; **logP**, partition constant; **LPS**, Lipopolysaccharide; **MBC**, Minimal Bactericidal Concentration; **MHB**, Mueller Hinton Broth; **MIC**, Minimal Inhibition Concentration; **MoA**, Mechanism Of Action; **mPAO**, Maize Polyamine-Oxidase; **MRP2**, Multidrug Resistance Protein 2; **MRSA**, Methicillin-Resistant *Staphylococcus aureus*; **MS**, Mass Spectrometry; **NHS**, National Health Service; **NMR**, Nuclear Magnetic Resonance; **NPN**, 1-*N*-phenylnaphthylamine; **ONP**, *o*-nitrophenol; **ONPG**, *o*-nitrophenyl-β-D-galactoside; **PAMPA**, Parallel Artificial Membrane Permeability Assay; **PAO**, Polyamine Oxidase; $\mathbf{P_{app}}$, apparent permeability; **P-gp**, P-glycoprotein; **pKa**, acid dissociation constant; **RFU**, relative fluorescence unit; **SAR**, Structure-Activity Relationship; **SD,** standard deviation; **SDS**, sodium dodecylsulfate; **TEA**, *N,N, N*-triethylamine; **TFA**, trifluoroacetic acid; **THF**, tetrahydrofuran; **TLC**, thin layer chromatography; **TRIS**, tris(hydroxymethyl)aminomethane; **VRE**, Vancomycin-Resistant *Enterococci*; **VRSA**, Vancomycin-Resistant *Staphylococcus aureus*; **WHO**, World Health Organization.

Introduction

1. The pathogens: bacteria.

1.1. Bacterial cell.

Bacteria are unicellular microorganisms, characterized by small size (0.5-6 μm x 2 μm) and weight (1 pg, with an estimated dry mass of 0.2 pg). They are prokaryotic cells endowed with an anatomy that is different from the eukaryotic ones and is summarized in **Figure 1**.

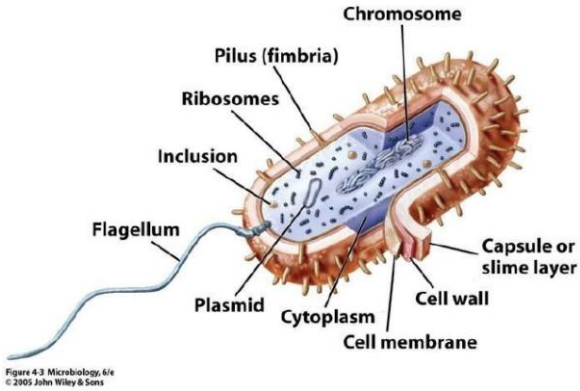


Figure 1. Bacterial cell anatomy.

In brief, bacterial cells are surrounded by a selectively permeable *cell membrane* mostly made of phospholipids, proteins, and, in lieu of eukaryotic sterols, hopanoids, whose function is the stabilization of membrane structure. The integrity of cell membranes is essential to create concentration gradients and charge separation, also known as proton motive force. This latter is required for producing the energy used to uptake nutrients and perform metabolic processes.[1] None of the *organelles* of eukaryotic cells are present in bacteria, such as nucleus and mitochondria. Small *ribosomes* are found in the cytoplasm and are the site of protein synthesis. They are referred to 70S and consist of a 50S larger unit and a smaller 30S subunit. The bacterial *genome* is a haploid DNA and is typically a single, double-stranded, circular chromosome compacted in an unspecific cytoplasmatic area called *nucleoid*. Accessory mobile genomic material called *plasmid* can be present in the cell and it consists of a double-stranded circular DNA, able to replicate independently inside the host and is responsible for resistant gene transfer. Also, bacteria are provided of a capsule or *envelope*, a complex and dynamic structure composed by a *peptidoglycan* mesh-like layer, characterized by repeating units of the disaccharide *N*-acetylglucosamine β-1,4-*N*-acetylmuramic acid and tetrapeptide stems, coupled through glycosidic bonds to form linear strands.[2] Differences in the bindings and thickness of peptidoglycan are recorded for bacterial species. In the end, some bacteria are surrounded an outer membrane and a dense array of negatively charged polymers, the lipopolysaccharides (LPS), composed of a lipid A portion and a core oligosaccharide linked to an *O*-antigen polysaccharide side chain.
Moreover, bacteria often secrete sticky materials on their surface or in the extracellular environment. Some of the most relevant ones are the *capsule*, a rigid, thick matrix of polysaccharides that allows the adhesion of bacteria to surfaces and is involved in bacterial toxicity and biofilm formation and the *slime* layer, an easily removable external structure. Most of bacteria can also produce the *biofilm*, a complex microbial architecture composed by self-produced extracellular polymers (polysaccharides, extracellular DNA and proteins) that allow bacteria to stick to surfaces, to form aggregates or micro-colonies and to become more resistant to antibacterial treatments, harsh environments, and human immunity. Also, accessory protein filament, called *fimbriae* and *pili*, have a role in enabling bacterial adhesion to surfaces. Pili are longer than fimbriae and are also involved in genetic exchange among microorganisms but also in cell motility.[1]

1.2. Classification of bacteria.

Bacteria are characterized by enormous biodiversity. In fact, they can be classified for their morphology (cocci as *Staphylococcus aureus*, bacilli as *Escherichia coli*, spirillia as *Helicobacter pylori* and several other shapes); their life conditions (necessity of oxygen: anaerobes, aerobes, capnophilics, aerotolerants; pH environment: acidophiles, neutralophiles alkaliphiles; temperatures: psychrophiles, mesophiles, thermophiles; presence of salts: halophiles, non-halophiles); their metabolism (autotrophs or heterotrophs, lactose-fermenters or no, reductive or oxidative metabolism) and their characteristic features (pigment production, growth rate, presence of specific enzymes).[3,4] However, the main classifications among bacteria are three. First of all, the behavior towards humans: if they are microbiota, commensal, pathogenic or no. Then, the response to antibiotics: if they are susceptible or resistant for nature or for acquired mechanisms. In the end, the structural differences in the cell wall: it is absent in mycoplasma, is composed by ether-linked lipids and a pseudopeptidoglycan in archaeobacteria and it has peptidoglycan and ester-linked lipids in standard bacteria. These latter have been in-depth studied by Hans C. Gram who invented a staining protocol that is able to make an important subclassification. Microorganisms that are stained during the procedure were named Gram-positive, the others Gram-negative bacteria. The first ones are stained by crystal violet, a primary stain that is retained also after the treatment with a decolorizer, while the Gram-negative strains lost the purple color and are stained by safranin, another lighter dye. This different behavior is caused by the architecture and composition of their cell wall: Gram-positive bacteria retain crystal violet stain due to their ubiquitous and thick multilayered peptidoglycan (30-100 nm), while Gram-negative strains do not, since they have a thinner peptidoglycan (few nanometers) and an additional outer lipid membrane (**Figure 2**).[3]

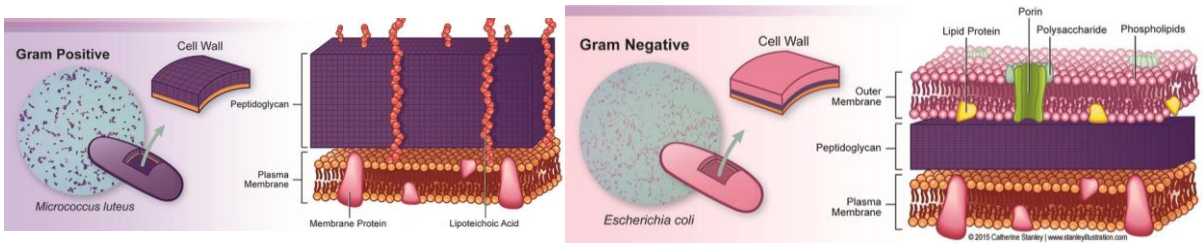


Figure 2. Comparison of Gram-positive (left) and Gram-negative (right) cellular membranes.

To make clearer and simplify the reading of this work, Gram-positive strains are always reported in violet and Gram-negative bacteria in purple.

1.3. Bacterial infection pathogenesis.

Human and animal bodies are not sterile and are always exposed to countless microbes. Many of them grow on and in it, without provoking any pathologies, and are known as microbiota or normal commensals, are beneficial, but others are an actual threat to the health.[3]

Microbial pathogenesis is referred both to the bacterial infection mechanisms and to the mechanisms by which the disease can develop. It begins with the exposure to the pathogen, its adhesion to host cells, invasion, colonization, growth, and host damage. The first step of infection is the attack on the host by a pathogen, which adheres to the host, invade his tissue and multiply. In general, the host can be more or less susceptible to the infection, according to his immunity and the bacterium features and properties. The host physiologic and immunologic conditions can cause defense mechanisms against the pathogen. Healthy individuals are less prone to be infected than individuals with defective immune responses, such as infants, elder people, immunosuppressed (cancer chemotherapy-receiving, AIDS) or post-trauma patients.

Moreover, each bacterium has its own ability to cause disease and this is the so-called virulence, based on the production of toxins, the presence of surface cell coats that inhibit phagocytosis, and surface receptors that bind to host cells. Some examples are fimbriae and pili, capsules and biofilm. The entry into the host body, the initiation of the pathogenesis, occurs through adhesion to mucosal membranes, such as skin, oral cavity, respiratory, gastrointestinal and urogenital tracts that represent some of the most important barriers of the body to the microbes. After the adhesion to the host, the bacterium invades the tissues, multiplying and colonizing them. Then, bacteria move by the lymphatic circulation, being tackled by the host immunity or remain localized in the entry site. This is the actual process of the infection. Some bacteria are able to enter the blood, causing bacteremia and generating a systemic infection, the septicemia, leading to a massive inflammation, septic shock, and even death. During this phase, bacteria can exert their toxicity towards the host through the release of preformed exotoxins, endotoxins or lytic enzymes, that are virulence factors responsible for inducing fever and inflammation since they cause the production of cytokines and endogenous pyrogens by host cells. The host response is based on nonspecific mechanisms, such as polymorphonuclear neutrophils and macrophage clearance, and specific immunity, developed later, such as the production of antibodies.

2. Antibiotics.

2.1. Definitions.

2.1.1. Antibiotic, antibacterial, chemotherapeutic

The term antibiotic was coined from the word *antibiosis* which literally means *against life* and was derived from the French words *antibiose* and *antibiotique* introduced by the mycologist Vuillemin in the late nineteenth century to explain the fight between microorganisms.[5] It was originally used in the English language as an adjective for *agent* or *activity* until 1947, when Selman A. Waksman, the discoverer of streptomycin and a pioneer in the screening of soils for the presence of biologicals, published a definition of *antibiotic* as a noun. His original definition described the microbes as organisms able to produce substances against other microbes: *An antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms.* In this definition, he excluded active molecules produced by plants, animals, and chemical syntheses. He continued with explaining the spectrum of action: *The action of an antibiotic against microorganisms is selective in nature*, *some organisms being affected and others not at all or only to a limited degree; each antibiotic is thus characterized by a specific antimicrobial spectrum.* In the end, he addressed the selectivity: an antibiotic is clinically useful if can kill pathogenic microbes without damaging host tissues: *The selective action of an antibiotic is also manifested against microbial vs. host cells.*[4]

Penicillin, discovered by Alexander Fleming and introduced during the Second World War, was the first antibiotic in the history and it was considered as a miracle drug but it was not called an antibiotic until the discovery of streptomycin by Selman A. Waksman, Albert Schatz and Elizabeth Bugie.

Contemporary usage makes no such distinction on the origin and nowadays *antibiotic* is often used as a synonym for *antibacterial*, including drugs produced partly or wholly through synthetic means, in order to simplify the communication. In fact, an authoritative, encyclopedic volume about antimicrobial agents, *Antimicrobial Agents*, *Antibacterials and Antifungals,* published by the American Society for Microbiology in 2005 wrote: *An antibiotic is a derivative produced by the metabolism of microorganism that possesses antibacterial activity at low concentrations and is not toxic to the host. The antibacterial agents obtained by total synthesis are synthetic antibacterial agents and not antibiotics. However, usage has meant that any substance possessing antibacterial activity that is not*

toxic to the host is referred to as an antibiotic.[5,6] Moreover, the World Health Organization (WHO) stated: *the term antibiotic is used as a synonym for antibacterial used to treat bacterial infections in both people and animals*.[5]

In the past, all antibacterial agents were antibiotics, molecules derived from the microbial origin. Starting from the discovery of arsphenamine by Paul Ehrlich, antibacterial infections could be treated through chemical compounds. Thus, there was a need for a new term to identify antibacterials from synthesis: *chemotherapeutic antibacterial*. In fact, Ehrlich introduced the concept of *chemotherapy*, referring to the use of a physiologically active chemical to selectively treat the disease while not damaging or only minimally harming the patient. In contemporary use, this chemotherapeutic agent usually describes a cytotoxic drug used in the treatment of malignant tumors.

2.1.2. Other commonly used terminologies.

Antimicrobials: substances that kill or inhibit the growth of microorganisms (fungi, bacteria, …). They include three subgroups:[5]

- *Antibiotics*, used to kill or inhibit microorganisms within the body.
- *Disinfectants*, used to kill or inhibit microorganisms on non-living objects. They are non-selective, being as toxic for human cells as they are for bacteria, so they cannot be used in humans or animals.
- *Antiseptics*, applied on living tissue to prevent infection. They are often from modifications of disinfectants, particularly by reducing their concentration. They are far less toxic and can be applied to the body surfaces, such as the skin and those areas where there is a low rate of adsorption. Despite their reduced toxicity, they are still too harmful for systemic use.

Anti-infectives: it includes the antivirals in the definition of antimicrobials.

Sterilization: killing microorganisms in liquid media or on solid objects by using chemical means such as oxidizing agents or physical means such as heat or high energy radiation.

Sanitizing agent: this term is used interchangeably with disinfectant, antiseptic and sterilizing agent.

Antibacterial: chemical that kills or inhibits the growth of bacteria. However, today it is often used synonymously with disinfectant as in *antibacterial soap.*

Chemotherapeutic: chemical, that's to say drug of synthetic origin, used to cure diseases, in particular, microbial infections. However, today this term usually refers to anti-cancer drugs.

2.2. The ideal antibiotic.

The ability to kill bacteria is a necessary but not sufficient condition for making *druggable* a compound. An antibiotic, to be referred to as *ideal*, should have the following other properties:[5,6]

- *Selectivity.* The antibiotic must kill or inhibit the infecting microorganism, causing minimum harm to the host cells. The selectivity can be achieved in two different ways: the antibiotic target is present in the infecting pathogen but not in the host or it is present in both bacterium and host but it is structurally or mechanistically different enough such that the antibiotic cannot affect the host. Thus, contrary to disinfectant, an antibiotic must have the essential property of selective toxicity that allows its systemic use in treating infections. This may be quantified by the *therapeutic* or *selectivity index*, which is the ratio of the toxic dose to the effective dose. In general, the larger the ratio the safer the drug. Some antibacterials have a very broad selectivity index and can be given in very high doses without toxic or side effects (such as penicillin), but others may produce serious toxicity at concentrations that are not much higher than those active that are required for treatment of infection (such as colistin). However, a completely safe antibiotic does not exist.
- *Few side reactions*, such as possible allergic reactions and negative interaction with food or other drugs.

- *Water solubility*. This feature is essential for effectiveness: the antibiotic must solubilize and be transported through body fluids (blood) to the infected sites.
-*Stability*: both shelf and biological. The antibiotic should have a long shelf life to be economically useful and storable. In fact, it should preferably be stable at room temperature, avoiding the need to be stored in a refrigerator. After the administration, it should remain unaltered in the body fluids for sufficient time to be able to carry out its action, being slowly degraded in the liver or eliminated through the urine unless the target is the urinary tract (such as for fosfomycin).
- Low cost for pharma industry and for patients.
- Slow resistance development.

2.3. Classification.

Antibiotics can be classified in several ways, according to target and mode of action, molecular structure, administration route (topical, oral or injectable) and spectrum of activity.
Based on this latter classification, we can distinguish *broad-spectrum* antibiotics, that treat a wide variety of infections, being active against many bacterial groups, and *narrow-spectrum* antibiotics, that are very specific and effective against only some selective bacterial strains. These two terms were coined in the 1950s for a comparative sense, without any clear definition.[7] Doctors usually prescribe a narrow-spectrum antibiotic when the infecting bacterium has been identified and it is more advantageous than the use of a broad-spectrum one because it is very selective and it does not kill nonpathogenic bacteria resident in the body (flora), not allowing superinfections and resistant bacteria selection. While broad-spectrum antibiotics are the best choice for empiric therapies when the treatment is urgently needed and the bacterium responsible for the disease has not been identified yet.
A distinction between the different mode of action results in *bacteriostatic*, *bactericidal* and *bacteriolytic agents* (**Figure 3**). A bacteriostatic inhibits replication of bacterial cells without decreasing their viable count. Thus, during the acute phase of the infection, the bacteriostatic merely provided control on bacterial multiplication and the cure should be provided by the patient's immunity system. A bactericidal agent leads to the death of the bacterium, without causing the lysis of the cells. In this case, the count of the total cells is constant, whereas the viable cells number decreased. It is actually defined as a drug that reduces the viable bacterial density by 99.9% in 18–24 hours. Hence, penicillins, vancomycin, and fluoroquinolones can be considered bactericidal for *S. aureus* and *S. pneumoniae* and bacteriostatic against *Enterococci*.[8] The bactericidal response can be dependent on the concentration of the antibiotic or on the time the bacteria are treated with the antibiotic. To understand this distinction, one needs to know what is *MIC*. It is the acronym for Minimum Inhibitory Concentration and it is the way to indicate the efficacy of an antibacterial since it is the lowest concentration able to inhibit the visible growth of a microorganism after overnight incubation. Moreover, another important value related to the activity is *MBC*, the Minimum Bactericidal Concentration, the lowest concentration of an antimicrobial to prevent the growth of a microorganism when subcultured on to antibiotic-free media. Usually, we refer to antibacterial behavior when the ratio between MBC and MIC is greater than or equal to 2. When the killing of bacteria is dependent upon the concentration of the drug, the most important factor is its absolute concentration, so one single large dose should be sufficient to eradicate all the pathogen. In this case, the rate of kill and the drug concentration are directly proportional. If administrating the drug is at concentrations above the MIC and the rate of kill is constant, the most important factor is the time of contact between drug and pathogen, so the drug has a time-dependent bactericidal action. A bacteriolytic agent can kill the microorganism, causing the lysis of the bacterial membrane, so the total and the viable cells numbers decrease. Bactericidal drugs are preferable to bacteriostatic ones because to reduce the bacterial numbers into the infection site

may decrease the risk of resistance onset. However, this distinction is mostly relative, since a bacteriostatic antibiotic can become bactericidal at higher concentration, whereas some bactericidal drugs may only be bacteriostatic under certain circumstances.[9] In general, antibiotics that target cell wall (penicillins and cephalosporins) or membranes (polymixins) have a bacteriolytic mode of action, the ones that interfere with key bacterial enzymes (quinolones and sulfonamides) are bactericidal, whereas those that inhibit protein synthesis (aminoglycosides, macrolides, and tetracyclines) are usually bacteriostatic.[10]

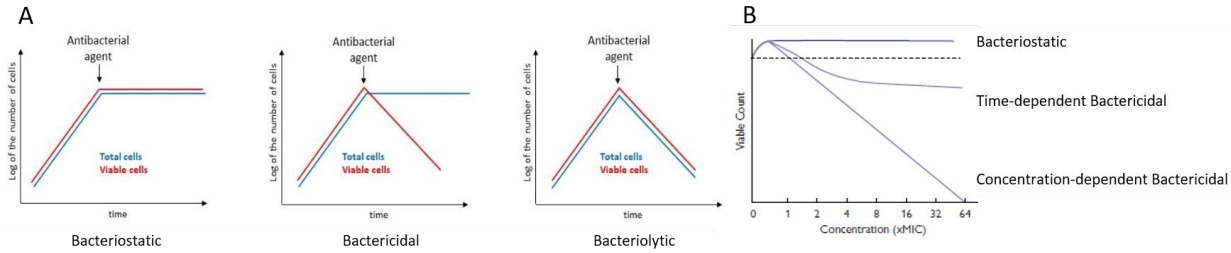


Figure 3. A. Total and viable cell counts for bacteriostatic, bactericidal and bacteriolytic agents. B. Dose-response for bacteriostatic and bactericidal mode of action. Images adapted from *Chemistry and related drugs*.[11]

2.4. Current treatments: an overview.

The most used way to classify antibiotics is based on their *biological targets* (**Figure 4**). Accordingly, herein the major modes of action:

- Inhibitors of bacterial cell wall synthesis;
- Disruptors of cellular membrane integrity;
- Inhibitors of DNA replication;
- Inhibitors of RNA transcription;
- Inhibitors of key metabolic pathways;
- Inhibitors of protein synthesis.

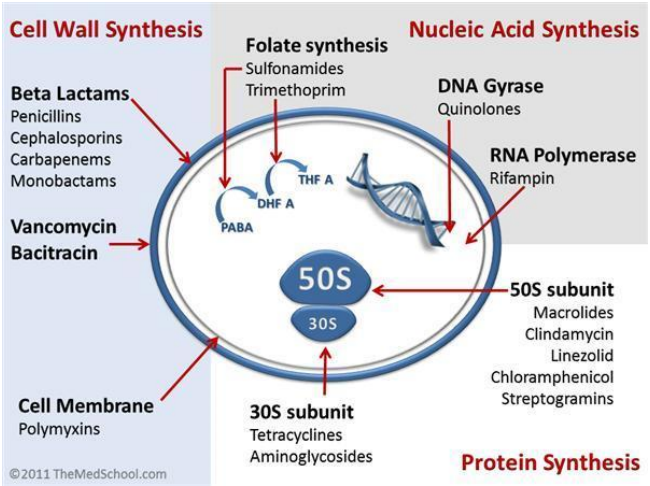


Figure 4. Main targets of antibiotic classes commonly used in therapy.[12]

Current and traditional antibiotics are organized for chemical classes, according to their chemical structure. A brief overview is here reported, highlighting the drugs chemical features, specific mechanism of action, resistance mechanism. Descriptions and details about each antibiotic class are not provided since they go beyond the scope of this research thesis.

- Inhibition of bacterial cell wall synthesis:

1) β-lactams.

Structure: four-membered β-lactam ring, azetidin-2-one.

Examples: Penicillins (Penicillin G), Cephalosporins, Carbapenems, Monobactams.

Spectrum of activity: broad.

Pharmacodynamics: suicide inhibitors of the penicillin-binding proteins (PBPs) transpeptidases, inhibiting the formation of peptidoglycan cross-links, bactericidal.

Resistance: hydrolysis of the β-lactam ring by β-lactamases, PBP modifications, expression of efflux pumps, downregulation of porins, alteration of outer membrane permeability.

Clinical use: combination therapy with β-lactamase inhibitors, such as clavulanic acid and sulbactam, first-line antibiotics.

2) Glycopeptides.

Structure: macrocyclic peptides with aromatic moieties and saccharide side chains.

Examples: Vancomycin and Teicoplanin.

Spectrum of activity: Gram-positive strains and *C. difficile.*

Pharmacodynamics: inhibition of transpeptidases and transglycosylases, preventing the synthesis of long polymers of N-acetylmuramic acid and N-acetylglucosamine and inhibiting the formation of peptidoglycan cross-links; depolarization of bacterial membranes, bactericidal.

Resistance: Formation of an altered peptidoglycan precursor with lower affinity for vancomycin (*vanA* and *vanB* resistant phenotype), re-organization of peptidoglycan biosynthesis.

3) Polypeptides.

Structure: non-protein polypeptide chain.

Examples: Bacitracin.

Spectrum of activity: narrow, Gram-positive strains.

Pharmacodynamics: Interference with the dephosphorylation of the C55-isoprenyl pyrophosphate.

4) Fosfomycin.

Structure: phosphonic acid derivatives.

Spectrum of activity: Gram-negative strains.

Pharmacodynamics: suicide inhibitor of phospho-enoyl-pyruvate transferase, an enzyme involved in. Inhibiting the *N*-Acetyl-muramic acid biosynthesis.

5) Cycloserine.

Structure: analogs of D-alanine.

Spectrum of activity: Gram-positive and Gram-negative strains and *Mycobacterium tuberculosis*.

Pharmacodynamics: inhibition of the enzyme alanine racemase and D-alanine ligase

- Disruptors of cellular membrane integrity:

1) Lipopeptides.

Structure: cyclic depsipeptides with a peptidyl side chain and a saturated alkyl tail

Examples: Daptomycin.

Spectrum of activity: Gram-positive strains.

Pharmacodynamics: insertion of their lipid tails into the cytoplasmic membrane, altering its curvature and creating holes that leak ions (potassium efflux), depolarization and disruption of structural integrity of membranes.

Resistance: upregulation of enzymes involved in cell membrane homeostasis, alteration of the target.

2) Polymyxins.

Structure: cyclic peptides with a hydrophobic tail.

Examples: Colistin.

Spectrum of activity: Gram-negative strains.
Pharmacodynamics: Binding to LPS through electrostatic interactions and displacement of divalent cations involved in the stabilization of the LPS membrane, impairment of the outer membrane integrity, release of LPS, changes in membrane permeability
Resistance: alteration of the charge properties of the outer membrane through expression of modified LPS, resulting in a decreased binding affinity; expression of efflux pumps.
Clinical use: last line antibiotics.

- Inhibition of nucleic acid biosynthesis:

1) Quinolones.
Structure: 4-oxo-1, 4-dihydroquinoline scaffold.
Examples: Ciprofloxacin.
Spectrum of activity: broad, Gram-negative and some Gram-positive strains.
Pharmacodynamics: Inhibition of DNA gyrase, binding to topoisomerase II, DNA gyrase and topoisomerase IV and intercalation into DNA, physically blocking DNA ligation and catalytic functions of DNA gyrase and topoisomerase IV, bactericidal.
Resistance: target modifications (acetylation), weakening the binding affinity to the targets; expression of protection proteins (*qnr* phenotypes), drug-inactivating acetylating enzymes, expression of efflux pumps and downregulation of porins.

2) Rifamycins.
Structure: macrocycles with aromatic moieties.
Examples: Rifampicin.
Spectrum of activity: most Gram-positive strains.
Pharmacodynamics: Inhibition of the bacterial DNA-dependent RNA polymerase, through the binding to the β subunit, bactericidal.
Resistance: target alteration, ADP-ribosylation, expression of efflux pumps.

3) Macrolactones.
Structure: modified macrolide structure.
Examples: Fidaxomicin.
Spectrum of activity: *C. difficile* and related infections.
Pharmacodynamics: inhibition of RNA polymerase, bactericidal.

4) Sulfonamides.
Structure: arylsulfonamide scaffold.
Examples: Sulphamethoxazole.
Spectrum of activity: Gram-negative bacilli, *Enterococcus.*
Pharmacodynamics: antimetabolite analogs of *p*-aminobenzoic acid (PABA) and competitive inhibitors for the enzyme dihydropteroate synthetase (DHPS). Blocking the tetrahydrofolic acid (THF) synthesis and DNA synthesis, bacteriostatic.
Resistance: expression of efflux pumps, target alteration through mutation of genes encoding for DPHS, overexpression of DHPS and DHFR.
Clinical use: combination therapies with 2,4-diaminopyrimidines (synergy).

5) Aminopyrimidines.
Structure: 2,4-diaminopyridine core.
Examples: Trimethoprim.
Spectrum of activity: Gram-negative bacilli, *Enterococcus.*
Pharmacodynamics: inhibition of the enzyme dihydrofolate reductase (DHFR), blocking the tetrahydrofolic acid) synthesis and DNA synthesis, bacteriostatic.

Resistance: expression of efflux pumps, target alteration, overexpression of DHPS and DHFR.
Clinical use: combination therapies with sulfa drugs (synergy).

6) Metronidazole.

Structure: nitroimidazoles.
Spectrum of activity: anaerobic strains.
Pharmacodynamics: disruption of DNA, causing the strand breakage of DNA and the extent of DNA, bactericidal.

- Inhibition of protein synthesis:

1) Aminoglycosides.

Structure: aminosugars linked through glycosidic bonds to an aminocyclitol core.
Examples: Streptomycin.
Spectrum of activity: Gram-negative aerobic bacilli.
Pharmacodynamics: Binding to the small S12 rRNA of the 30S subunit of the bacterial ribosome with high affinity, blocking the translocation of tRNA from the A site to the peptidyl-tRNA site P and leading to mistranslation proteins, bactericidal.
Resistance: Phosphorylation, acetylation, nucleotidylation, expression of efflux pumps, target alteration, production of inactivating enzymes.

2) Amphenicols.

Structure: phenylpropanoid scaffold.
Examples: Chloramphenicol.
Spectrum of activity: broad.
Pharmacodynamics: Inhibition of the peptidyl transferase activity of the 50S bacterial ribosome subunit, bacteriostatic.
Resistance: Acetylation, expression of efflux pumps, target alteration, production of chloramphenicol acetyltransferase.

3) Macrolides.

Structure: macrolactone rings with deoxy- and amino-sugars.
Examples: Erythromycin.
Spectrum of activity: Gram-positive strains, sometimes broad.
Pharmacodynamics: Interference with aminoacyl translocation by binding to the 50S ribosome subunit, hindering the peptide exit tunnel and 23S RNA, bacteriostatic.
Resistance: target alteration, expression of *Erm*-encoded methylases for 23S RNA, glycosylation, phosphorylation, expression of *mef* type efflux pumps.

4) Ketolides.

Structure: modified macrolide core with a sugar replaced by a keto-group and a cyclic carbamate in the lactone ring.
Examples: Telithromycin.
Spectrum of activity: broad.
Pharmacodynamics: Binding to the bacterial ribosome at two sites and modification of the structures that mediated resistance.
Resistance: rare, ability to bypass macrolides resistance mechanisms thanks to the improved affinity for the ribosome subunit (binding to an additional site) the evasion of efflux pumps.

5) Lincosamides.

Structure: amino acid linked to sugar moieties.
Examples: Clindamycin.
Spectrum of activity: most Gram-positive strains, Gram-negative anaerobes.

Pharmacodynamics: Binding to the bacterial ribosome at two sites and modification of the structures that mediated resistance, bacteriostatic.

Resistance: cross-resistance to macrolides, nucleotidylation, expression of efflux pumps, target alteration.

6) Tetracyclines.

Structure: linear fused tetracyclic nucleus.

Examples: Tigecycline and the newer subclass of glycylcyclines.

Spectrum of activity: Gram-positive strains, *Mycoplasma*.

Pharmacodynamics: binding to the 30S ribosomal subunit and preventing the attachment of aminoacyl-tRNA to the A acceptor site, bacteriostatic.

Resistance: monooxygenation, expression of efflux pumps and new membrane transporters, peculiar ribosomal protection proteins (*tetM* and *tetO*) that alter the conformation of the active site and inactivating enzymes (*tetX*).

7) Fusidanes.

Structure: cyclopentanoperhydrophenanthrene framework.

Examples: Fusidic acid.

Spectrum of activity: Gram-positive strains, MRSA.

Pharmacodynamics: Inhibition of the transfer of amino acid from aminoacyl-sRNA to the protein on the ribosomes, by preventing the translocation of the elongation factor G (EF-G) from the ribosome, bacteriostatic.

8) Oxazolidinones.

Structure: oxazolidinone core with *N*-linked aryl and heterocyclic rings and short side chains.

Examples: Linezolid.

Spectrum of activity: Gram-positive strains.

Pharmacodynamics: binding to the P site on 50S subunit, inhibition of the formation of initiation complex and translocation of peptidyl-tRNA from the A acceptor site to the P donor site.

Resistance: rRNA mutation, *cfr*-mediated target modification, ribosomal proteins mutation and expression of efflux pump (*optrA* phenotype).

Clinical use: resistant infections.

9) Streptogramins.

Structure: cyclic peptides.

Examples: subclasses A and B.

Spectrum of activity: Pristinamycin IIA.

Pharmacodynamics: Prevention of the transfer of the tRNA bound at the A site of the rRNA complex. A derivatives group, binding to PTC site on 50S ribosomal unit; B derivatives group, binding to the peptide exit tunnel and inhibition elongation of the nascent polypeptide.

Resistance: expression of efflux pumps, inactivating *N*-acetyltransferases, *O*-nucleotidyltransferases, and *O*-phosphotransferases, carbon-oxygen lyase, acetylation.

10) Retapamulin.

Structure: pleuromutilin derivatives.

Spectrum of activity: *S. aureus* and *S. pyogenes*.

Pharmacodynamics: inhibition of the translation by binding to the PTC on the 50S ribosomal subunit.

Resistance: unaffection by resistance to other major classes of antibiotics.

11) Nitrofurantoin.

Spectrum of activity: Gram-negative strains, *Enterococcus*., bactericidal.

Pharmacodynamics: inhibition of the ribosomal protein synthesis.

Current approaches to overcome the antibiotic resistance rise can be the clinical use of drugs from different classes in combination (mutilin-quinolone or rifamycin-quinolone)[13] or the administration of antibiotics with an alternative drug delivery system that enable them to reach high concentrations in the target tissues (inhaled drugs for lung infections).[14,15]

Moreover, new targets and strategies of recently developed antibiotics are:[16]

- Interference with protein post-translational modification (inhibitors of peptidyl deformylase);[17]
- inhibition of fatty acid biosynthesis, as for example targeting enoyl-ACP-reductase FabI or keto-acyl-carrier-protein reductase FabF;[18]
- interference with the bacteria cell-to-cell communication, namely the quorum sensing pathway;[19]
- induction of cellular SOS response, which is a conserved regulatory response because of cellular stress conditions that can cause serious genome damage;
- anti-virulence approaches, such as the inhibition of bacterial toxin release;[20–22]
- bacteriophage therapy alone or in combination with traditional antibiotics;
- passive immunization, such as radio-immunotherapy,[23,24]
- vaccination,[25–27]
- administration of antibodies;[28–30]
- targeting antibiotic resistance elements, such as efflux pumps[31] or β-lactamases.[32]

3. Bacterial resistance.

3.1. A complex phenomenon.

Antimicrobial resistance (AMR) can be defined as the acquired ability of a microorganism to resist to cytotoxic concentrations of an antimicrobial agent to which it was at first sensitive. It is a natural evolutionary phenomenon in accordance with the Darwinian selection process: it consists in the adaptation of the microbe to harsh environments in order to survive to external toxic substances (our drugs). Thus, bacteria develop a *resistome*,[33] creating, collecting and transferring resistant genes that make them safe and insensitive to drugs. After the introduction in the market of a new antibiotic, they develop some strategies to overcome its activity, becoming resistant to it in a period variable from few years, such as linezolid, or longer times, like in the case of erythromycin. As a result, the timeline for introduction and resistance development for some antibiotics shows a worrying rapid rise of AMR and a very short duration of drugs effectiveness (**Figure 5**).

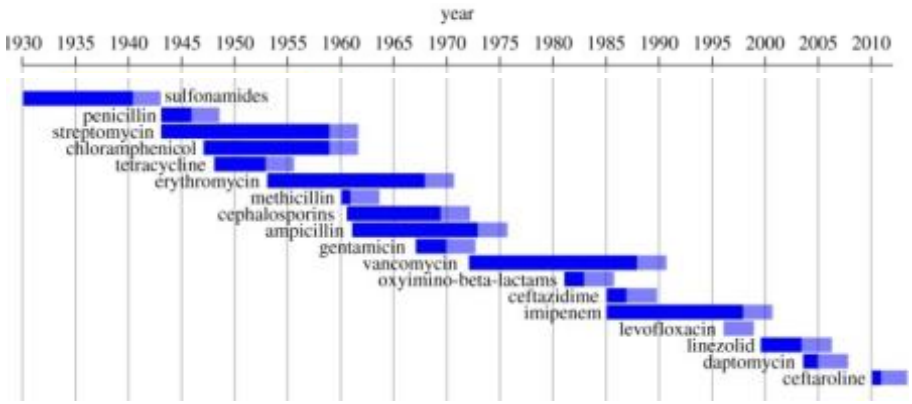


Figure 5. Times of drug introduction and the subsequent evolution of resistance, indicated by the ends of the bars.

In the last years, AMR has emerged as a major global public health issue that was identified as one of the three greatest threats to mankind in the XXI century by WHO.[34] In fact, the associated rates of morbidity and mortality are very high and make all the governments declare an unquestionable state of emergency. Moreover, the enormous economic burden due to the prolonged hospitalization and decreased productivity is nonquantifiable. As an example, only the costs for MRSA infection hospitalization in 2007 were estimated as 44 million euros for 31 countries that have participated in the European Antimicrobial Resistance Surveillance System.[35] In 2014, the last WHO Global Report on Surveillance of Antimicrobial Resistance reported that some Gram-negative strains have become resistant to more than 50% of commonly used antibiotics.[36] In fact, the European disease control and prevention Surveillance of antimicrobial resistance in Europe showed a relevant increase in the detection of resistant clinical isolates in Europe. **Figure 6** shows, for example, data for resistant *Acinetobacter species* isolates in 2012 and 2017 and the percentage of them that are susceptible or resistant to common antibiotic classes.[37,38]

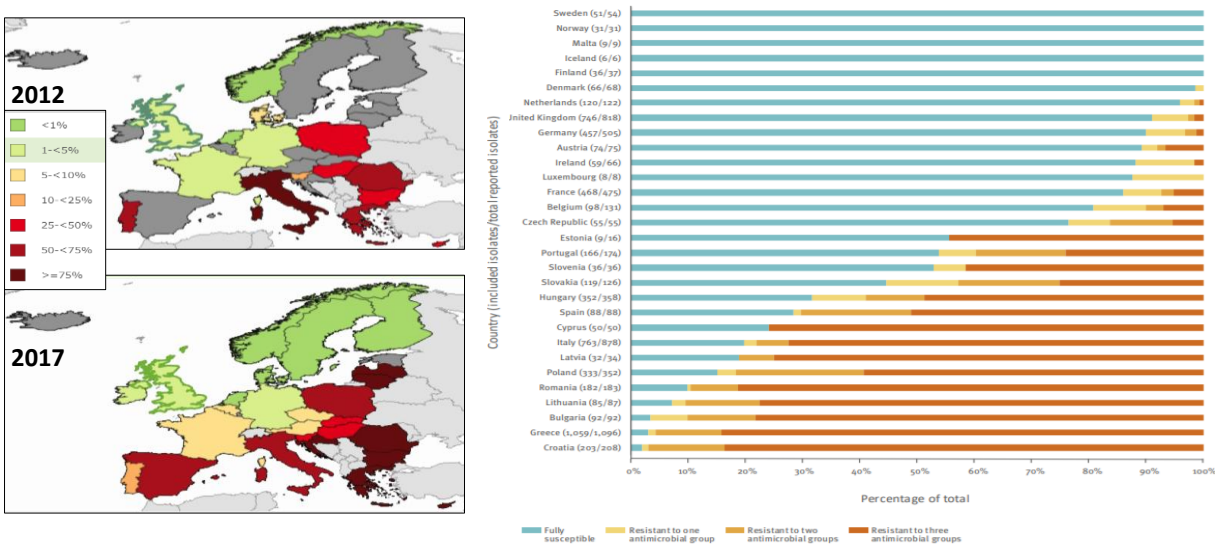


Figure 6. *Acinetobacter spp.*: left, percentage of invasive isolates with combined resistance (fluoroquinolones, aminoglycosides and carbapenems) by country, EU/EEA countries, in 2012 and 2017; right, distribution of isolates: fully susceptible and resistant to one, two and three antimicrobial groups (among isolates tested against fluoroquinolones, aminoglycosides and carbapenems), EU/EEA countries, in 2017. [37,38]

Moreover, the center for disease control and prevention reported that 2 million of MDR infections per year were recorded in the USA and 23,000 of them died in 2013 (**Table 1**).[39]

Table 1. Resistant bacterial strains and associated deaths for bacterial infections per year.

Bacterial strain	Infections per year	Deaths per year
Multidrug-resistant *P. aeruginosa*	6,700	440
Vancomycin-resistant *Enterococcus* (**VRE**)	20,000	1,300
Methicillin-resistant *S. aureus* (**MRSA**)	80,461	11,285
Extended-spectrum beta-lactamase (**ESBL**) producing *Enterobacteriaceae*	26,000	1,700
Multidrug-resistant *Acinetobacter species*	7,300	500
Drug-resistant *S. pneumoniae*	1.2 million	7,000
	hospitalizations per year >19,000	
Vancomycin-resistant *S. aureus* (**VRSA**)	Cases 2002-2013: 13 in 4 states	
Summary totals for antibiotic-resistant infections	2,049,442	23,488

The infectious disease society of America coined the acronym *ESKAPE* pathogens in order to easily refer to the six bacterial species (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) that collectively caused around 2/3 of all US nosocomial infections.[40,41] Although their resistance mechanism is not the same, all these pathogens share the common ability to escape the antibiotic treatments.[42,43] In February 2017, WHO published a list of bacterial strains that urgently require the development of new antibiotics. In particular, carbapenem-resistant *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and ESBL-producing *Enterobacteriaceae* have been classified as critical pathogens, with priority one,[44] in fact, they represent the so-called *superbugs*, that are recognized as not only drug- or multidrug-, but extensivelydrug- or totallydrug-resistant pathogens.[45] The Center for Global Health assumed that superbugs kill 700,000 people per year globally[46] and an important review on antimicrobial resistance made a prediction about the scenario in 2050, estimating 10 million of deaths per year (more than people who die for cancer), 1 death every 3 seconds and 66 trillion USD attributable to AMR infections (**Figure 7**).[46]

Figure 7. Comparison between the deaths attributable to AMR and other main causes of death in the world today and prevision for the deaths caused by AMR in 2050.[46]

3.2. Classification and mechanisms.

Resistance can be classified as intrinsic or acquired. The first one is the innate ability of a microorganism to be unaffected by the action of a particular antimicrobial agent by virtue to its structural or functional properties encoded by its natural genome. For example, Gram-negative bacteria are intrinsically resistant to vancomycin, since their outer membrane makes them impermeable to voluminous and hydrophobic antibiotics. This *insensitivity* causes a resistance phenotype already present in the bacterium, before the first exposure of the antimicrobial.[47]

The acquired resistant phenotype is developed by some cells belonging to a subpopulation of a bacterial species previously susceptible to an antibiotic only after the exposure to it. Bacteria with mutant genome survive to the antibiotic action, selecting resistant strains. Furthermore, since resistant genes are not eliminated or reversed by bacteria, they can be accumulated over time, conferring an MDR phenotype.

The acquired resistance can take place by point mutations or by resistance gene acquisition, so it is further classified into chromosomal and extra-chromosomal subtypes. The chromosomal or endogenous resistance is due to a rare and spontaneous mutation in structural or regulatory chromosomal genes and is transferred during the cell division in a vertical manner (**Figure 8A**). These mutations can also generate a simultaneous resistance to several antibiotics belonging to the same or different classes, provoking the so-called *cross-resistance*. The extra-chromosomal or exogenous resistance is caused by a horizontal genetic transfer via mobile genetic elements, like plasmids, transposons or integrons, which act as vectors between bacteria.[48,49] Horizontal gene transfer occurs *via* three main strategies (**Figure 8B**): transformation, transduction, and conjugation. Bacteria, that acquire external genome through transformation, incorporate short fragments of naked DNA by naturally transformable bacteria. In the transduction mechanism, resistant genes are transferred from one bacterium into another *via* bacteriophages, while the gene transfer in conjugation occurs *via* sexual pili and requires cell-to-cell contact.

Examples of what resistant genes cause to overcome to the action of antibiotics are the modification of the target and the reduced in cell accumulation (**Figure 8C**):[50]

- Modification of the antibiotic binding site in the target with a consequent decrease in its affinity, through changes in amino acid composition or in protein conformation.
-Inactivation of the antibiotic: production of enzymes that inactivate or destroy the antibiotic, such as β-lactamases.
- Reduced entry of the antibiotic into bacterial cell: mutations of protein channels (porins).
- Enhanced export of the antibiotic out of the cell: production and expression on the cell surface of efflux pumps.
- Alteration of microbial proteins that transform pro-drugs into the effective moieties.
- Development of alternative pathways to those inhibited by the antibiotic

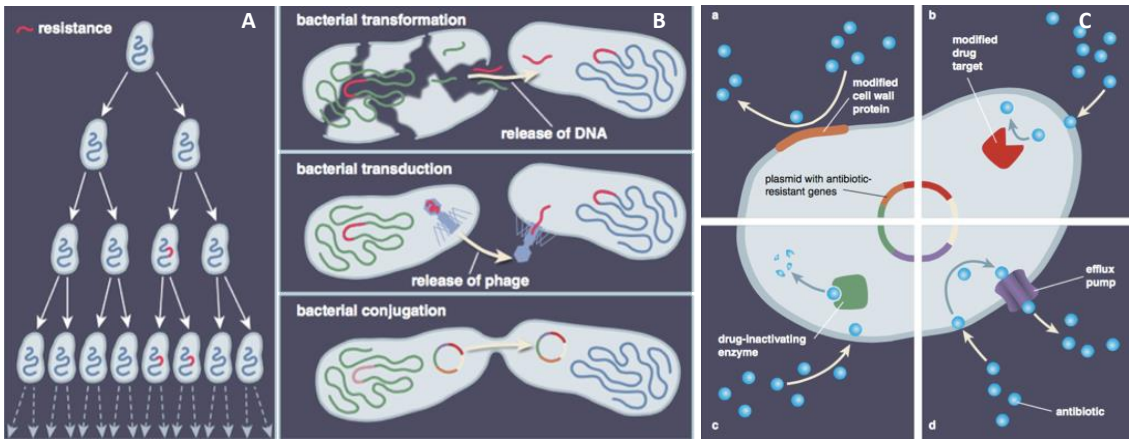


Figure 8. Representation of vertical (A) and horizontal (B) gene transfer between bacteria and (C) schematic representation of the major mechanisms of antibiotic resistance: a, alteration of antibiotic-binding target; b, modification of antibiotic target; c, production of antibiotic efflux pumps; d, production of antibiotic-inactivating or -destroying enzyme.

3.3. Causes and consequences.

Although antibiotic resistance is the above-described natural adaptation process of bacteria, it is considered a multifactorial issue. Actually, several events play an important role in its fast development and all these factors, taken together, are involved in the *perfect storm*[51] antibacterial resistance, such as the widespread overuse and misuse of antibiotics, the decrease in research and production of new antibiotics, especially those with an innovative mechanism of action.

The main causes of resistance rise are presented in **Figure 9** and herein briefly discussed.

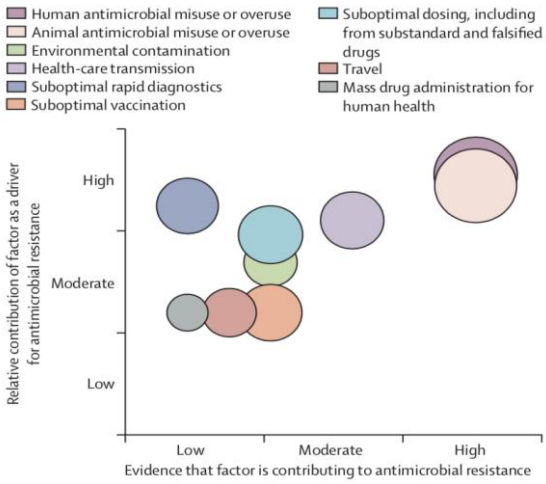


Figure 9. Conceptual framework of modifiable causes of AMR. [52]

1) Human overuse and misuse of antibiotics.

It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then, there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant. Here is a hypothetical illustration. Mr. X. has a sore throat. He buys some penicillin and gives himself, not enough to kill the streptococci but enough to educate them to resist penicillin. He then infects his wife. Mrs. X gets pneumonia and is treated with penicillin. As the streptococci are now resistant to penicillin the treatment fails. Mrs. X dies. Who is primarily responsible for Mrs. X's death? Why Mr. X, whose negligent use of penicillin changed the nature of the microbe. Moral: If you use penicillin, use enough.

A. Fleming, Nobel lecture, December 11, 1945.[53]

In this speech, A. Fleming claimed that inappropriate use of penicillin can lead to *mutant forms,* and, later, in a New York Times interview, he warned that *public will demand [the drug and]… then will begin an era… of abuses,* [53] making a prediction about the future antibiotic resistance rise and its causes. Unfortunately, what he expected, has become a reality.

The main causes are the following:

- Inappropriate antibiotic prescriptions lead to an exponential overprescription, for example, the estimated annual prescriptions in the USA were 154 million in 2010-2011 and at least 30% of systemic antibiotics were considered unnecessary.[54] Moreover, broad-spectrum antibiotics are prescribed at a higher rate than the narrow-spectrum one, since they provide a fast treatment, not requiring for diagnostic analysis.[55]
- Self-medication is quite recurrent through the practice of medication-sharing between friends and families.[43] This is caused by the incorrect drug packaging, that highlights a relevant mismatch between the number of doses within the pack size and doses required for the actually prescribed treatment.[56] In fact, the use of leftover doses is estimated as 30% of the total consumption and causes 87% of the non-prescription use in Italy.[57] Thus, it results in an improperly shorter or longer duration of the treatment cycle and in wrong dosing than as required. For these reasons, the Italian Ministry of Health together with the Italian Agency of Drugs (AIFA) and other foreign institutions worked hard to establish a more rational and *ad hoc* antibiotics packaging.[58,59]

Furthermore, even if antibiotic dispensing is limited by a medical prescription, the purchase of these drugs is sometimes allowed regardless of the presence of it, threating to the patient health, since drug incompatibilities, side effects or allergies remain unchecked and antibiotic effectiveness.[60] Although the role of pharmacists in stewardship is undeniably important to discourage incorrect use, they sometimes dispense un-prescribed antibiotics.[57,61] This is recorded as an ordinary event in certain developing countries, reaching over-the-counter antibiotic use up to 90% in some cases, since the antibiotic prescription is less controlled.[62,63] Nonetheless, several online pharmacies give the possibility to purchase antibiotics without a prescription.[64,65]

Moreover, because of public misconception, antibiotics have become a sort of *panacea of medicine*, used also to treat non-bacterial diseases, like cold and flu (**Figure 10**, left).[62] In fact, also a European survey conducted in 2016 showed a severe lack of knowledge about this field: about half the population answered that it is true that antibiotics kill viruses. Moreover, a significant discrepancy between the countries emerged, respondents from Northern Europe gave more correct answers than those from Greece and Bulgaria. Italians were in the lower ranking positions (**Figure 10**, right).[66]

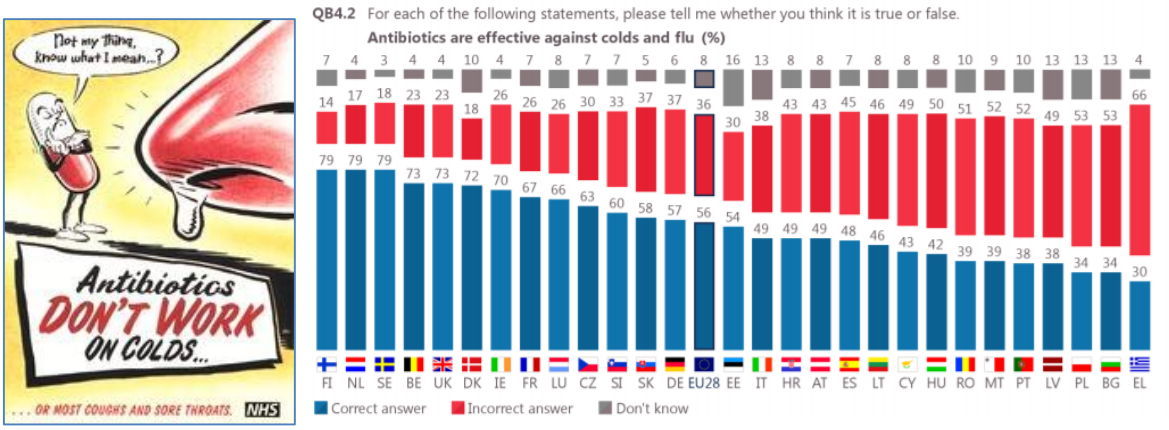


Figure 10. Left, postcard by the UK National Health Service (NHS);[67] right, answers to "can antibiotics kill viruses?" from EU survey in 2013. [66]

- The antibiotic misuse in crops and food industries has been becoming alarming since, through the food chain, animals can transfer resistant strains to humans. In the past years, antibiotics were used extensively for non-curative uses but as growth promoters. Nowadays, this use and the extra-label administration in livestock have been banned, like the addition of other antibiotics in food animals.[68,69]
- Current data underline that also environmental contamination and pollution have a key role in the resistance increase, in particular, the application of farm waste on lands, hospital effluent, discharges of pharmaceutical factories.[70]

In general, the consumption of antibiotics in the world is very high. Observing data collected by the antimicrobial consumption interactive database (ESAC-Net) (**Figure 11A**), the same north-south divide highlighted by the EU survey is reflected in the defined daily doses of their inhabitant and these data are directly related to the resistant strains isolated in areas: lower levels were observed for Netherlands and Sweden than those for Greece.[46,71,72] This is the mirror of their policies for a more careful use of antibiotics in clinics, crops, and animals (**Figure 11B**).[55,73]

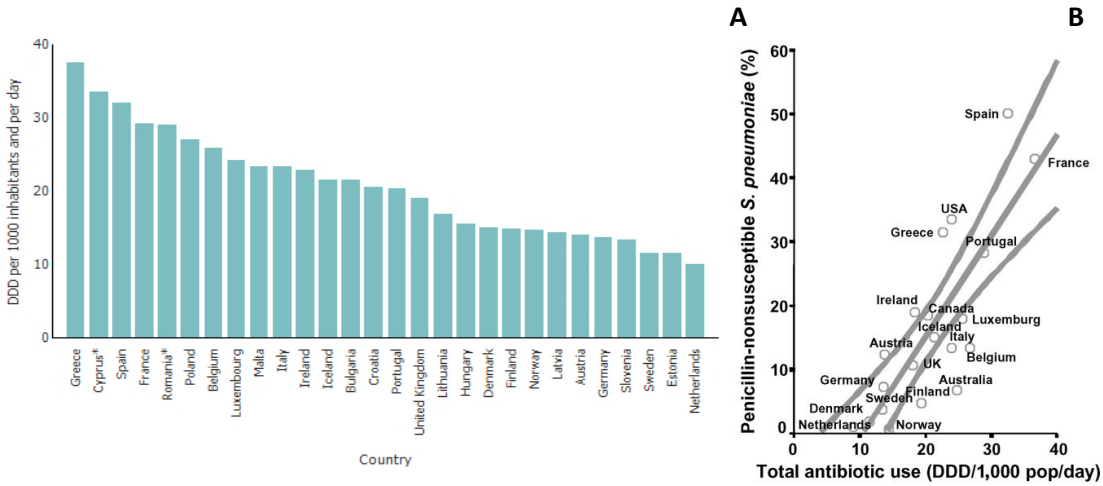


Figure 11. A, Consumption of systemic antibiotics in the community (primary care sector) in Europe for the year 2017;[74] B, Total antibiotic use in the outpatient setting versus prevalence of penicillin-nonsusceptible *S. pneumoniae* in twenty industrialized countries.[75] DDD is defined as daily doses.

In summary, a new approach toward antibacterial therapy should be taken, involving, first of all, the reduction of antibiotic prescriptions and un-prescribed use, the cyclic rotation between classes, preferably avoiding broad spectrum and last-resort antibiotics or drugs combination. Furthermore, media campaigns have been already started, such as the institution of the Antibiotic Awareness day on November 18th with the scope to raise public awareness about drug-resistance.[76–78]

2) Decrease in antibiotic production.

Nowadays, although antibiotics are among the most important saving-life drugs, research and development in this field are stationary. Antibiotics are in a cornerstone of pharma industries for several factors. In fact, they are classified with a very low net present value (NPV). This metric is a risk-adjusted measure of the expected best avenue of investments for a pharmaceutical project, starting from the initial research studies to other development expenses. Antibiotics are considered unattractive and not lucrative, compared to drugs for cancer or chronical disease treatments: while, for example, diabetes drugs are administrated for the whole life,[51] antibiotic therapy usually takes few days, causing a rapid pathogen eradication.[73] Moreover, long times and big costs are required for getting drugs from the lab bench to the market, especially when the drug is endowed with antibacterial activity. In fact, although antibacterial drugs have a better chance than the others at the beginning of drug development thanks to the great predictability of the activity assays (30% of antibiotics that enter Phase 1 are approved, while only 20% of the other drugs), they have a very high risk to fail in the latest and most expensive stages. Thus, pharma companies have to apply very high market prices.[79] Another important risk in the research for new antibacterials is that, like for all the antimicrobials and antivirals against pathogens with high mutation rates, pharma companies cannot predict the rise of resistant strains epidemics and of specific resistance elements. Moreover, several little pharma companies departed from the business or consolidated among them and many large industries curtailed antibiotic R&D divisions, decreasing from eighteen in 1990 to four in 2013 the number of new antibiotics introduced in the market (**Figure 12**, left).[51] As results, an innovation gap between the introduction of cephalosporins (1960) and linezolid (2000) in clinics was recorded: none new antibiotic scaffold was discovered (**Figure 12**, right).

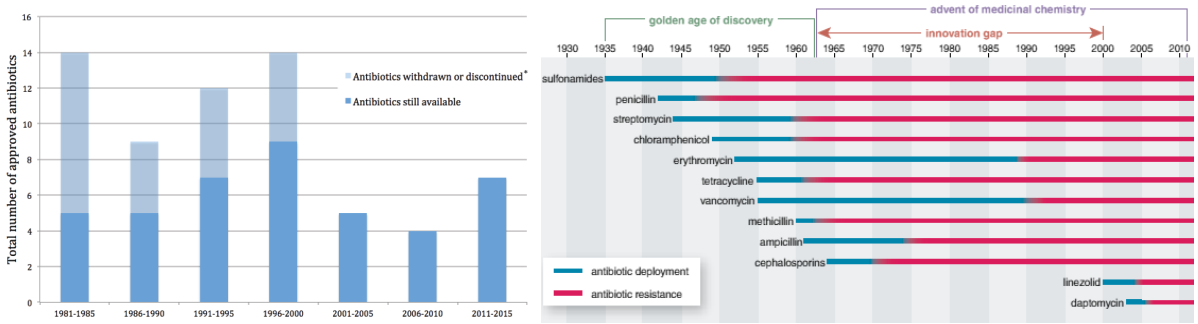
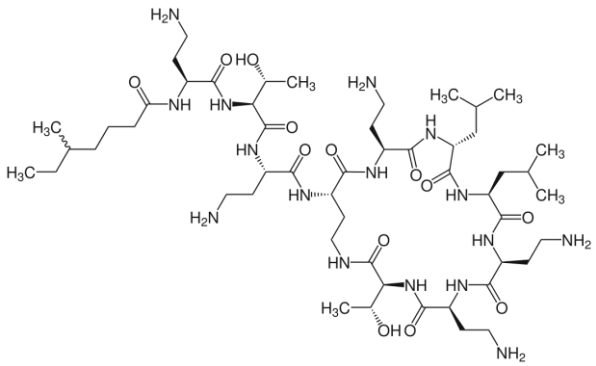


Figure 12. Left, number of new antibiotics FDA approvals versus years; right, innovation gap.[80]

No action today means no cure tomorrow was the theme given by the WHO to a World Health Day, in fact, new action plans are urgently needed to fight the resistance issue. Fortunately, several programmes for infection, prevention, and control have been already created. They promote basic hygiene practices, environmental cleaning, prophylaxis, and topical decolonization in order to limit the spread of infections and more detailed clinical studies and careful drug-administration surveillance.[46,81,82]

Moreover, WHO established the Global Action Plan, calling on all governments and institutions to increase awareness on the correct antibiotic use, to improve prevention measures, and to incentivize industries in investing in R&D projects and diagnostics.[83] A US national action plan was promoted in 2015 with the aim of reducing the overuse of antibiotics by 50% in 2020 and the inpatient setting by 20%.[84] Also in Europe and in the whole world, important initiatives in this fields were taken by the Innovative Medicines Initiative,[85] the Joint Programming Initiative on AMR[86] and other institutions,[87–90] in addition to media campaigns.[91–93]

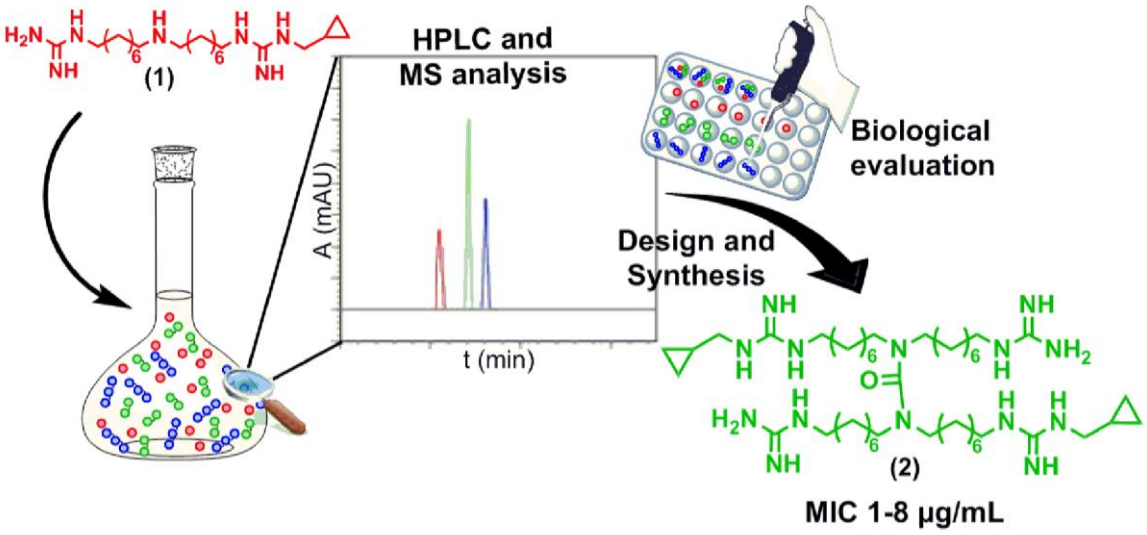
3.4. Last-resort Colistin.



Colistin (on the left) is an old antibiotic belonging to the polymyxin class. It was discovered in the late 1940s and used in clinics in 1950. Reports of its nephro- and neuro-toxicity issues deterred its prescription for years and it was set aside, not being used until the 1990s, when the threatening emergence of large-scale AMR of Gram-negative pathogens caused a renewed interest in its therapeutic use. Nowadays, colistin represents not only an important therapeutic option but, in some cases, also the only one still effective against MDR Gram-negative pathogens including *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*.[94] Colistin is available in two forms, colistin sulfate (topical) and colistimethate sodium (parenteral), both administrable for inhalation. This latter is the anionic prodrug, characterized by lower toxicity than the effective colistin. The target of polymyxins is the bacterial cell membrane. In fact, colistin can associate to the membrane through electrostatic interactions between their cationic moieties and the anionic LPS in the Gram-negative outer membrane and can also displace divalent ions (Mg^{2+} and Ca^{2+}) whose role is the stabilization of the membrane from the negatively charged LPS. The absence of these ions and the perturbation of the membrane electric potential lead to a local disturbance and derangement and to increased permeability, resulting in cell death. Moreover, colistin can bind to released LPS exerting an anti-endotoxin activity.

In the last years, despite being not a common event, alarming cases of bacterial resistance to colistin have been observed and reported. It develops either through mutational or by adaptive mechanisms. For example, an alteration of the lipid A, the phosphoethanolamine, the LPS composition, resulting in a change in the outer membrane superficial charge, can occur with a decreased binding affinity for colistin. Additional resistant elements, such as expression of efflux pumps or overexpression of outer membrane proteins were also observed.[95–98]

State of the art



1. Guanidine moiety in medicinal chemistry.

In the last years, chemists focused their attention on guanylated polyamines, a particular class of polyamines characterized by one or more of their amino moieties as part of guanidine functions. The guanidine group is a common structural key element in several natural and synthetic compounds, characterized by a vast chemical diversity and a broad biological activity.[99,100]

The replacement of amine with guanidine in polyamines involves changes in the biological and chemical properties of the molecule, that are amplified if compared to the correspondent amine: guanidine is strongly basic (pKa of guanidinium ≈ 13.5),[101] indicating that at physiological or basic pH, is protonated forming the guanidinium cation. For example, the guanylation of the amino moiety in neomycin B and kanamycin A resulted in restoring and extending the activity toward gentamicin-resistant *P. aeruginosa* and MRSA.[102]

Proteins and enzymes use the guanidinium moiety in the lateral chain of the amino acid arginine to recognize and to bind anions, such as carboxylates, phosphates, and metals, through ion pairing and hydrogen bonding, resulting in potency and selectivity.[103] Moreover, the biophysical properties of polyalkylguanidines make them excellent drugs or lead compounds because they are highly soluble in water and bioavailable. In fact, during the period 2008-2012, numerous guanidine-containing compounds emerged for their potent biological activities, making guanidine scaffold one of the most widely investigated scaffolds in medicinal chemistry for small peptides, peptidomimetics, and low molecular weight drugs.[104]

Compounds bearing guanidine functions have been studied for years as drugs for several therapeutic applications:[105] for central nervous system or cardiovascular (clonidine and amiloride), disorders, ischemic, inflammatory and autoimmune diseases, diabetes (biguanides as metformin), ulcer and stomach acidity (cimetidine and famotidine), and also as anticancer and antimicrobial (streptomycin, bleomycin, capreomycin and proguanil) agents.[104,106] Some examples of guanidine-related drugs are shown in **Figure 13.**

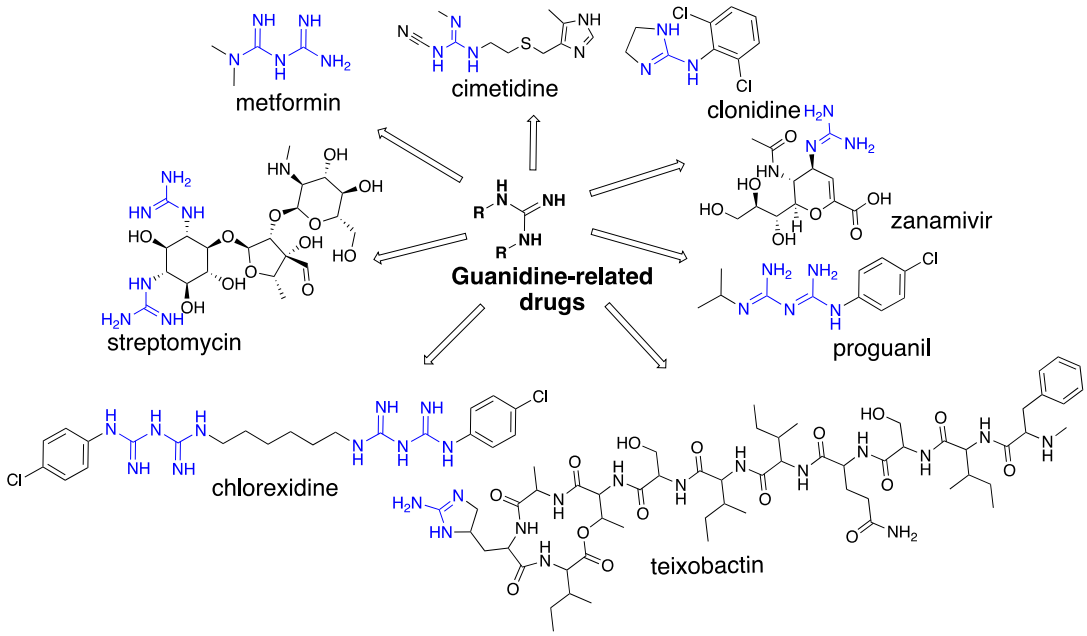


Figure 13. Examples of guanidine-related drugs.

Among a series of natural and synthetic guanidines, guanidine hydrochloride, synthalin A, galegine, pterogynidine, and teixobactin showed interesting antibacterial profiles. Thus, several research groups are now working at developing new guanidine derivatives as antibacterial agents.[42,105,107]

Several guanidine-related compounds like bisbiguanides (chlorhexidine, **Figure 14**) and bisamidines (hexamidine) have emerged as broad-spectrum antibiotics, although their narrow selectivity index allows only an antiseptic use for disinfection of skin and surgical instruments.

In particular, chlorhexidine, the oldest known guanylated drug, is bacteriostatic at low concentrations and bactericidal at higher ones. Its mode of action involves in electrostatic interactions between its cationic biguanidino groups and the negatively-charged bacterial surface, resulting in cell membrane disruption, cytoplasm leakage and osmotic cell lysis of the cell (**Figure 14).** Since it can inhibit the biofilm formation, it is also used as oral antiseptics, preventing dental caries, periodontitis, endocarditis, and cystic fibrosis pneumonia.

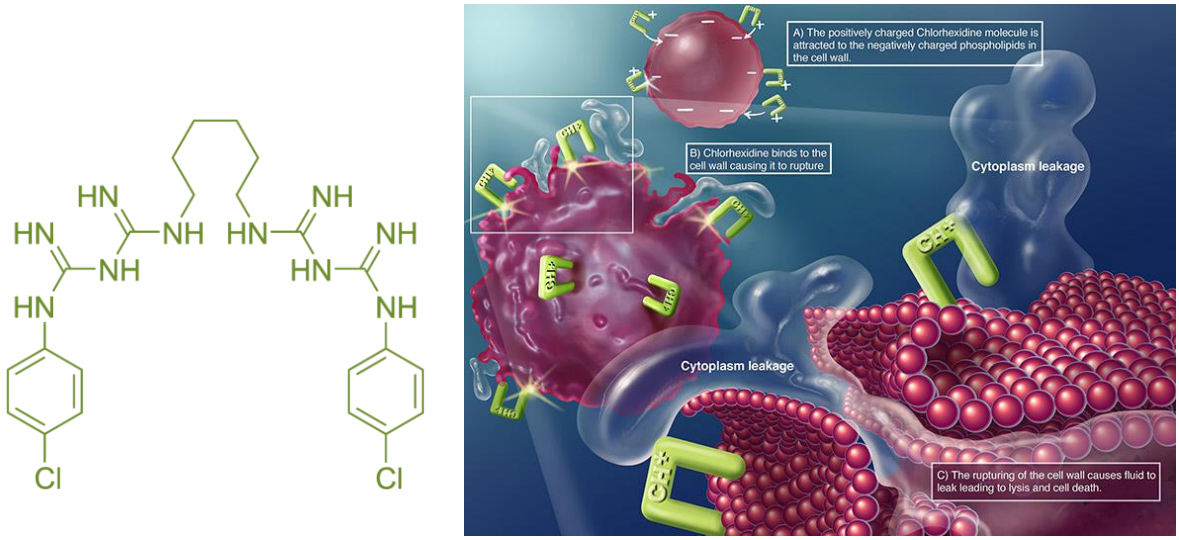


Figure 14. Mechanism of action of chlorhexidine (CH+) and its chemical structure.[108]

Also, organic chemists focused their attention on guanylated compounds as electrophilic catalysts due to their ability to form several hydrogen bonds. The guanidine moiety, being protonated in physiological environments is able to weakly interact through cation-π with planar electron-rich moieties such as phenyl rings. In fact, having several π electrons in the bonding orbitals, guanidinium species have several resonance forms due to the conjugation between the lone pairs of the nitrogen atoms and the imine double bond, resulting in the delocalization of the positive charge over the entire functional group known as *Y aromaticity*.[109–111] The electron delocalization causes also the above-mentioned highly basicity and the planarity of the nitrogenous backbone which can determine the conformation of substituted guanidines and the possibility to interact with aromatic systems in both biochemical (amino acids and nucleic acid bases) and chemical fields.

2. From the crops to the lab bench: guazatine, a potent inhibitor of PAOs.

For years, the research group of Prof. M. Botta has been involved in the study of Polyamine Oxidases (PAO)[112–115], a family of FAD-dependent enzymes that catalyze the catabolism of polyamines. Polyamines are ubiquitous small aliphatic hydrocarbon molecules bearing several amine groups endowed with a net positive charge at physiological pH. Polyamines have key roles in cell proliferation, differentiation and apoptosis in prokaryotic and eukaryotic cells (**Figure 15**, left), the most known physiological polyamines are shown in **Figure 15** (right).[116] In general, polyamine pathway modulators have become of great interest in medicinal chemistry as agents for treatment or prevention of cancer:

they can cause an intracellular accumulation or a lack of polyamines, leading to DNA fragmentation or to an increased production of cytotoxic reaction products, such as aminoaldehydes and H_2O_2, strong inducers of apoptosis.[117] Thus, polyamines analogs as inhibitors of polyamine transporters or of PAO activity have been studied as agents for treatment or prevention of cancer.[118–122]

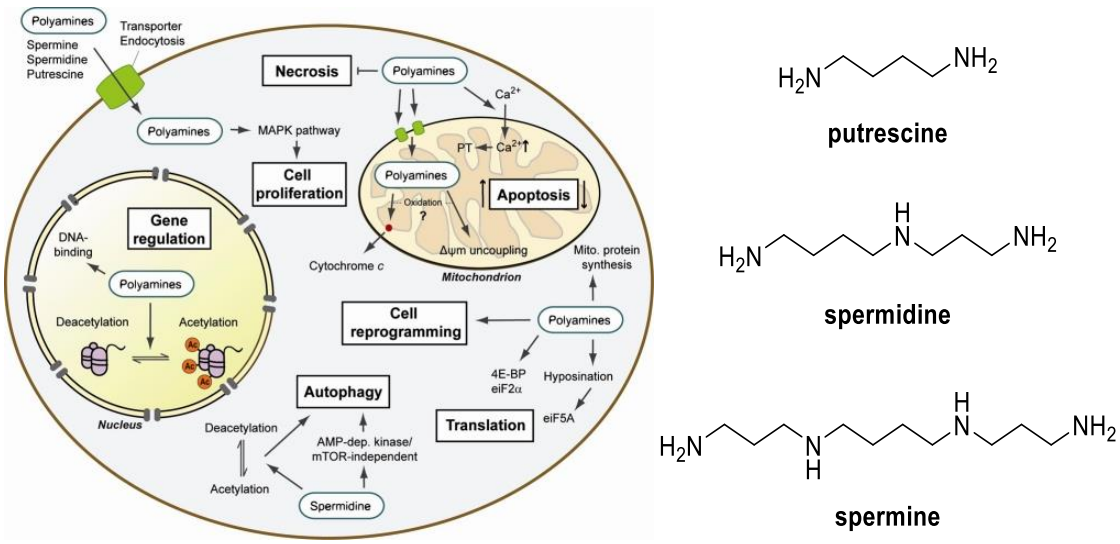


Figure 15. Left, summary of the cellular mechanisms of action of polyamines;[123] right, chemical structures of some physiological polyamines.

With the aim of discovering the potential human use of guazatine, a non-systemic fungicide widely used in agriculture, the research group of Prof. M. Botta in-depth analyzed its composition. In fact, guazatine is not a pure compound, but a mixture of reaction products from polyamines and polyguanidines. Thus, Prof. Elena Dreassi, from my research group, identified its main components by means of LC and LC-MS studies, discovering iminoctadine, a bis-guanylated triamine (structure reported in **Figure 16**) as the most abundant chemical entity.[124] Hence, iminoctadine was tested as antifungal, resulting to have very potent activity against *Candida albicans*, responsible for most of the nosocomial infections,[124] and it was found to inhibit some members of PAO family through a competitive binding and a very high affinity.[112–114,125] Encouraged by these findings, a library of iminoctadine analogs characterized by changing the guanidino terminals was synthesized and its affinity to *Zea mays* PAO (MPAO) was evaluated. Data unlighted subnanomolar inhibitory activity for the whole library (**Figure 16**).[126]

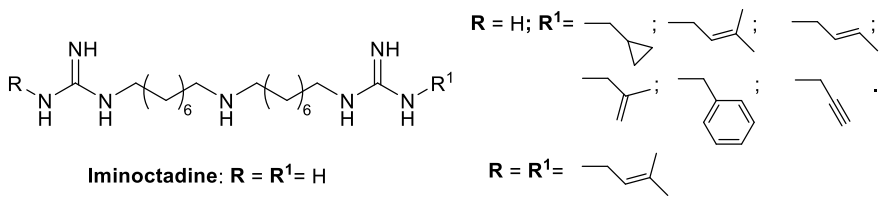


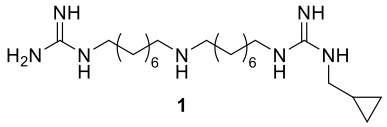
Figure 16. Chemical structure of iminoctadine and anti-PAO synthesized library.

3. A compound with notable antibacterial activity.

From literature, it was known that aliphatic amines are membrane disruptors and compounds characterized by the presence of one or more guanidino functions in their structure are strongly adsorbed by fungi membrane and active against microorganisms.[42,127–130] Moreover, in our research group, a class of cyclic compounds bearing guanidine moieties were developed as antifungals and a

connection between these functions to the antimicrobial activity had already emerged in other publications.[131–133] Thus, we decided to enlarge the small anti-PAO library and to evaluate their antibacterial profile on a panel of representatives Gram-positive and Gram-negative strains, including drug-resistant clinical isolates. Surprisingly, only one compound (**1**, **Table 2**) among the series emerged as a potent broad-spectrum antibacterial agent, with MIC values ranging from < 0.125 to 32 µg/mL (**Table 2**).[134]

Table 2. Chemical structure of compound **1** and its putative biological activity on wild-type and mutant strains.



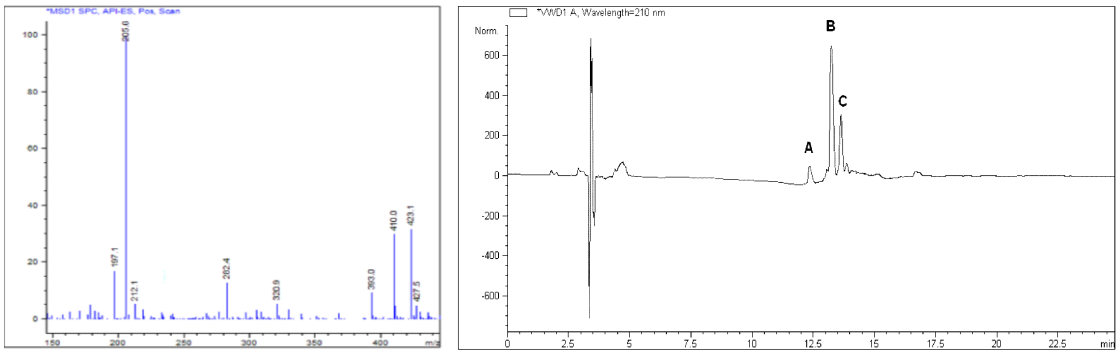
1

Bacterial strains	MIC (µg/mL)	Resistant bacterial strains	MIC (µg/mL)
A. baumannii ATCC 17978	4	*A. baumannii* AC-54/97	4
A. hydrophila ATCC 7966	8	*A. xylosoxidans* AX 22	4
E. meningoseptica CCUG 4310	32	*A. faecalis* 424/98	4
E. coli CCUG^T	0.5	*E. cloacae* VA-417/02	2
K. pneumoniae ATCC 13833	1	*K. pneumoniae* 7023	2
P. aeruginosa ATCC 27853	8	*P. aeruginosa* 101/1477	8
B. subtilis ATCC 6633	0.5	*P. aeruginosa* VR-143/97	8
E. faecalis ATCC 19433	1	*S. maltophilia* 634/08	4
S. aureus ATCC 25923	4	*S. hominis* SI-7/2011	2
S. epidermidis ATCC 14990	0.5	*S. aureus* ATCC 43300 (MRSA)	4
S. pyogenes ATCC 12344	<0.125	*S. aureus* ATCC 700699 (Van A)	4
		S. haemolyticus SI-6/2011	2
		S. warneri SI-5/2011	4

4. A serendipitous discovery: the oligomers.

In order to perform further analyses, compound **1** was re-synthesized. The biological evaluation of its newly synthesized batches surprisingly showed a significant reduction of the antibacterial activity, when compared to that of the original batch (**Table 3**).[134]To understand the causes of this experimental discrepancy, the original batch was reconsidered and in-depth analyzed.

The mass spectrum obtained by direct injection of a sample of the original batch of compound **1** (**Figure 17**, left), detected signals attributable to **1** and heavier molecular entities. In fact, the original batch turned out to be a mixture of different components, as emerged by the HPLC-UV-MS analysis (**Figure 17**, right).[135]

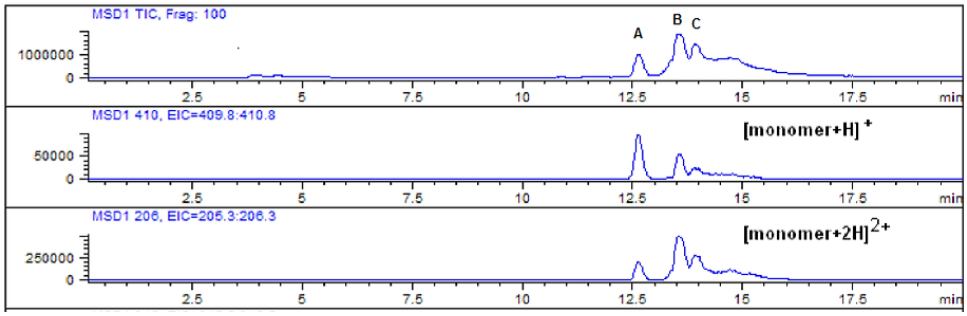


Reprinted (adapted) from Zamperini C et al.[136]

Figure 17. Left, mass spectrum obtained by direct injection of a sample of the original batch of compound **1**; right, chromatographic profile of a sample of the first batch by LC-MS.

The UV chromatogram showed three main components, as the UV signals A, B, and C, corresponding respectively to compound **1** and two other larger species, with *m/z* values approximatively twice and three times higher than **1**. Our analytical team was able to successfully isolate only the eluate **A**, while analytes **B** and **C** were collected together since their similar retention times. By means of MS and ^{1}H NMR spectra, we identified **A** as the pure compound **1**.

In order to gain some information about eluates **B** and **C** identities, preliminary fragmentation studies obtained by changing the fragmentor voltage were performed on a sample of the mixture, revealing at higher fragmentation energy (100 mV) the presence of fragments of compound **1** in all the three chromatographic peaks (**Figure 18**). This demonstrated that the unknown **B** and **C** could be derivatives of compound **1**.[135]



Reprinted (adapted) from Zamperini C et al.[136]

Figure 18. Extracted-ion chromatograms (EIC). Total ion current (TIC) chromatogram obtained from a chromatographic run conducted at 100 mV was shown in the first line. The m/z values attributable to 1 (410.1 and 205.5 m/z) were detected also in correspondence of the other TIC signals (B and C).

Then, the two fractions **A** and **B + C** were tested for their antibacterial profile, revealing that **A** had the same activity of the freshly synthesized batches, while **B + C** showed activities comparable with that of the original mixture, suggesting that contained the chemical entities responsible for the antibacterial action (**Table 3**). [135]

Unfortunately, it was not possible to carry out NMR experiments of eluates **B** and **C**, because they were not isolated but collected in a single fraction. However, even if we had performed the NMR spectrometry, the spectra would have shown a high population of signals in a narrow range of ppm, making the assignment of each peak very difficult. Moreover, the obtained amount of this fraction was not enough to investigate the chemical nature of its components. Thus, to elucidate the chemical formula and the structure of **B** and **C**, accurate mass measurements and empirical formula calculations for the molecular ions were performed by using LTQ-Orbitrap XL mass spectrometer. Thanks to the expertise on properties and reactivities of di-Boc-guanidino moieties and their byreactions,[137,138] we

hypothesized that **B** and **C** could consist of the structure of **1** (monomer) repeated twice or three times and linked through a carbonyl group, giving oligomeric compounds, a dimer, and a trimer. Thus, two possible structural isomers for each oligomer were designed, a symmetric and an asymmetric one, as reported in **Figure 19**. We refer to the symmetric structure (**2** and **4**) when the central amines of the two monomers are involved in a urea group, whereas the asymmetric structure (**3** and **5**) when the monomers connection involves the central amine of one monomer and the guanidine group of the other, generating an amidinourea moiety. [135]

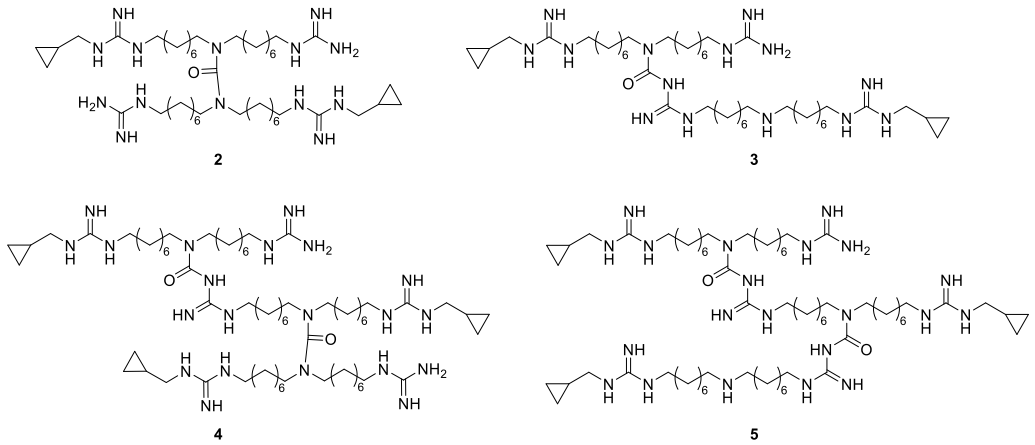
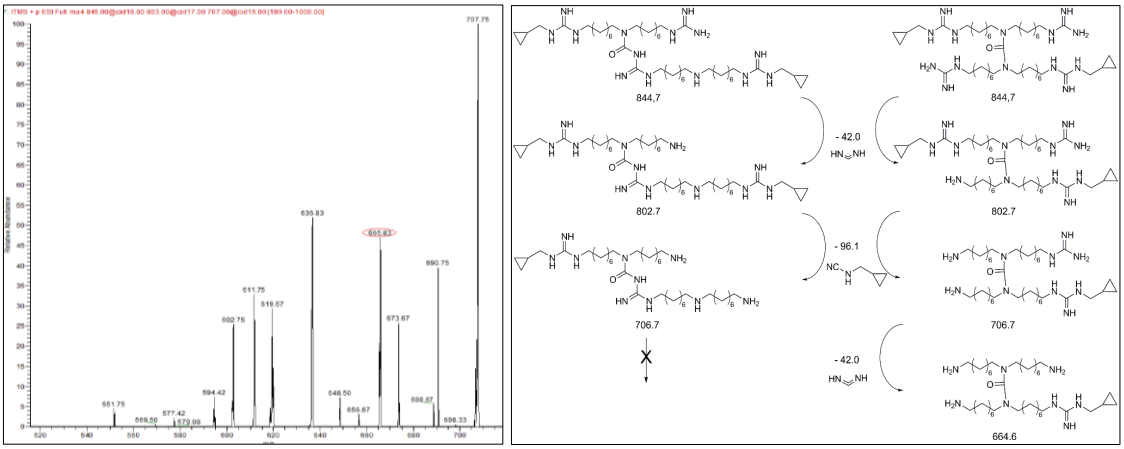


Figure 19. Structures of dimers (isomers **2** and **3**) and trimers (isomers **4** and **5**).

To establish the actual structure of dimer and trimer between the two hypothesized isomers, the mixture was analyzed through the per infusion MS^n technique, using an ion trap coupled with the Orbitrap mass analyzer. Due to the structural similarity of **1** to guazatine components,[124] a similar fragmentation pattern has been hypothesized and the MS^4 spectrum obtained from the precursor ion 845.7 *m/z* showed the signal at 665.8 *m/z* which is characteristic of the symmetric isomers (**2** and **4**), since this fragmentation is not possible for the asymmetric dimer (**3**), as reported in **Figure 20**. The per infusion MS^n technology allowed to record the presence of a symmetric moiety that could belong to the dimer or the trimer. [135]

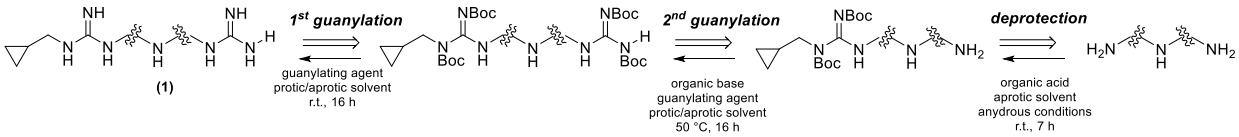


Reprinted (adapted) from Zamperini C et al.[136]

Figure 20. Left, zoom of MS4 spectrum of the mixture (845.7 → 803.9 → 707.8 m/z); right, proposed fragmentation pattern for the structures of dimeric moieties and their calculated exact mass.

Although the factors favoring the formation of these derivatives are still unclear, we assumed that the generation of this mixture occurred during the storage of the sample in DMSO solution before the biological evaluation, especially considering that the characterization analysis of compound **1**,

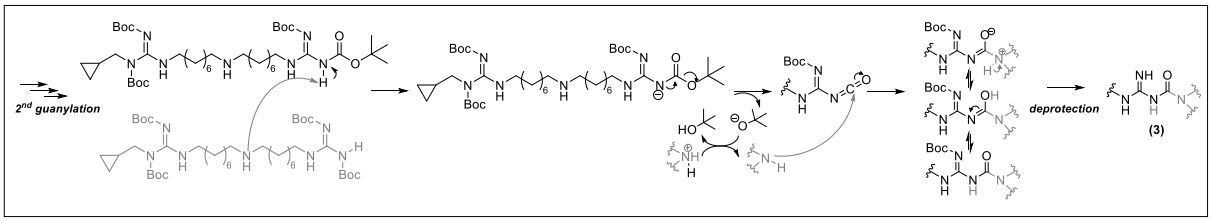
performed immediately after its synthesis, confirmed its high rate of purity and authenticity. [135] Likely, small impurities were formed as byproducts in the synthetic route (previous retrosynthesis of **1** shown in **Scheme 1**) and/or reacted again during the sample storage.



Scheme 1. Previously described retrosynthesis of compound **1**.

As regard as the possible mechanisms of formation of oligomers, we need to distinguish between the symmetrics and the asymmetrics and the key chemical moieties that characterize them. In particular, the asymmetric oligomers (**3** and **4**, **Figure 20**), but also the symmetric trimer (**4**, **Figure 20**), contain the amidinourea function between two monomeric building blocks.

What we suppose is that this chemical function was generated during the second guanylation step of the monomer synthesis through an intermolecular reaction between the central amine of a first Boc-protected monomer **1** (shown in grey in **Scheme 2**) and the carbonyl group of a Boc belonging to the diBoc-guanidino moiety of a second one (in black, **Scheme 2**). In brief, the proposed reaction mechanism proceeds via an isocyanate intermediate that is formed from the Boc protecting group on the guanidine function. This E_{1cB} elimination occurs in basic condition for the same monomer. The deprotonation of guanidino nitrogen allows an intramolecular rearrangement with the consequent loss of *tert*-butoxide. The latter contributes to the deprotonation of the previously protonated amine (in grey) which can act as nucleophilic agent. Then the tautomerization through proton transfer produces the amidinourea moiety (**Scheme 2**).[137,139] However, we hypothesized another possibility: the same byreaction could occur in the batch storage between the deprotected monomer (**1**) and its mono- or di-Boc intermediates resulted from the not completion of the final deprotection step, even if the amine function is present as a salt.



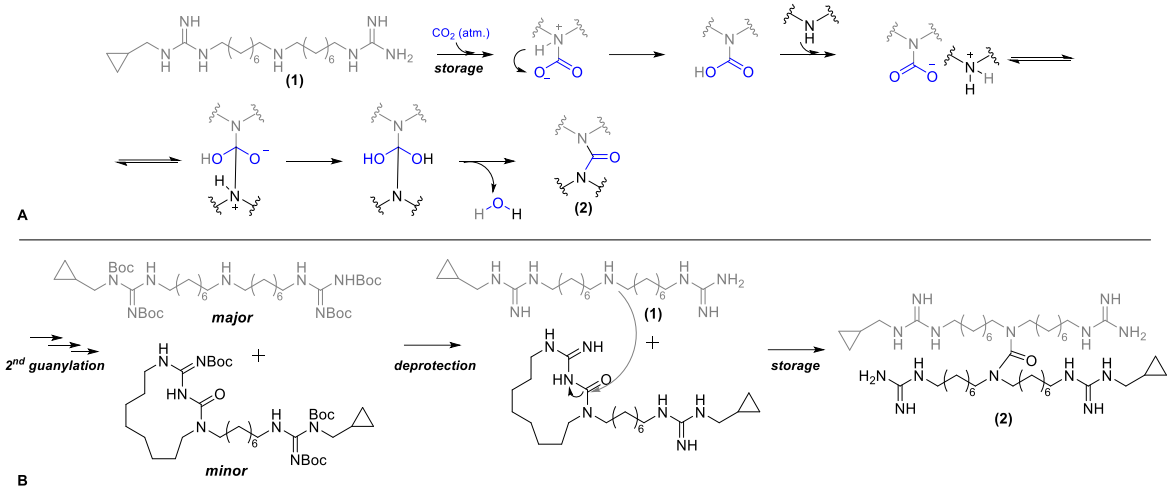
Scheme 2. One of the proposed mechanisms of amidinourea moiety formation, the characteristic function of the asymmetrics, herein the formation of the asymmetric dimer **3** is reported.

Considering that the symmetrics are endowed with a urea moiety, two possible mechanisms were proposed to explain the source of the carbonyl group that links the central amine of the monomers (**1**).

According to the first hypothesis (**Scheme 3A**), a reaction between the central amine of **1** (in grey, **Scheme 3A**) in the storage solution and atmospheric carbon dioxide occurred: the nucleophilic addition was followed by a proton transfer furnishing the corresponding carbamic acid that reacted with another molecule of **1** (in black, **Scheme 3A**). The dehydration of the obtained adduct gave the urea moiety. This transformation has been exhaustively described as a convenient strategy to capture waste carbon dioxide through the linkage with amine functional adsorbents.[140]

The second proposed mechanism (**Scheme 3B**) involves a macrocyclic derivative, whose formation could occur during the second guanylation step in the monomer synthesis via an intramolecular reaction according to the same mechanism showed in **Scheme 2** and already reported in our previous

work.[137] The concomitant presence of the monomer (in grey, **Scheme 3B**) and this macrocyclic byproduct in the storage solution could allow the nucleophilic addition of the central amine of **1** to the carbonyl group of the amidinourea macrocycle. The following ring opening furnished the urea moiety.



Scheme 3. Hypothesized mechanisms of urea moiety formation involving atmospheric carbon dioxide (**A**) and a macrocyclic byproduct (**B**). Here in the formation of the symmetric dimer (**2**) is reported.

However, to confirm the structure of **B** and **C**, we turned to the synthesis of all the possible isomers **2-5** (**Figure 19**), with the aim to compare their retention time and accurate mass and to discover the actual responsible for the biological activity. The preparation of the target molecules was challenging and sometimes the yields were very low but allowed us to obtain and characterize them. The chromatograms of **2-5** obtained by the same HPLC method showed a perfect correspondence between the two symmetric isomers (**2** and **4**) and the eluates **B** and **C** of the mixture. Furthermore, we performed a quantitative analysis of the fraction containing eluates **B** and **C** with the same separation method above mentioned and it revealed that the **B/C** ratio, corresponding to the molar ratio of compound **2**/compound **4**, was 7/3. This molar ratio was extrapolated from appropriate standard calibration curves of compounds **2** and **4** obtained through HPLC-UV/MS signals.

In the end, all the synthesized oligomers **2-5** were tested on a panel of eight representative bacteria, showing a notable antibacterial activity, especially on Gram-positive organisms but showed a different biological profile according to their isomerism: the symmetric isomers (**2** and **4**) were more active than their asymmetric counterparts (**3** and **5**). The symmetric dimer (**2**) exhibited the most potent activity on all the tested organisms, with MIC values ranging from 0.5 to 8 µg/mL, instead the asymmetric dimer (**3**) apparently lost most of its activity on Gram-negative ones. The symmetric trimer (**4**) was moderately active against all the tested species, while the asymmetric one (**5**) had a good activity on Gram-positive pathogens, in particular, *E. faecalis* (**Table 3**). Taking into account that the accepted methodological variation in this kind of biological assays is ± 2 twofold MIC dilutions (for example, a MIC value of 2 µg/mL could correspond to an interval ranging from 0.5 to 8 µg/mL), data for the eluates **B + C** can be considered comparable to the ones of compound **2** and compound **4**, because the difference between them is included in the accepted range of experimental error. However, we could not exclude a synergistic activity between the two compounds in order to explain the slightly higher activity of the eluates **B + C**, which has been observed in our laboratory with some compounds belonging to this chemical series. In conclusion, these antibacterial activity data overall support that the two symmetric oligomers were most likely the active components of the original mixture, thus confirming our study. [135]

Table 3. MIC determination for the initial mixture, eluate **A**, **B+C,** and compounds **1-5**.

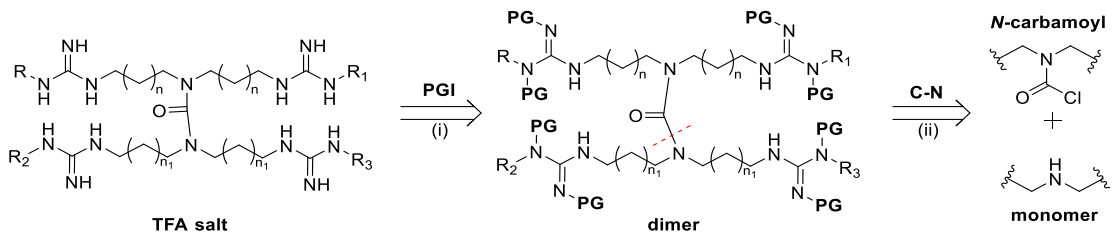
Test samples	MIC (µg/mL)[a]							
	E. coli CCUGT.	*K. pneumonia* ATCC 13833	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 17978	*S. epidermidis* ATCC 14990	*E. faecalis* ATCC 29212	*B. subtilis* ATCC 6633	*S. aureus* ATCC 25923
Initial mixture[b]	0.5	1	8	4	0.5	1	0.5	4
Eluate **A** (**1**)	64	> 64	64	> 64	64	> 64	64	> 64
Eluates **B+C**	1	2	16	16	0.5	1	0.5	-
1 – monomer[c]	64	> 64	> 64	> 64	> 64	> 64	64	> 64
2 – sym. dimer	2	2	8	8	1	2	0.5	2
3 – asym. dimer	>64	>64	>64	>64	8	8	<0.125	4
4 – sym. trimer	4	4	16	8	4	4	4	8
5 – asym. trimer	8	8	32	32	4	<0.125	8	8
Colistin	0.5	0.5	0.5	1	-	-	-	-
Vancomycin	-	-	-	-	2	1	0.5	1
Daptomycin	-	-	-	-	0.25	1	1	0.5

Colistin, vancomycin, and daptomycin are reported as control antibiotics. -: not determined. [a]MICs are expressed as the average values calculated from experiments performed at least in triplicate. The first four tested strains are Gram-negatives (*E.c., K.p., P.a, A.b.*), the last four ones are Gram-positives (*S.e., E.f., B.s., S.a.*). [b]First batch of **1** as published that turned out to be a mixture of eluates A, B, and C.[134,135] [c]Newly synthesized batches of **1**.

5. Extension of the chemical class of symmetric dimers.

The encouraging results obtained from this serendipitous discovery[135] led to the design of some derivatives, structurally analogous to compound **2**, in order to explore preliminary SARs by changing alkyl chains length, type and number of substituents.[141]

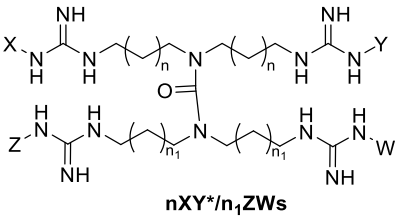
The general synthetic pathway used to prepare the dimeric series (**Scheme 4**) consisted in the connection between two (bis)guanidino building blocks: a monomer and an appropriate carbamoyl derivative, as reported in our previous work.[136]



Scheme 4. General retrosynthetic approach for dimers. (i) protecting groups, (ii) *C-N* disconnection.

A coding system to uniquely identify the dimers and their chemical structure was adopted (**Figure 21**). In particular, dimers are named with an alphanumeric code consisting of three parts. The first number indicates the number of carbon atoms in the alkyl chains, while the following two letters identify the substituents present on the two guanidine moieties of the monomer. The carbamoyl derivatives are identified through the code of the corresponding monomer with an asterisk; the dimers with the

respective codes of their constituting building blocks separated by a slash and the correspondent trifluoroacetate salts have the dimeric code followed by the letter *s*. For example, dimer **2** is identified with the code: **8CH*/8CH s**.



X, Y, Z, W: different groups and combinations, such as:
H: hydrogen; **C**: cyclopropylmethyl;
E: ethyl; **M**: methyl; etc.
***:** carbamoyl intermediate
s: trifluoroacetate salt

Figure 21. Generic scaffold of dimeric derivatives and legend of the code adopted.

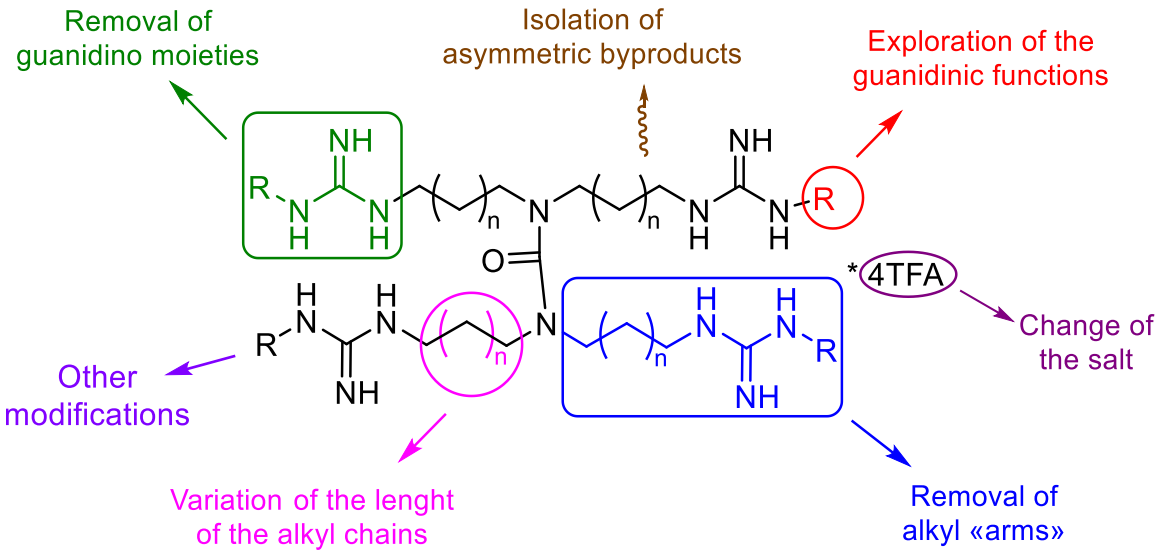
Dimer **6** (**10CH*/10CHs**), characterized by the increase of two carbon atoms in the alkyl linker, was synthesized and biological data highlighted a modest reduction of the activity (**Table 4**).
Then, the cyclopropylmethyl substituents of **2** were replaced with methyl or ethyl groups (compounds **7** (**8MH*/8MHs**) and **8** (**8EH*/8EHs**), which were chosen as substituents because of their smaller alkyl size. These compounds retain the activity trend of **2**. In particular, by comparing the MIC values of these compounds, we observed that in general the activities of these compounds were a bit worse than the one of **2** but the difference was never higher than one or two dilutions. The activity of **7** (**8MH*/8MHs**) and **8** (**8EH*/8EHs**) against the Gram-positive *S. aureus* resulted to be higher than that of **2**. In the end, dimers with a substitution on all the guanidino functions **9** (**8EE*/8EEs**) and **10** (**8CC*/8CCs**) emerged as an active compound but with higher MIC values than the hit compound **2**.

Table 4. MIC values of some dimeric derivatives of the series and control antibiotics against selected strains of Gram-positive and Gram-negative bacteria.

Cpd	Cpd Code	MIC (μg/mL)[a]							
		E. coli CCUG[T]	*K. pneumoniae* ATCC 13833	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 17978	*S. pyogenes* ATCC 12344	*E. faecalis* ATCC 19433	*B. subtilis* ATCC 6633	*S. aureus* ATCC 25923
2	**8CH*/8CHs**	2	2	8	8	1	2	0.5	2
6	**10CH*/10CHs**	16	8	64	64	4	4	2	8
7	**8EH*/8EHs**	2	2	16	16	0.5	1	1	1
8	**8MH*/8MHs**	1	4	8	8	0.5	1	0.5	0.5
9	**8EE*/8EEs**	2	4	32	16	0.25	1	0.5	0.5
10	**8CC*/8CCs**	4	4	64	8	2	4	8	16
	Colistin	0.5	0.5	0.5	1	-	-	-	-
	Vancomycin	-	-	-	-	0.5	1	0.5	1
	Daptomycin	-	-	-	-	0.125	1	1	0.5

Colistin, vancomycin, and daptomycin are reported as control antibiotics. [a]MICs are expressed as the average values calculated from experiments performed at least in triplicate. The first four tested strains are Gram-negatives (*E.c., K.p., P.a, A.b.*), the last four ones are Gram-positives (*S.p., E.f., B.s., S.a.*).

Results and Discussion



1. Aim of the work

Encouraged by the good biological data conducted on the oligomers **2-5** (**Table 3**) and in particular on the dimeric analogues **6-10** (**Table 4**), in my PhD project, we decided to better investigate this new chemical class, focusing our efforts on the study of the mode(s) of action and the structure-activity relationships (SARs). To do this, compound **2** was re-synthesized on a large scale and new derivatives obtained by little changes in the chemical structure of **2** were rationally designed and synthesized. In brief, SAR based on the length of the carbon atoms in the linker that connects the urea function to the guanidino moiety was investigated by synthesizing dimers with six carbon atoms (**11**, **6CH*/6CHs**) and hybrid dimers composed by a 6-membered and 8-membered monomers (**12**, **8CH*/6CHs** and **13**, **8CC*/6CCs**) (**Figure 22**).

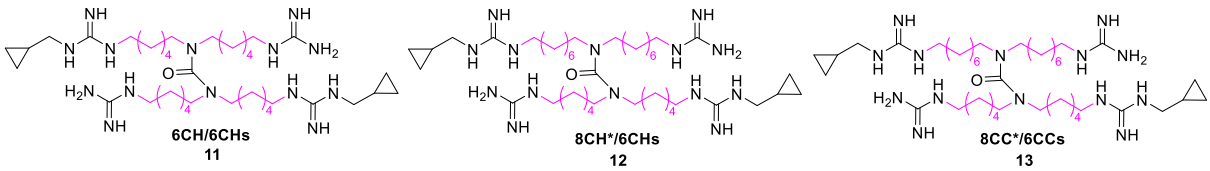


Figure 22. Structures of dimer derivatives **11-13** obtained by changing the linker length.

As regard as the substituents on the guanidino moieties, in order to perform little changes in terms of pKa and lipophilicity, compounds with two or four benzyl groups were synthesized (**14**, **8BH*/8BHs** and **15**, **8BB*/8BBs**, **Figure 23**). Also, in this case, we added the letter **B** for benzyl in the dimer identity code.

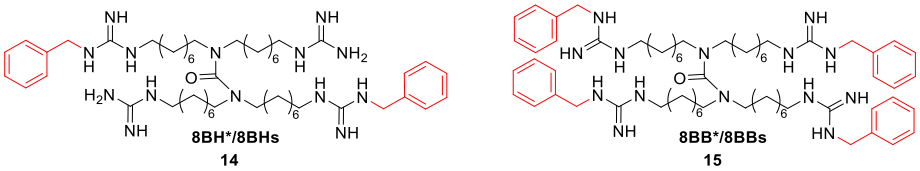


Figure 23. Structures of dimer derivatives **14** and **15** obtained by changing the substituent on guanidino moieties.

Then, a compound with free guanidine functions (**16**, **8HH*/8HHs**) was synthesized to understand the importance of the substitution. Moreover, we prepared dimer **17**, **8CC*/8HHs**, the isomer of **2**, being characterized by a symmetry axis instead of a plane and obtained by assembling two symmetric building blocks, one completely substituted, while the other no, to evaluate if this structural inversion would affect the activity. In the end, a dimer with three of these alkyl groups was prepared (**18**, **6CC*/6CHs**) (**Figure 24**).

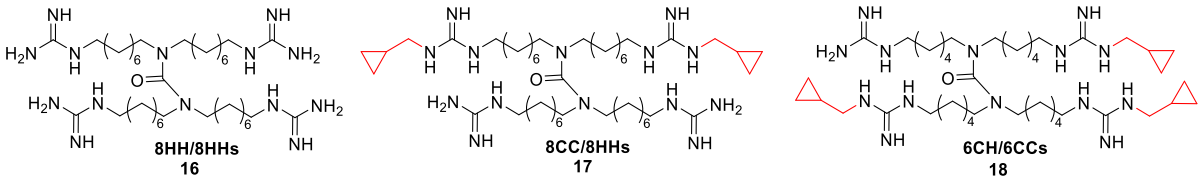


Figure 24. Structures of dimer derivatives **16-18** obtained by changing the number of cyclopropylmethyl substituents on guanidino moieties.

Furthermore, since preliminary cytotoxicity assays revealed a narrow selectivity index for dimer **2**, the most active derivative of the series, and trifluoroacetate salts are endowed with toxic behavior at high

concentrations, we prepared the dimer **2** as guanidinium hydrochloride salt (**19**), while attempts to prepare the stearate salt and the free-base derivative failed (**Figure 25**).

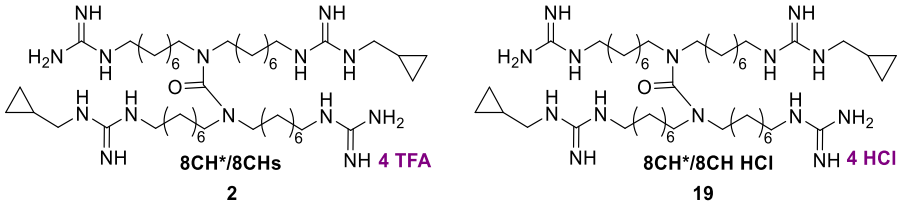


Figure 25. Structure of dimer as trifluoroacetate (**2**), hydrochloride (**19**) salts.

Furthermore, in literature toxicity and drug promiscuity are often attributable to high lipophilicity and high basicity of the molecule. Thus, we decided to prepare a dimer (**20**) characterized by the replacement of two guanidino moieties with amidinourea groups, that have lower predicted pKa (5.8) compared to that of guanidines (12.8).[142] This prediction was confirmed by computational studies on the tautomeric equilibrium between the two species **A** and **B** of the amidinourea. They demonstrated that the tautomeric state **B** is more stable since it can form a 6-member ring system by means of an intramolecular hydrogen bond (**Figure 26**) between the guanidino nitrogen and the ureic oxygen. The ring system plays a key role in the physicochemical properties, in fact stabilizing the molecule, it hampers the nitrogen protonation and consequently decreases the pKa.[142] Hence, the identity code of dimers was enriched by a new letter: **U**, which represents the $CONHCH_3$ moiety.

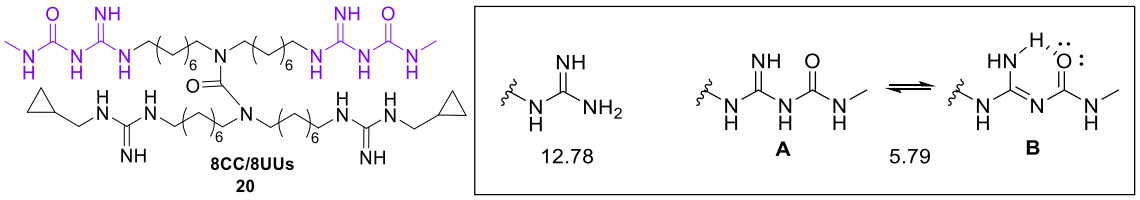


Figure 26. Left, structure of *N*-methyl amidinourea dimer (**20**); right, pKa values predicted through Marvin Sketch for guanidino and amidinourea moieties showed in the two major transitional states **A** and **B**.

Furthermore, during the synthesis of some monomers in large scale, very little amount (yield of 1-0.8%) of two asymmetric dimeric byproducts were isolated, deprotected **(21**, **Asy6CHs** and **22**, **Asy8HHs**, **Figure 27**) and, finally, sent to biological evaluation.

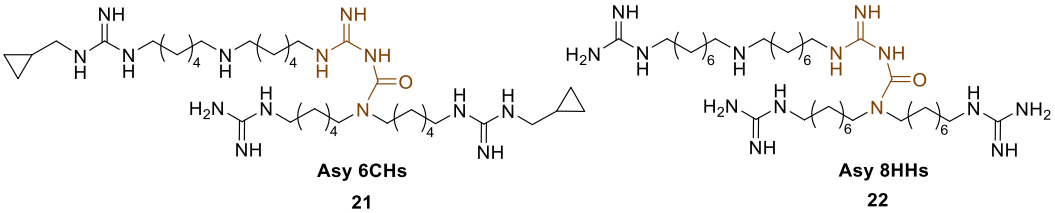


Figure 27. Structures of isolated asymmetric dimers **21** and **22**.

To better understand the pharmacophore features of the dimers, starting from compound **10** (**8CC/8CCs**), characterized by a faster preparation, some changes in the chemical structure were performed. In particular, we prepared derivatives characterized by the removal of one, two or three *arms* which are the whole linker that connects the urea to the guanidine moiety, obtaining *N,N,N'*- and *N,N-'* or *N,N*- polysubstituted urea derivatives (**23-25**, **Figure 28**).

For these new derivatives, the identity code of dimers was implemented to report different types of building block. In this series, the derivatives are identified by reporting them with **8C** (number of carbon atoms and letter of guanidine substituent) which is one linker on the urea moiety and the urea

terminal(s) in brackets such as **(NH)** or **(NHMe)**. For example, derivative **23**, **8CC*/8C(NH)s**, is a compound with three *N*-substitutions of 8 carbons on the central urea bearing a cyclopropylmethyl guanidino function (**8CC** and **8C**).

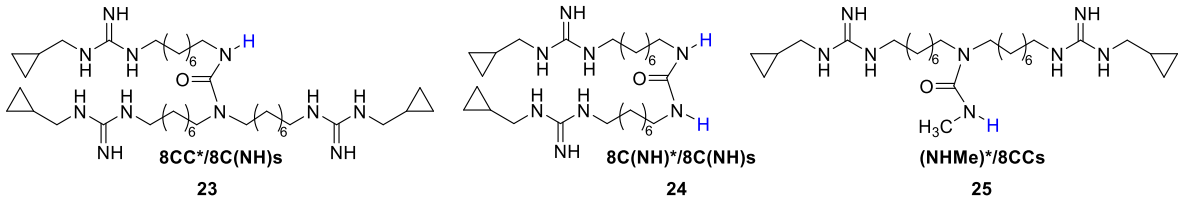


Figure 28. Structures of derivatives **23-25** obtained by removing *arms* from the central urea moiety.

Then, the investigation of the SAR led us to prepare some derivatives characterized by the *shut-down* of the guanidino group, by removing the whole function. Hence, derivatives which bear one (**26**), two (**27** and **29**) or three (**28**) *turned off* guanidines were synthesized by changing some steps in the synthetic pathway (**Figure 29**). In this case, the code reports both the linkers in the building block (number of carbons and letter of the substituent), adding a dash (**-**) when the guanidino function is absent. For example, derivative **26**, **8C8-*/8CCs** is endowed with three standard *N*-substitutions of an 8-membered alkyl chain bearing a cyclopropylmethyl guanidino moiety and another of 8 atoms without the guanidine (**8-**).

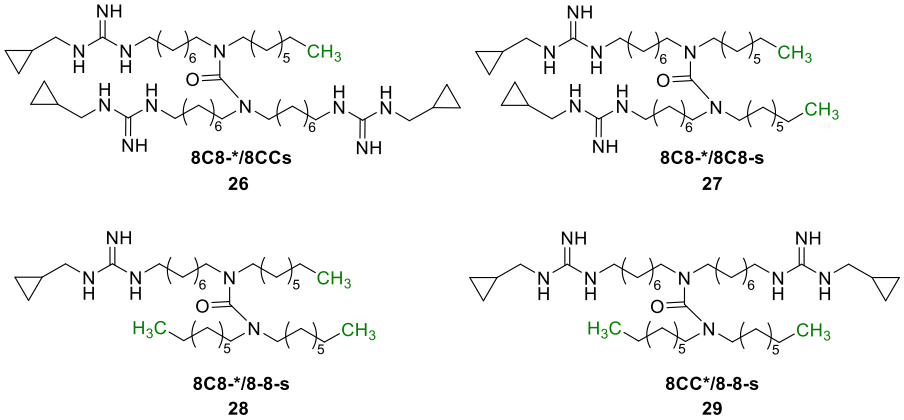


Figure 29. Structures of derivatives **26-29** obtained by *turning off* guanidine functions.

In the end, good data on selectivity index highlighted by compound **26** drove us to the synthesis of three derivatives obtained by changing the carbon atom length of the linker (**30-32**, **Figure 30**).

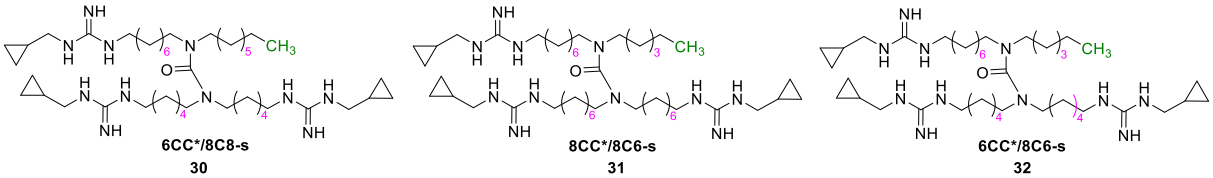


Figure 30. Structures of derivatives **30-32** obtained by changing the length of the linker in the mono-*turned off* guanidine derivative.

In the end, new monomers were prepared with the purpose to synthesize a larger library of self-coupled and hybrid dimeric derivatives, such as the homologs of compound **33** (**8CH)** with three, seven, and ten carbon atoms in the linker (**34**, **3CH**; **35**,**7CH** and **36**, **10CH**), a monomer with a longer alkyl (such as the octyl terminal, **37**, **8OH**) or alkenyl (such as the geranyl terminal, **38**, **8GH**) terminal on the guanidine (**Figure 31**). Here again, letters **O** and **G** were added to the dimer code, meaning octyl and

geranyl respectively. Future self-coupled or hybrid derivatives based on these new monomers will be prepared.

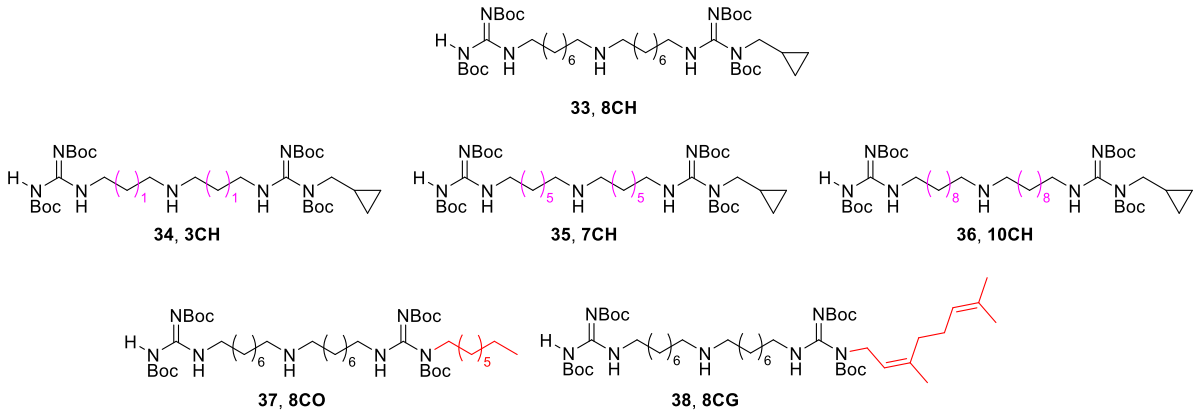


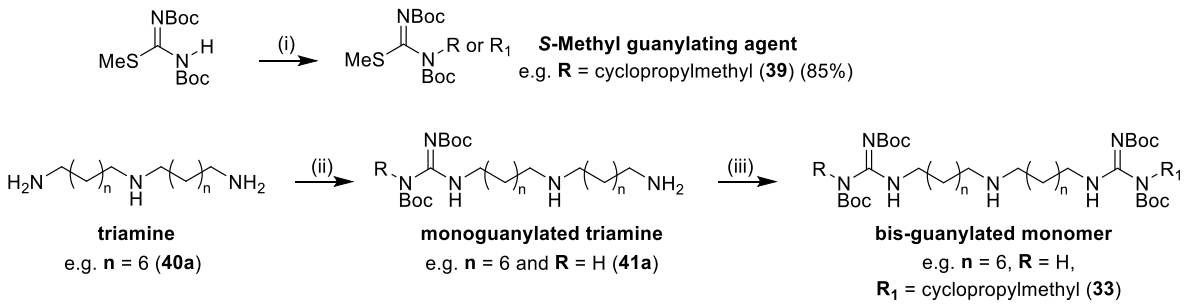
Figure 31. Structures of **33**, **8CH** and new monomers **34-38** for future dimer preparation.

All the above-mentioned modifications are discussed in detail in the following paragraph.

2. Chemistry

Preparation of monomers as building block for dimers.

The synthetic procedure for the preparation of the monomeric building blocks reported in the literature[126] (**Scheme 5**) was based on the subsequent two guanylation steps with appropriately *N*-substituted *S*-methyl guanylating agents (e.g. **39a** bearing a cyclopropylmethyl group) on a **triamine** derivative with a different number of carbon atoms (e.g. **40a** with n = 6) through a previously prepared and appropriately substituted guanylating agent. Unfortunately, this approach was not easily accessible for 8-membered monomers since the starting material 1,17-diamino-9-azaheptadecane (**40a**) was no longer commercially available.



Scheme 5. Previous synthetic approach to prepare the monomeric building block. *Reagents and conditions*. (i) ROH, MeOH, DIAD, PPh_3, dry THF, 0 °C to reflux, 16 h; (ii, iii) **Appropriate guanylating agents**, THF/MeOH, 50 °C, 16 h.[126]

Thus, initially, we chose to set up a synthetic approach to prepare the monoguanylated triamine **41a** as a useful intermediate for **33** (**8CH**) and the asymmetric monomers, which are the molecules characterized by two different guanidino terminals, a proton (**H**) and a carbon substituent (**C**, **B**, **O**, **G**). Indeed, the above-described strategy was not taken into account for monomers with 8 carbon atoms because the first guanylation step of the triamine derivative **40a** (step ii, **Scheme 5**) involved a vast excess of this latter compared to the guanylating agent and a consequent enormous waste of the synthesized starting material. The stoichiometrical ratio of 3/1 was necessary for this reaction (ii) to obtain the guanylation on only one of the two reactive groups (the primary amines) identical to each other.

Thus, in our research group different synthetic approaches were performed: although the preparation of secondary amines has been widely investigated, the traditional procedures are often problematic and challenging because of harsh reaction conditions, poor yields, low chemical selectivity, a vast range of similar byproduct formation, difficult or not-possible purifications.[143,144] Moreover, in our case, the oily physical state of almost all the intermediates of the synthetic pathways did not help the isolation procedures, making traditional chromatography often necessary also for very polar compounds. We tried unsuccessfully to convert the oily amines into solids through, when possible, the addition of different acids but, unfortunately, also the corresponding salts of these compounds were found to be oils. The major traditional synthetic strategies to prepare secondary amines can be classified as reported in **Figure 32**.

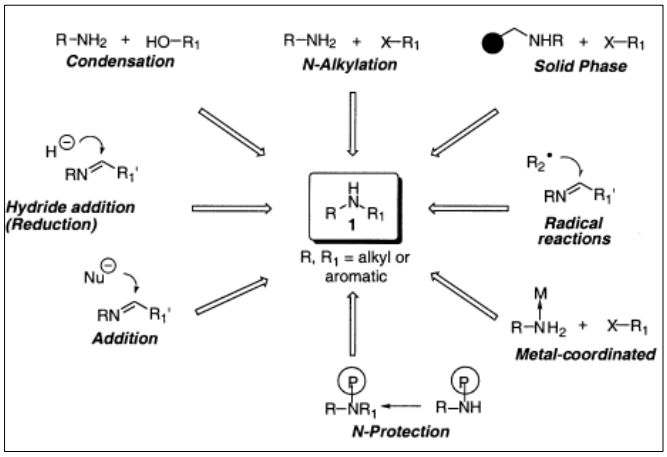
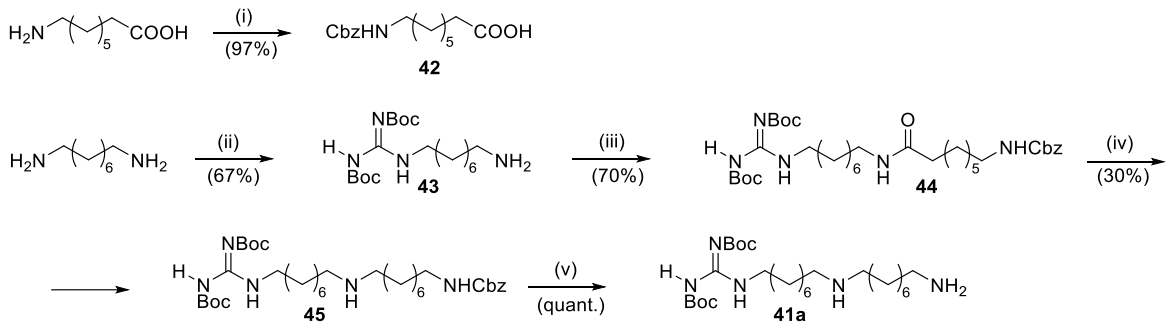


Figure 32. Synthetic approaches for the preparation of secondary amines adapted from Salvatore et al.[143]

During my PhD, we turned to the following strategies:
1) preparation of the amide precursor and reduction,
2) *N*-monoalkylation of primary amines,
3) *N*-alkylation of benzylamine and *N*-benzyl removal.

The first approach, reported in **Scheme 6**, was adapted from Sanguinetti et al.[145]
The amino group of 8-aminooctanoic acid was protected with Cbz in order to avoid the intramolecular amidation in the following step, yielding compound **42**. Then, 1,8-diaminooctane was monoguanylated with *N,N'*-diBoc-1*H*-pyrazole-1-carboxamidine. This guanylating agent was preferred to 1,3-Bis(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea to avoid the heating of the reaction mixture. In fact, to carry out the reaction at temperatures higher than room temperature can cause the formation of side products for intermolecular cross-reaction between the amino group of **43** and the carbonyl group of the Boc belonging to another molecule of **43**, furnishing an amidinourea moiety. Then, **43** was coupled with the carboxyl acid of **42** giving the linear amide **44**. The reduction of **44** was challenging and furnished the amine **45** with a very poor yield.[145] However, attempts by using LAH or $NaBH_4$ after amide activation with triflic anhydride, as reported in the literature[146,147] failed. In the end, the reductive cleavage furnished **41a** in quantitative yield. Although the synthetic pathway was straightforward, the amide reduction impaired the yield significantly.

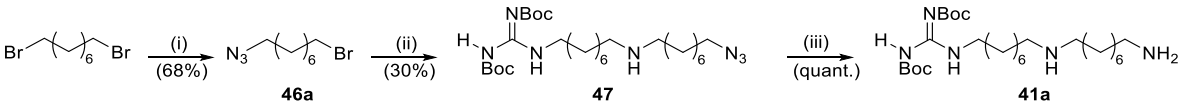


Scheme 6. Synthetic approach for compound **41a** through the amide precursor. *Reagents and conditions:* (i) Benzylchloroformate, K_2CO_3, THF, r.t, 3h; (ii) *N, N'*-diBoc-1*H*-pyrazole-1-carboxamidine, DIPEA, r.t., CH_3CN/MeOH 9/1, 16h; (iii) **42**, EDC, HOBt, DIPEA, DMF, 0 °C to r.t., 16 h; (iv) DIBAL(H), DCM, -78°C to r.t., 16 h; (v) H_2, Pd/C, HCl, *i*-PrOH, r.t., 16 h.

Another approach was tried by performing a monosubstitution of a primary amine function, leading to the secondary amine of monoguanylated derivative **41a**. The synthetic pathway is reported in **Scheme 7** and starts from the nucleophilic substitution of 1,8-dibromooctane by sodium azide to yield **46a**. This reaction was carried out in a diluted DMF solution and different stoichiometrical ratio between the two starting material were tried in order to avoid the generation of the diazido byproduct. Sodium azide/bromide 1:0.60 was found to furnish the product in higher yield. Moreover, increase of the temperature (70°C in lieu of 50°C) resulted in favoring the disubstitution, while when the time of reaction was extended to 24 h in lieu of 12 h, the product yield was increased.

The use of sodium azide and organic azido derivatives could raise concern since they are known to be potentially toxic, explosive and shock-sensitive compounds. In fact, sodium azide is toxic, absorbed through the skin and reacts violently with heavy metals, acid solutions, and chlorinated solvents. Thus, standard safety precautions must be taken, such as avoiding a high scale use. Furthermore, the stability of organic azides depends on their chemical structure and in order to predict their manipulability the *rule of six* is particularly useful: there must be six carbon atoms every energetic functional group (diazo, azido, nitro, etc.) in the molecule to provide sufficient stability, while less than six carbons per functional group may result in being explosive. [148] Also, the number of nitrogen atoms must not exceed that of carbons and the ratio of the sum of the number of carbon and oxygen atoms over that of nitrogen must be major or equal to three, never lower than one.[149] However, the amount and the type of compounds synthesized in our laboratory were safe and did not raise any major concern, taking the appropriate care. For example, according to the previously mentioned rules, the octyl derivative **46a** has a C/N ratio of 2.7, thus can be safely isolated and stored.

Then the resulted 1,8-bromoazido derivative **46a** underwent another substitution by the monoguanylated derivative **43**. In order to avoid multiple *N*-alkylations, forming tertiary amine and quaternary salt as major products, cesium hydroxide was used as base and coordinating metal (**Figure 33**).



Scheme 7. Synthetic approach for compound **33** through Cesium Hydroxide. *Reagents and conditions:* (i) NaN_3, DMF, 50 °C, 24 h; (ii) **43**, $CsOH{\cdot}H_2O$, molecular sieves, dry DMF, r.t., 24 h; (iii) PPh_3, H_2O, THF, r.t., 16 h.

Cesium hydroxide exerts opposing reactivity towards primary and secondary amines, favoring high chemoselectivity in nucleophilic substitution. While the hydroxide ion acts as a base, the cesium ion forms a complex with a primary amine in dry conditions by virtue of its behavior as a Lewis acid. The metal coordinates to primary amine via a soft acid-soft base interaction that makes the amine protons acidic. Thus, they are abstracted by the hydroxide and the desiccant activity of molecular sieves causes

the removal of water and the formation of the cesium amide. This intermediate reacts rapidly with the bromide, generating the secondary amine, that, being a strong base, coordinates to cesium more strongly than the corresponding primary amines, furnishing a relatively more stable and sterically hindered complex. The reaction solvent is important, in fact, this interaction can occur only in a polar aprotic solvent (DMF) since cesium is weakly coordinated to the hydroxide anion (**Figure 33**).[150]

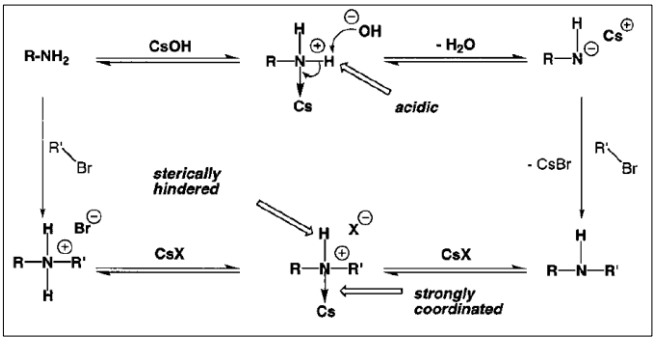


Figure 33. Mechanism of *N*-monoalkylation of primary amine in presence of cesium hydroxide. Adapted from Salvatore et al.[150]

Although the reaction was carried in flame-dried glassware in an inert atmosphere, the recorded yield of secondary amine **47** was very low (30%). In the end, the azido moiety was converted into amine via Staudinger reaction, giving compound **41a**.

Staudinger reaction allows the conversion of the azido group to amine in milder condition than reductions: the mechanism of reaction is based on the attack of the lone pair electrons of triphenylphosphine towards the terminal nitrogen atom of the azide, furnishing a linear phosphazide intermediate. This intermediate undergoes intramolecular rearrangement via a four-membered ring transition state to give the azaylide or iminophosphorane with the concomitant loss of N_2. The addition of water causes the hydrolysis of azaylide, yielding the final amine and triphenylphosphine oxide (**Figure 34**). [151–153]

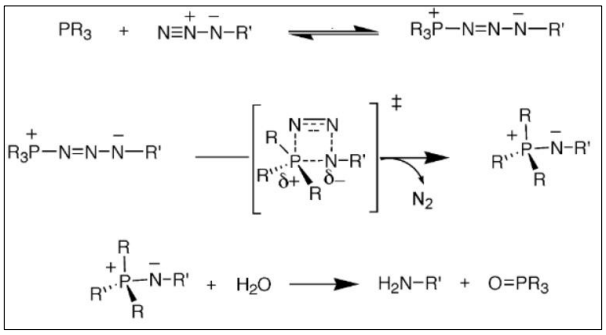


Figure 34. General mechanism of Staudinger reaction. Adapted from Lin et al.[153]

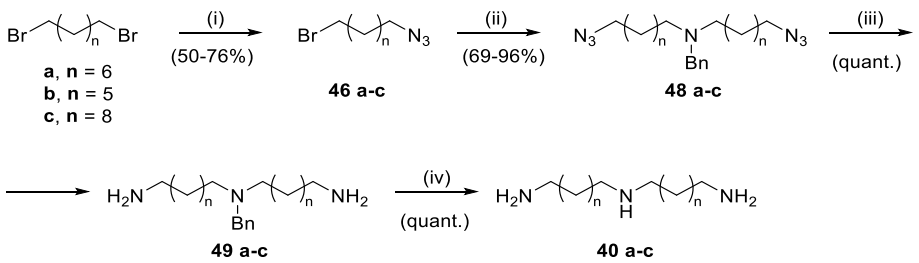
However, all these procedures, albeit no high-yielding, allowed us to obtain fast the 8-membered monoguanylated triamine **41a**, an important intermediate for the preparation of several monomers.

To prepare the other monomers, like those with the same guanidino terminals, and to compare the whole pathway yields to the previously described procedure, we decided to synthesize 1,17-diamino-9-azaheptadecane (**40a**).

Published procedures for the straightforward synthesis of symmetric triamine derivatives involved 1,8-diaminooctane as starting material and use Raney Nickel[154,155] or nitric acid at high temperature, [156] to perform the amine dimerization through the loss of ammonia. According to the first method, the addition of Raney-Nickel to 1,8-diaminooctane in refluxing dry benzene allowed the conversion of

primary to secondary amines, affording the desired triamine with low yield (28%). This protocol was also adapted to 4-, 5-, and 6-carbon chain diamines which were unable to produce the correspondent triamines in these conditions. Thus, they were previously monoacetylated in acetylacetate at 100 °C that reacted fast with the catalyst and, in the end, hydrolyzed to the triamines.[154,155] The second approach was based on the heating at 200°C of 1,8-diaminooctane in presence of concentrated nitric acid.[156] However, both the strategies are not safe and reproducible in academic laboratories, also for economic and sustainability reasons. In fact, in the first case, the use of benzene is not a safe alternative because of its carcinogenicity and the catalyst cannot be recycled, since it has a progressive loss of catalytic activity after the first cycle of reaction. As regard as the second procedure, it is a very low-yielding (12%), implying a big waste of starting material. Furthermore, in both the cases the described purification method is a high-temperature distillation in vacuum (200-220°C/4mmHg), not accessible through academic instruments. Similar one-pot attempts had been unsuccessfully performed in our research group, starting from 1,8-diaminooctane by catalysis of Pd/C 10% and microwaves.

In the end, a new cheap and convenient method was set up to make the whole synthetic process more accessible and versatile. It was based on a straightforward four-steps route and represents a modification of a recently published procedure.[157] We preferred azido groups, in place of the cyano moiety used by Zhang et al., in order to insert the terminal amino functions. We thus obtained the final product under milder conditions, higher yields and avoiding the use of a high amount of dangerous sodium cyanide and expensive Raney Nickel, used as a catalyst for cyano groups reduction. The development of this synthetic approach is fairly significant considering its versatility. The new procedure was easily readapted for the ten and seven carbon atoms derivatives by starting with the appropriate 1,n-dibromoalcane (**Scheme 8**).



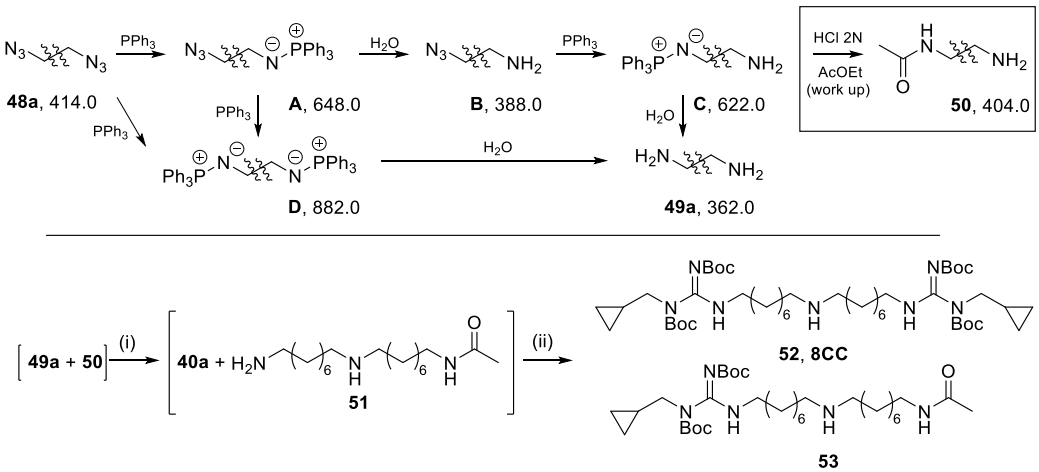
Scheme 8. Synthesis of triamine **40a-c**. *Reagents and conditions*: (i) NaN_3, DMF, 50 °C, 24 h; (ii) Benzylamine, KI, K_2CO_3, *n*-BuOH, 115 °C, 24 h; (iii) PPh_3, H_2O, THF, r. t., 16 h; (iv) H_2, Pd/C, AcOH, *i*-PrOH, r. t., 16 h.

A bis-alkylation of benzylamine with bromoazide **46a-c**, synthesized through the previously described procedure starting from the appropriate dibromoalcane,[135] was performed to obtain diazido derivatives **47a-c**. Unsuccessful attempts to reduce the azido functions and remove the *N*-benzyl group of compound **48a** simultaneously in just one reaction step to yield the triamine **40a** were made. In fact, being both functional groups sensitive to hydrogen, we tried to perform catalyzed hydrogenations by using palladium or palladium hydroxide on carbon also in large quantity (up to 1 eq.) in presence of the excess of different types of acid, such as concentrated HCl and glacial or aqueous acetic acid.[158] In fact, the increase of the amount of catalyst in the mixture should correspond to an improvement in the reaction in term of product yield.[159]

Thus, we resorted to a different procedure based on a two steps-one pot conversion of azides into amines **49a-c** via Staudinger reaction and *N*-debenzylation via acid-catalyzed hydrogenolysis. Both the reactions were quantitative, thus no other attempts to shorten the synthetic route were performed.

The above-described Staudinger reaction to obtain diamino derivative **49a** gave a side product when ethyl acetate was used as solvent during the work up. In brief, when MS(ESI+) monitorization of the

reaction seemed to highlight its completion, the crude was treated with ethyl acetate and hydrochloric acid 2 N to allow a separation between the product in the aqueous layer and the triphenylphosphine oxide resulting from triphenylphosphine in the organic counterpart. In this way, the polar diamine **49a** could be isolated through back-extraction for sodium hydroxide 2 N. In the first separation step, since the presence of an ester (the solvent ethyl acetate) and a strong acid (hydrochloric acid), little amount of unreacted monoamine-azaylide **B** was probably hydrolyzed by the resulting acetic acid giving the acetylated amine **50** (**Scheme 9**, top). Unfortunately, efforts on isolation of the acetylated byproduct from the reaction mixture failed since **49a** and **50**, being polyamines, are very polar compounds and were retained by both silica gel also by using very polar eluents with the addition of TEA or ammonia during the chromatographic purifications. Also, alumina chromatography was unable to separate the two compounds. Hence, the *N*-benzyl cleavage and the guanylation step on the other free amine group, though the bottom-described protocol, was carried out on the mixture of **49a** and **50**, reaching the isolation of **52** (**8CC**) and compound **53** that was characterized by NMR spectra (**Scheme 9**, bottom). In summary, ^{1}H NMR spectra in CD_3OD showed the characteristic signal of the methyl singlet of the acetyl function at 1.89 ppm and also in ^{13}C NMR carbonyl group and methyl group were found at 171.63 and 21.09 ppm and respectively. However, to avoid the formation of acetylated byproducts, ethyl acetate was replaced by diethyl ether during the work up.



Scheme 9. Top, possible mechanism of reaction of diazido compound **48a** and formation of acetylated side product **50** and $[M + H]^+$ signals detected during MS(ESI+) reaction monitorization; bottom, synthesis of acetyl monomer **53**. *Reagents and conditions*: (i) H_2, Pd/C, AcOH, *i*-PrOH, r. t., 16 h; (ii) cyclopropylmethyl guanylating agent, DIPEA, THF, r.t., 16 h.

The subsequent reductive *N*-benzyl cleavage was conducted with palladium on carbon with the addition of acid. This was necessary since nitrogen atoms and amines can poison the electrophile metal catalyst, resulting in the failure of the reaction, thus the use of acid additive, such as acetic acid, makes the Bn-*N* σ bond more polarized and more electrophilic for surface hydride attack in hydrogenolysis.[160,161] In fact, when the acid was added in a large amount (100 eq.), the reaction yield dramatically increased compared with those without or with a small amount of acid (0.1-10 eq.). We selected isopropanol as reaction solvent since we obtained a mixture of product and *N*-methyl or *N*-ethyl byproducts when methanol and ethanol respectively were used. Yet, MS signals of *N*-isopropyl byproducts were sometimes detected, but the ^{1}H NMR spectra showed their presence as very small impurities not quantifiable. A plausible mechanism of *N*-alkylation involves three steps: the alcohol used as solvent is first converted into the corresponding aldehyde or ketone by Pd/C-catalyzed dehydration, then it reacts with the amine, allowing the acid-catalyzed formation of the imine and, in the end, Pd/C-catalyzed hydrogenation yields the corresponding amine. Obviously, the byreaction proceeds faster for primary alcohol, such as methanol and ethanol, than for the secondary one.[162,163]

The guanylation reactions needed the preparation of appropriately substituted guanylating agents. Among the several possibilities,[103,164] we chose two guanylating agents already studied and used in our research group: *N,N'*-di-Boc-*S*-methylisothiourea and *N,N'*-di-Boc-*1H*-pyrazole-1-carboxamidine. As shown in **Figure 35**, this latter is one of the most effective, while the reaction with *S*-methylpseudothiourea occurs only after heating.

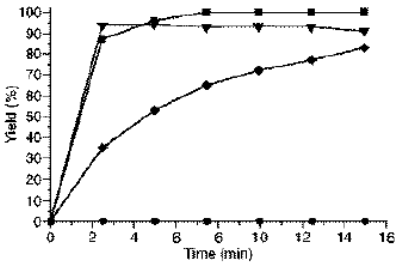
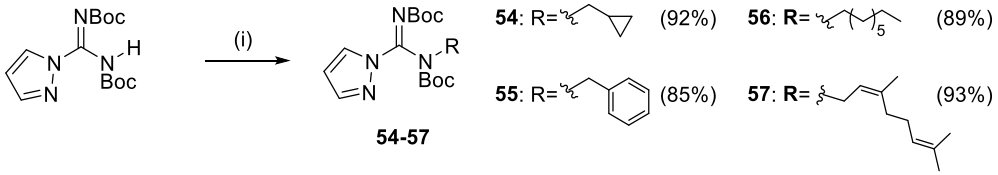


Figure 35. Rates of guanylation of benzylamine with (circle) *N,N'*-di-Boc-*S*-methylisothiourea, (triangle) *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine, (rhombus) *N,N'*-di-Boc-thiourea with Mukaiyama Reagent, (square) *N,N'*-di-Boc-2-triflyl-guanidine.[165]

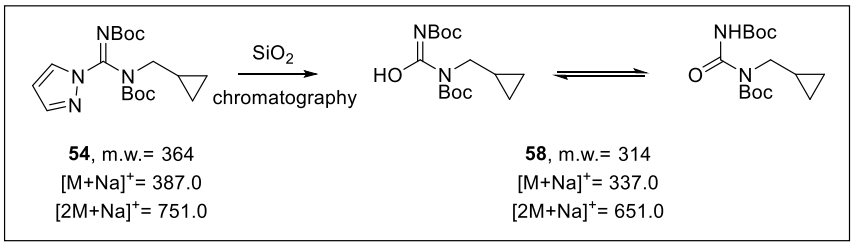
However, all the *N*-substituted guanylating agents were obtained through a Mitsunobu reaction between *N,N'*-bis(tert-butoxycarbonyl)-*S*-methylisothiourea (as shown in **Scheme 5**) or *N,N'*-di-Boc-1*H*-pyrazole-1-carboximidamide and the appropriate alcohol with DIAD and triphenylphosphine, as reported in **Scheme 10**.[136]



Scheme 10. Synthesis of *N,N'*-Di-Boc-pyrazole guanylating agents **54-57**. *Reagents and conditions*: (i) appropriate alcohol, DIAD, PPh_3, dry THF, 0 °C to reflux, 16 h.

The isolation of the products **54-57** was performed in a fast silica gel flash chromatography because the products were found to be not stable in silica if they were retained for a long time. Despite 2D TLCs did not show any degradation when we spend too much time for the chromatographic isolation of **54**, the reaction yields decreased and a different compound was obtained. ^{1}H NMR spectrum showed signals attributable to the alkyl fragments but the lack of those of pyrazole.

In particular, from MS analyses we found that the obtained byproduct, probably, had a molecular weight of 314 g/mol. In fact, the MS spectrum of the product **54** shows signals of the sodium adduct and the sodium double mass and the byproduct MS peaks were 337.0 and 651.0 (**Figure 36**). Hence, we supposed that silica, being weakly acid, could hydrolyze the imidazole, a good leaving group, from the guanidine, giving the urea **58**. However, this hypothesis should be confirmed by in-depth studies and by the synthesis of compound **58** to match the characterization data.



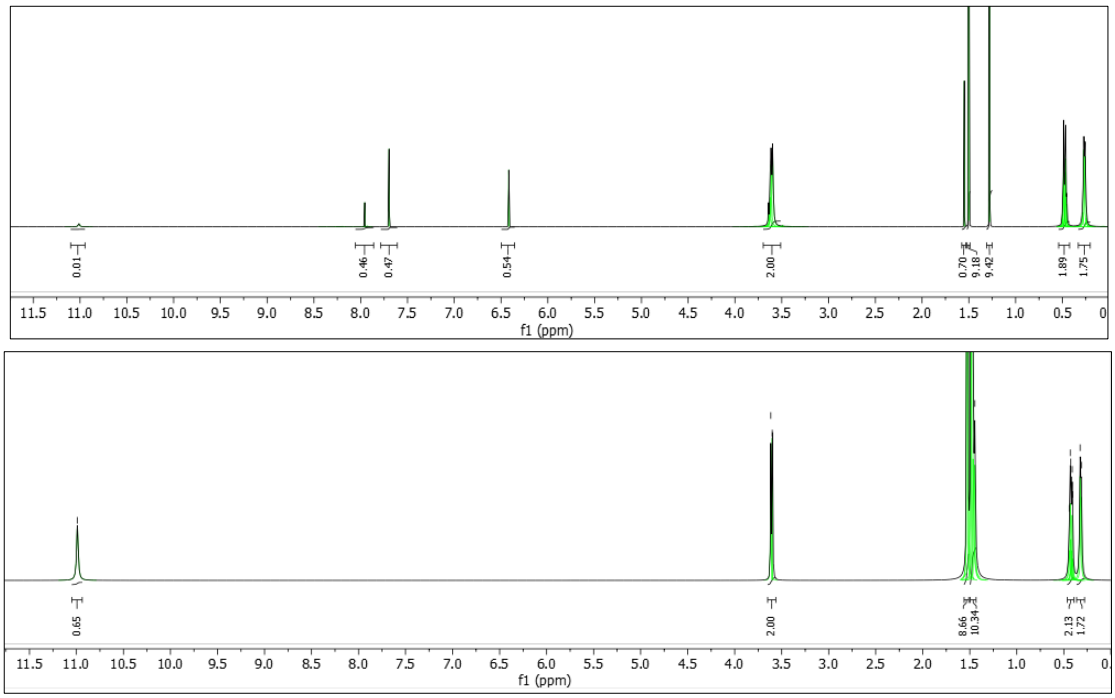
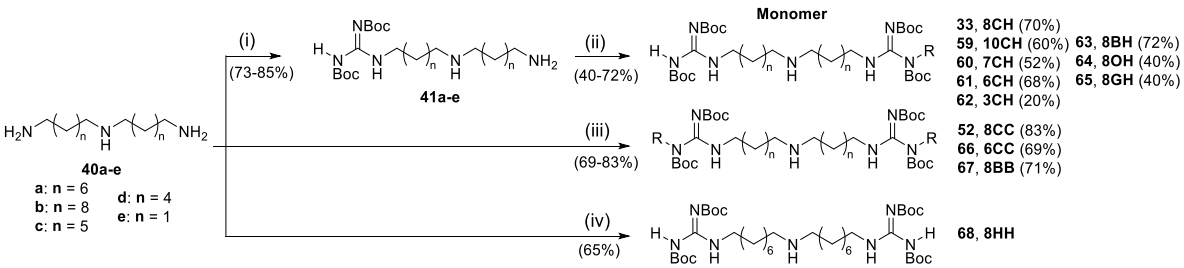


Figure 36. Scheme of the conversion of **54** in the hypothesized byproduct **58** and zoom of their ^{1}H NMR spectra obtained for solvent subtraction, **54** on the top and **58** on the bottom.

The obtained triamine **40a-c** or the commercial triamines with six (**40d**) and three carbon atoms (**40e)** were thus guanylated as shown in **Scheme 11**. The appropriate commercial or synthesized guanylating agents were used in the right stoichiometric ratio, in order to obtain monomers with the alkyl substitution on one (i, ii), two (iii) or no (iv) guanidine functions (**Scheme 11**).



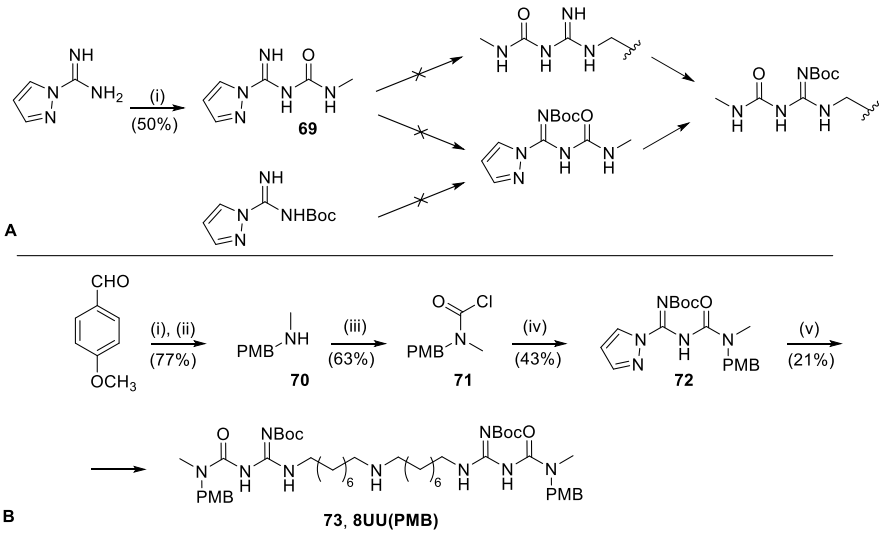
Scheme 11. Synthesis of monomers **33**, **52**, **59-68**. *Reagents and conditions*: (i) 1,3-Bis(tert-butoxycarbonyl)-*S*-methyl-2-thiopseudourea, THF/MeOH 5/3, 45 °C, over 16 h; (ii) appropriate guanylating agent **54-57**, DIPEA, THF, r.t., 16 h; (iii) *N,N'*-di-Boc-*N*-alkyl-1*H*-pyrazole-1-carboxamidine **54** or **55**, THF/MeOH, r.t., 16 h; (iv) *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine, THF/MeOH,r.t., 16 h.

In brief, to obtain the monoguanylated triamine **41a-e** and to avoid the bisguanylated byproduct, very slow addition of *N,N'*-bis(tert-butoxycarbonyl)-*S*-methylisothiourea to a diluted solution of the appropriate triamine **40a-e** in large excess was conducted by means of a syringe pump or a dropping funnel. The reaction mixture was stirred for 10 hours after the addition was completed and the heating was necessary to allow the reaction to proceed, as mentioned before. The workup of the reaction was conducted with care, taking into consideration the formation of methanethiol as a byproduct.

Methanethiol is an extremely flammable and toxic gas, characterized by a distinctive putrid smell. Thus, when the reaction was complete, the exhaust gas from the reaction was run through a trap with bleach that is able to oxidize and quench the thiol group. In the end, a filtration on silica-packed column allowed to isolate the product. Only a little percentage of the unreacted triamine **40a** was recovered when the crude was purified through alumina gel chromatography because of its very high polarity.

The second guanylation was performed by using the appropriately *N*-substituted *N,N'*-di-Boc-*1H*-pyrazole-1-carboximidamide (**Scheme 11**); when the correspondent *N,N'*-bis(tert-butoxycarbonyl)-*S*-methylisothiourea (**39**) was employed, it was necessary to heat the reaction mixture at 40-50 °C to favor the substitution and the release of methanethiol as leaving group. However, any substitution occurred at r.t., while the heating allowed the isolation of the products only in lower yields, as observed for the synthesis of **33** (35% in lieu of 70%).

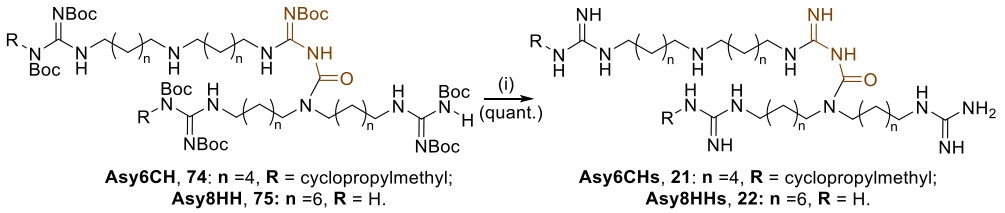
With respect to the preparation of the symmetric bisguanylated monomer, such as **52**, **66**-**68**, the reaction mixture was very concentrated and, in this case, the limiting reagent was the triamine **40a**,**b**. In order to synthesize an *N*-methyl amidinourea building block, the development of a new synthetic strategy was required. First, we prepared a specific ureic-guanylating agent **69**, yielded by a reaction between *1H*-pyrazole-1-carboxamidine hydrochloride and *N*-Succinimidyl *N*-methylcarbamate, a methyl isocyanate-mimicking agent. Unfortunately, the absence of the Boc group surrounding the carboxamidine makes the guanidino carbon less electrophile and less prone to undergo the nucleophilic attack by triamine **40a**. Efforts to insert the Boc group on **69** or to insert the *N*-methyl ureic moiety on *N*-Boc-*1H*-pyrazole-1-carboxamidine with the same above-mentioned procedure were unsuccessful (**Scheme 12A**). Thus, a different agent was designed and synthesized (**Scheme 12B**). In brief, starting from the *p*-anisaldehyde, reductive amination with the aqueous solution of methylamine was performed, giving the derivative **70**, which was reacted with triphosgene, furnishing the carbamoyl derivative **71**. This latter was coupled with *N*-Boc-*1H*-pyrazole-1-carboxamidine, furnishing **72**, that successfully reacted with the triamine **40a**, giving a PMB-protected *N*-methyl amidinourea monomer **73** (**Scheme 12**).



Scheme 12. Failed (A) and successful (B) attempts to prepare a monomer bearing the *N*-methyl urea moiety. *Reagents and conditions*: A, (i) *N*-Succinimidyl *N*-methylcarbamate, DIPEA, dry DCM, r.t., 16 h; B, (i) $MeNH_2$ aq. sol., MeOH, r.t., 1 h; (ii) $NaBH_4$, MeOH, 0 °C to r.t., 1 h; (iii) Triphosgene, DIPEA, dry DCM, 0 °C to r.t., 1h; (iv) *N*-Boc-*1H*-pyrazole-1-carboxamidine, NaH, dry THF, 0 °C to reflux, 12h; (v) **40a**, DIPEA, THF/MeOH, r. t., 16 h.

During a large-scale preparation of monomers **61** (**6CH**) and **68** (**8HH**) and, in particular, in the second guanylation step, besides the products (yields: 68 and 65%), we isolated the asymmetric derivatives **74** (**Asy6CH)** and **75** (**Asy8HH**) in very low yield (1.1-0.8 %), generated with the mechanism described

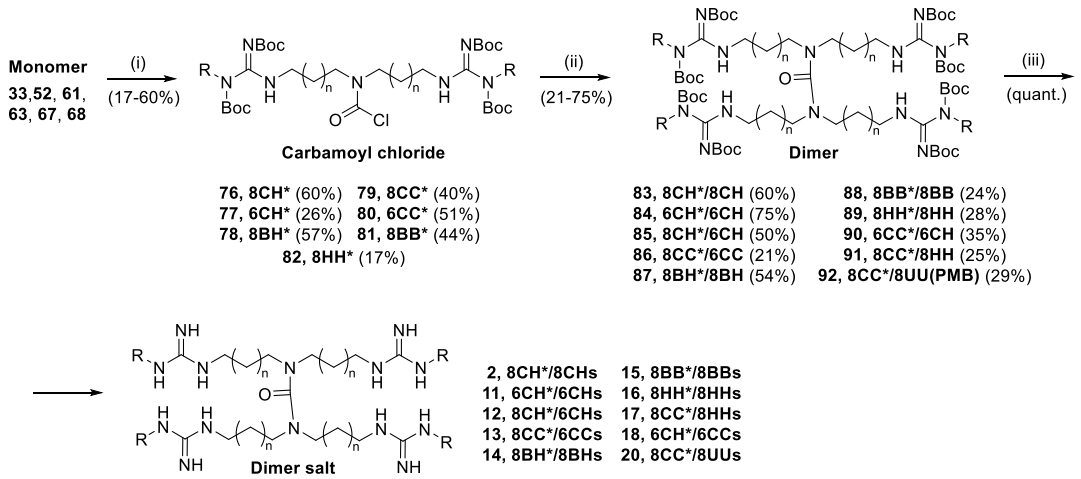
in **Scheme 2**. Then, they were deprotected, furnishing **21** and **22** and their antibacterial activity was evaluated (**Scheme 13**).



Scheme 13. Synthesis of asymmetric dimers **21** and **22**. *Reagents and conditions*: (i) TFA 20%, dry DCM, r.t., 7h

Preparation of dimers.

In our research groups, the coupling step between the central amines of two monomers was studied with several condensing agents, such as CDI,[166] but the resulting imidazole adduct did not react with the amine of the second monomer, probably due to the hindering by the alkyl chains. Hence, we sorted out by *N*-carbamoylating one monomer via triphosgene, a safer alternative than phosgene,[166] in dry conditions and then coupling this resulting carbamoyl derivative to a monomer, as reported in **Scheme 14**. In fact, as expected for *N,N*-disubstituted compounds,[167] carbamoyl chloride derivatives are stable and can be isolated and stocked in dry conditions, in contrast to the isocyanates derived from primary amines.



Scheme 14. General synthetic pathway for dimers. *Reagents and conditions*: (i) Triphosgene, DIPEA, dry DCM, 0 °C to r.t., 0.5-3 h; (ii) appropriate **monomer** (**33**, **52**, **61**, **63**, **67-68**), DIPEA, NaI, dry DCM, ref., sealed tube, 72 h; (iii) TFA 20%, dry DCM, r.t., 7h.

Initially, we tried to perform the dimerization step in a one-pot reaction unsuccessfully, either by employing few equivalents of triphosgene or by adding the second monomer after the complete carbamoylation of the first building block.[168–170] Probably these one-pot approaches failed because the optimal conditions for the two single reactions, as investigated later, are different. In fact, although both the reactions must be set up in anhydrous conditions, the carbamoylation must be carried out with a proper dilution and at low temperatures (0 °C to r.t.) to avoid the acid-catalyzed removal of Boc protecting groups, while the coupling can occur only in a concentrated environment and requires reflux temperature. We suppose that the long alkyl chains could cause a steric hindrance and the acid environment generated by triphosgene could provoke the protonation of the basic monomer, resulting in a salt and hampering the urea formation.

The procedure for the carbamoylation reaction was developed after several attempts. Initially, triphosgene was added portion-wise as solid, then different aprotic solvents, such as THF and DCM,

several molarities for triphosgene solution and the addition of different equivalents of a base were tried in order to achieve the optimal conditions. In particular, better results were obtained by using DIPEA as a base, in fact, being a tertiary amine, it is a good acceptor of hydrochloride acid which is released by triphosgene. Moreover, DIPEA allows full utilization of the starting material and a reduction of the equivalents of triphosgene used (mechanism of reaction showed in **Figure 37**).[167]

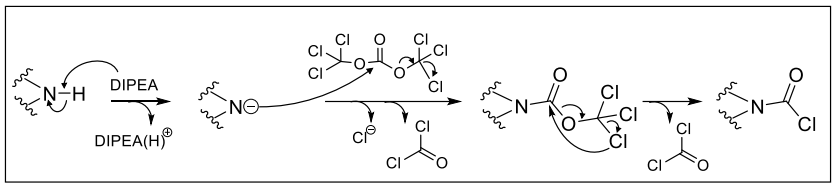


Figure 37. Mechanism of carbamoyl derivatives formation by triphosgene and DIPEA in dry DCM.

However, this reaction should be still improved because it represents the bottle-neck step of the whole synthetic pathway, generating several byproducts which are not always easy to identify in order to avoid their production. For example, to reduce and, in the end, to avoid the formation of Boc-cleaved side products, especially the more predominant mono-Boc ones, more diluted solutions were prepared to be added to the monomer. The monitorization of the reaction was conducted by means of TLC and MS(ESI+) setting the fragmentor at 0 mV and dissolving the sample in DMSO. In fact, when the analysis was performed at higher fragmentation energy (such as 70 mV), the spectra showed several mass spectrometry artifacts, such as compounds with cleaved-Boc or carbamic acid. Moreover, MS monitoration for direct injection needed the sample preparation in DMSO since the presence of MeOH can cause the conversion into the correspondent methyl carbamate (**Figure 38**).

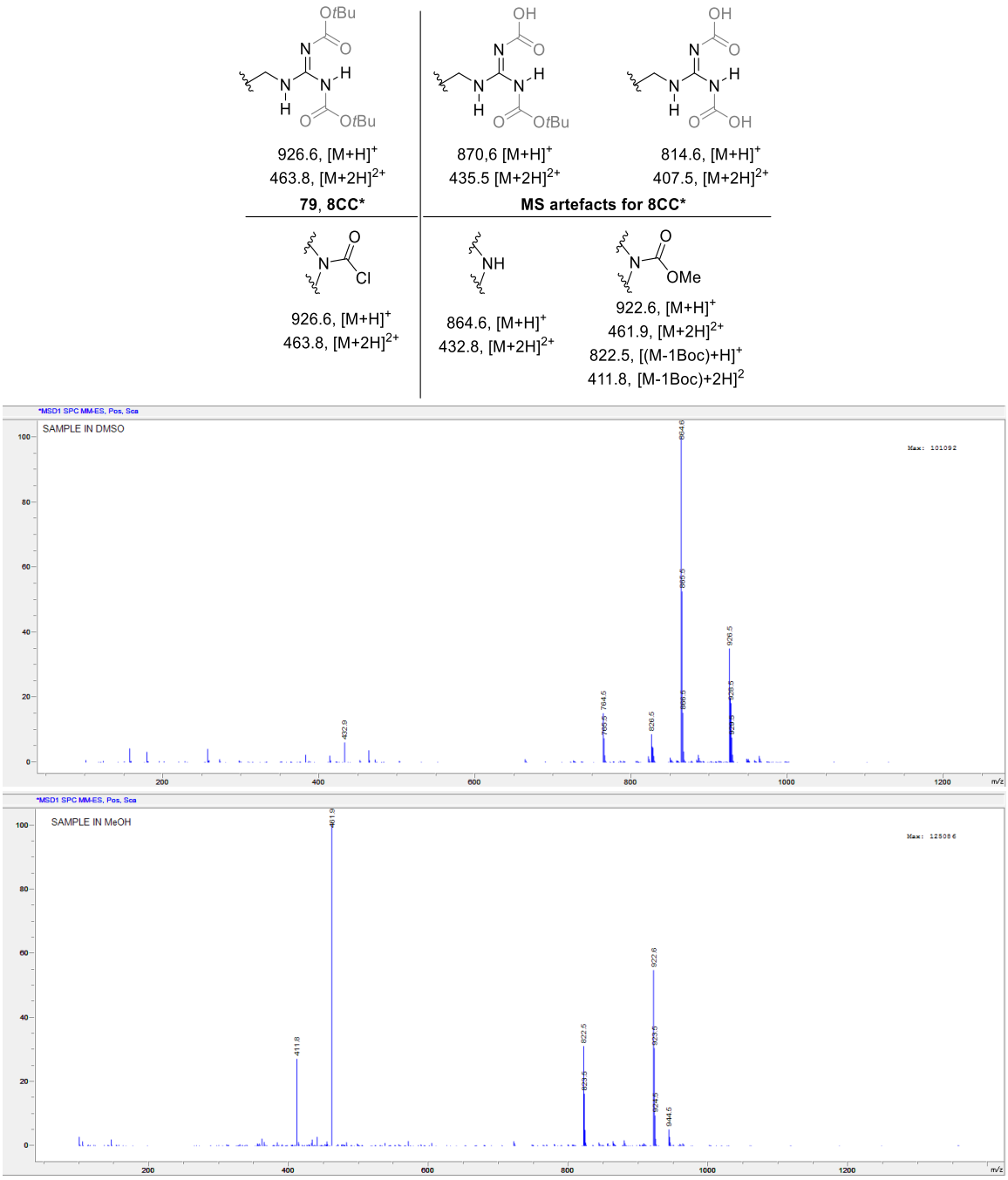


Figure 38. Structure of carbamoyl derivative **79**, **8CC*** and its artifacts at MS spectrometer and MS(ESI+) spectra of the same compound dissolved in DMSO (top) or MeOH (bottom).

In the end, the dimerization was carried out by refluxing the appropriate monomer and carbamoyl derivative in dry condition in presence of anhydrous DIPEA and a catalytic amount of NaI. In our laboratories, the addition of this salt was found to improve the yield in reaction with similar substrates. Indeed, iodide is widely used as catalyst in many single-step bimolecular nucleophilic substitutions (S_N2) to enhance the reactivity of alkyl or aryl halides (Finkelstein reaction).[171] Recent studies confirmed that also acid chlorides can be activated using a simple iodide source to undergo nucleophilic attack from relatively weak nucleophiles. The hypothesized mechanism of reaction involves a transient iodide intermediate that represents a better leaving group than chloride.[172] The addition of catalytic iodide ion causes the generation of a reaction equilibrium between the chloride and the iodide derivatives. This equilibrium is driven forward by taking advance of the different solubility of the metal halide salts in the solvent and, in accordance with Le Chatelier's principle, the substitution of chlorine atom by the iodide is favoured since chloride salts are insoluble or less soluble

than the correspondent iodide in dry organic solvents and are consequently removed from the equilibrium by precipitation. [171,173] In the dimerization case, the amine of the other monomeric building block, albeit being a weak nucleophile, could be able to react with the transient carbamoyl iodide, which is not very stable, via an addition and elimination reaction (**Figure 39**).[172]

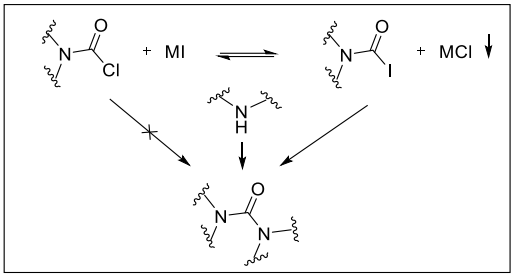


Figure 39. Mechanism of Finkelstein reaction adapted on *N*-carbamoyl derivative in the dimerization step.

The coupling procedure was changed by increasing the molarity of the monomer solution added to carbamoyl derivative and sodium chloride in dry DCM and by using a flame-dried sealed tube in lieu of the standard round-bottom flask. This latter was useful to maintain the reaction condition anhydrous, resulting in a relevant improvement in terms of yield.

In the end, the dimer derivatives were deprotected under acidic condition, furnishing the trifluoroacetate salt of the final products (**2**, **11-18**, **20**). The reaction was carried out at r.t. with a 20% solution of TFA in dry DCM. The proceeding of the deprotection is very slow,[174] probably because of the *N,N'*-diBoc-guanidino system is highly stable, being characterized by hydrogen bond pathways between the carbonyl oxygen atoms belonging to the Boc groups and the protons surrounding the guanidines[174] and the molecule is very flexible (**Figure 40**).

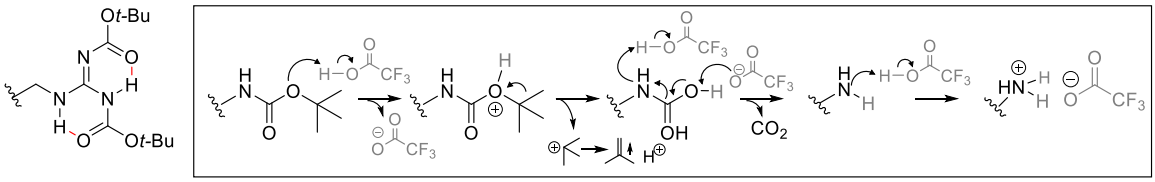
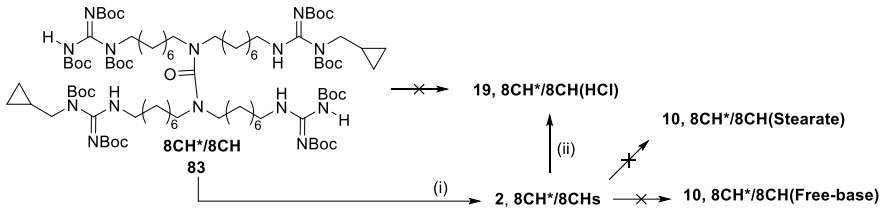


Figure 40. Left, *N,N*-diBocguanidino moiety and hydrogen bond pathway (red line); right, mechanism of Boc cleavage with TFA.

Preparation of different salts of dimer 2.

Unfortunately, a narrow selectivity index was found for dimer **2**, as representative of the whole series. Considering the toxicity of trifluoroacetic acid (TFA) and its salts in several cell lines[175–177] we decided to re-synthesize compound **2** by changing the anionic counterpart of the salt. Indeed, it is known that the presence of residual TFA or TFA anion can interfere with cellular assays by reducing cell proliferation or favoring cell viability. Furthermore, in view of *in vivo* experiments and potential uses in medicine, we developed a protocol to prepare more pharmaceutically suitable salts. Initially, we tried to perform the deprotection reaction on Boc-dimer **83** (**8CH*/8CH**) with concentrated hydrochloride acid in dry methanol or preparing the acid *in situ* through acetyl chloride in dry methanol but the obtained product did not respect the acceptable purity for biological evaluation (>95%), as detected by HPLC-UV-MS analysis. Thus, we converted the trifluoroacetate to the chloride salt by stirring a methanol solution of compound **2** at r.t. with an anion-exchange resin, Amberlite IRA 400 resin chloride form, allowing the isolation of the guanidinium chloride salt **19**. The completion of conversion was checked by ^{19}F NMR spectrometry which revealed the disappearing of the characteristic signal at 78.2 ppm in CD_3OD (**Scheme 15**).

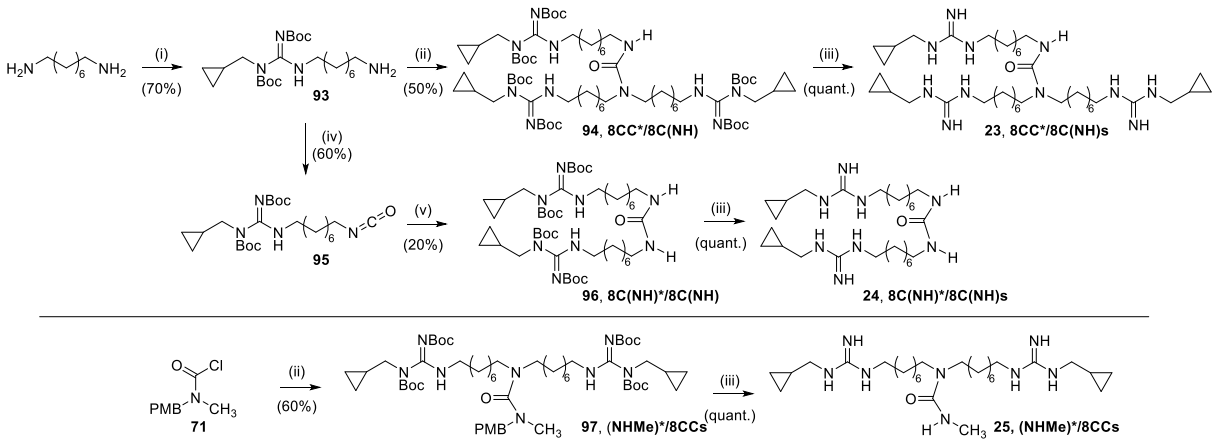
With the same purpose, we tried to prepare the stearate salt, being the stearate one of the safest counterparts. In fact, as confirmation, Ullmann's Fine Chemicals listed the toxicity of some guanidine salts, reported as the acute oral lethal dose (LD_{50}) in rats and it is 1120 mg/kg for guanidine hydrochloride and approximatively six-seven times higher for guanidine stearate (7500 mg/kg).[178] In literature we found a protocol in which the free-base amine of erythromycin was treated with an aqueous solution of stearic acid at 40-60 °C, yielding the correspondent stearate salt.[179] Thus, we tried to deprotonate the guanidinium groups of TFA salt **2** to obtain the free-base guanidines by stirring **2** with strong inorganic or organic bases, such as very concentrated sodium hydroxide aqueous solution and TEA or DBU in methanol. Also, the neutralization procedure based on the treatment of TFA salt **2** with the anion exchange Amberlyst A-26 OH^- form[180] or the addition of the resin directly on the reaction mixture after the completion of Boc removal in TFA failed (**Scheme 15**).



Scheme 15. Attempts to exchange or neutralize TFA salt for dimer **2**. Reagents and conditions: (i) TFA 20%, DCM, r.t., 9 h; (ii) Amberlite IRA 400 (Cl^-), MeOH, r.t. 72 h.

Preparation of *arms*-removed and *turned off*-guanidines derivatives

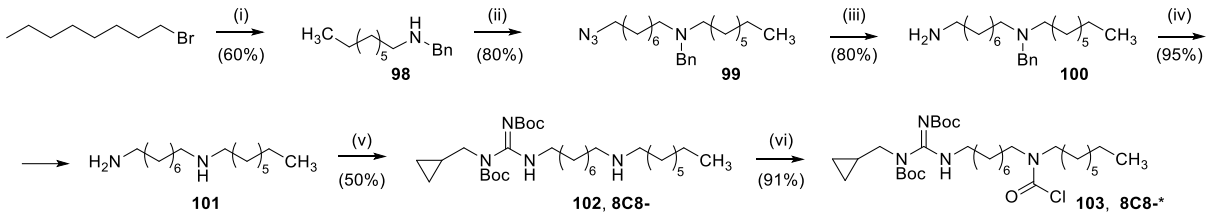
The investigation of the SAR led us to prepare some derivatives characterized by the removal of one or more *arms* bearing the guanidino function (**Figure 28**). To do so 1,8-diaminooctane was monoguanylated by the guanylating agent **54**. The resulted compound **93** was coupled with the carbamoyl chloride **79** (**8CC***) to prepare the three *arms*-composed compound **94** or converted into the correspondent isocyanate **95**, to be self-coupled, yielding the two *arms*-derivative **96**. Then, the already described compound **71** was coupled to **79** (**8CC***), furnishing the asymmetric two-*arms* derivative **97**. In the end, all the protected derivatives **94**, **96** and **97** were deprotected by TFA, furnishing the series **23-25** (**Scheme 16**).



Scheme 16. Synthesis of *arms*-removed derivatives **23-25**. *Reagents and conditions*: (i) **54**, DIPEA, THF/MeOH 3/1, r.t., 16 h; (ii)**79**, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h; (iii) TFA 20%, dry DCM, r.t., 4 h (vi) Triphosgene, dry DIPEA, dry DCM, 0 °C to r.t., 2 h; (v) **93**, DIPEA, NaI, dry DCM, sealed tube, ref., 24 h.

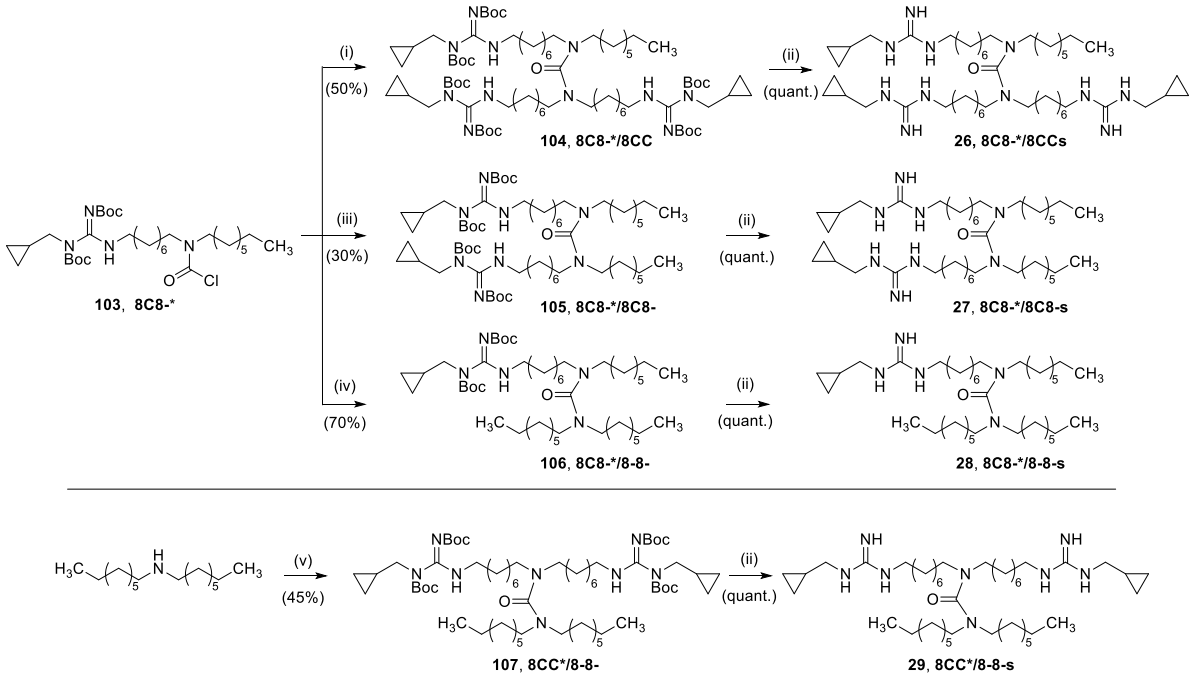
Furthermore, we synthesized compounds characterized by the *shut-down* of one or more guanidino groups, by removing the whole function. Hence, the preparation of derivatives which bear one (**X**), two

(**Y**) or three (**C**) *turned off* guanidines required the building block **8C8-** (**102**) that was synthesized by changing some steps in the previously described synthetic pathway (**Scheme 7**). In particular, this intermediate was obtained by two subsequent nucleophilic substitutions of benzylamine with the commercial 1-bromooctane (i, **98**) and 1,8-bromoazide derivative **46a** (ii, **99**), the azido function reduction (iii, **100**), the *N*-benzyl cleavage (iv, **101**), and the final guanlylation (v, **102**). In the end, the compound was *N*-carbamoylated, giving **103** (**8C8-***) in high yield (**Scheme 17**).



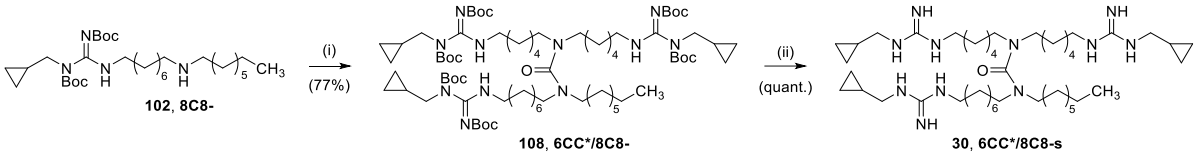
Scheme 17. Synthesis of the carbamoyl building block **8C8-*** (**103**). *Reagents and conditions*: (i) Benzylamine, K_2CO_3, *n*-BuOH, 115 °C, 24 h; (ii) Bromoazide **46a**, KI, K_2CO_3, *n*-BuOH, 115 °C, 16 h; (iii) PPh_3, H_2O, THF, r. t., 16 h; (iv) H_2, Pd/C, AcOH, *i*-PrOH, r. t., 16 h; (v) **54**, DIPEA, THF/MeOH 2/1, r.t., 16 h; (vi) Triphosgene, dry DIPEA, dry DCM, 0 °C to r.t., 4 h.

Then, compound **103** (**8C8-***) was coupled with monomer **52** (**8CC**), **102** (**8C8-**), and *N,N*-bisoctylamine to yield derivatives **104**, **105** and **106**, respectively. In the end, *N,N*-bisoctylamine was reacted with carbamoyl **8CC*** (**79**), furnishing a two *turned off*-guanidino derivative (**106**) which is an isomer of **105**. In the end, Boc groups of **104-107** were deprotected to obtain the guanidinium salts **26-29**, respectively (**Scheme 18**).



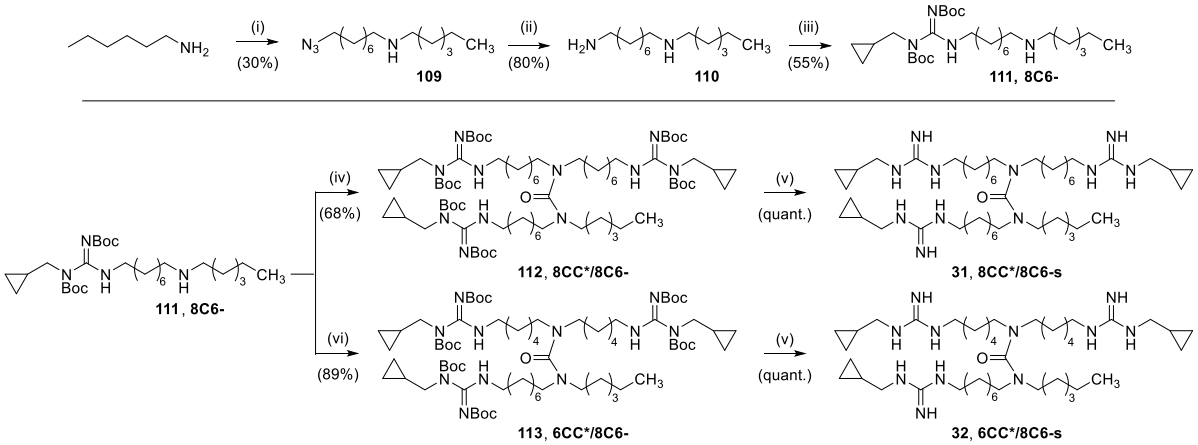
Scheme 18. Synthesis of turned off-guanidino derivatives **26-29**. Reagents and conditions: (i) **52**, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h; (ii) TFA 20%, dry DCM, r.t., 4-5 h; (iii) **102**, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h; (iv) *N,N*-bisoctylamine, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h; (v) **79**, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h;

Encouraged by a broader SI for compound **26** when compared to the other *traditional* dimers, derivatives with the same scaffold but a different length of the linker moieties were prepared. In particular, the **8C8-** monomer was coupled to **80** (**6CC***), yielding the analogous **6CC*/8C8-** (**108**), then deprotected to **27** (**6CC*/8C8-s**) (**Scheme 19**).



Scheme 19. Synthesis of *turned off*-guanidino derivatives **30**. *Reagents and conditions*: (i) **80**, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h; (ii) TFA 20%, dry DCM, r.t., 5 h.

The homologous monomer **8C6-** (**111**) was prepared by nucleophilic substitution of hexylamine with 1,8-bromoazide **46a** in presence of cesium hydroxide (i, **109**) and subsequent azido reduction (ii, **110**) and guanylation of the resulting amine (iii, **Scheme 20**, top). Thus, **8C6-** building block (**111**) was reacted with the carbamoyl derivatives **8CC*** (**79**) and **6CC*** (**80**), yielding the Boc-derivatives **112** and **113** that were finally deprotected, yielding **31** and **32** as TFA salts (**Scheme 20**, bottom).



Scheme 20. Synthesis of *tuned off*-guanidino derivatives **31** and **32**. *Reagents and conditions:* (i) 1,8 bromoazide **46a**, $CsOH{\cdot}H_2O$, molecular sieves, dry DMF, r.t., 24 h; (ii) PPh_3, H_2O, THF, r.t., 16 h; (iii) **54**, DIPEA, THF/MeOH 2/1, r.t., 16 h; (iv) **79**, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h; (v) TFA 20%, dry DCM, r.t., 5 h; (vi) **80**, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h.

3. Biology

Evaluation of antibacterial activity of the new derivatives.

The antibacterial activity of the newly synthesized compounds **2**, **11-32** was evaluated on a panel of representative microorganisms, including Gram-positive and Gram-negative type strains (**Table 5**). Although the compounds synthesized in this series were not enough to conduct an exhaustive SAR analysis, data reported in **Table 5** allowed us to do some preliminary but interesting observations, in terms of the length of the alkyl chains, the type and the number of the substituents on the guanidine functions and other significant modifications.

Table 5. MICs of the synthesized chemical library on representative Gram-positive and Gram-negative bacteria.[a]

Modification	Cpd	Cpd Code	MIC (µg/mL)[a]							
			E. coli CCUG[T]	*K. pneumoniae* ATCC 13833	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 17978	*S. pyogenes* ATCC 12344	*E. faecalis* ATCC 19433	*B. subtilis* ATCC 6633	*S. epidermidis* ATCC 14990
Hit compound	2	8CH/8CH s	1	1	4	4	1	<0.125	0.5	2
Lenght of linker	11	6CH*/6CHs	16	32	>64	>64	2	32	4	1
	12	6CH*/8CHs	8	4	16	16	0.5	4	2	1
	13	8CC*/6CCs	4	4	>64	64	1	8	4	2
N-substituent	14	8BH*/8BHs	4	2	8	8	-	2	2	-
	15	8BB*/8BBs	4	4	8	16	1	4	4	8
	16	8HH*/8HHs	4	32	32	16	1	2	32	-
	17	8CC*/8HHs	4	4	16	16	4	8	2	8
	18	6CC*/6CHs	8	16	>64	64	2	16	4	4
Salt anion	19	8CH/8CH HCl	1	1	4	4	1	0.5	0.5	2
N-function	20	8CC*/8UUs	4	4	16	8	2	4	4	2
Asymmetric	21	Asy6CHs	32	64	>64	>64	8	32	8	16
	22	Asy8HHs	1	2	16	32	0.25	1	0.5	8
	3	Asy8CHs	>64	>64	>64	>64	32	8	<0.125	8
Arm-removed	23	8C(NH)*/8CCs	8	8	64	64	1	8	1	1
	24	8C(NH)*/8C(NH)s	64	>64	>64	-	8	64	>64	>64
	25	(NHMe)*/8CCs	>64	>64	>64	>64	8	64	32	8
Turned off guanidine	26	8C8-*/8CCs	4	4	8	4	1	2	2	2
	27	8C8-*/8C8-s	>64	>64	>64	>64	8	32	>64	16
	28	8C8-*/8-8-s	>64	>64	>64	>64	16	>64	64	64
	29	8CC*/8-8-s	>64	64	>64	>64	4	>64	>64	>64
	30	6CC*/8C8-s	4	4	16	16	1	0.5	0.5	1
	31	8CC*/8C6-s	4	8	16	8	1	1	2	0.5
	32	6CC*/8C6-s	4	8	64	32	1	2	1	4
References		Colistin	0.5	0.5	0.5	0.5	-	-	-	1
		Vancomycin	-	-	-	-	0.5	1	0.5	-
		Daptomycin	-	-	-	-	1	1	1	-

Derivatives identity code. C: cyclopropylmethyl; **H**: hydrogen; **E**: ethyl; **M**: methyl; **B**: benzyl; **U**: $CONHCH_3$; **-** absence of the guanidino function; **NH** or **NHMe**: absence of the *N*-urea substitution. *****: carbamoyl intermediate; **s**: trifluoroacetate salt. Colistin, Vancomycin, and Daptomycin are reported as control antibiotics. [a]MICs are expressed as the average values calculated from experiments performed at least in triplicate. - : not determined. The first four tested strains are Gram-negatives (*E.c., K.p., P.a, A.b.*), the last four ones are Gram-positives (*S.p., E.f., B.s., S.e.*).

With regard to the alkyl chains, observing the reported biological data, the decreasing to six carbon atoms (**11**) resulted in a complete loss of antibacterial potency, in particular against Gram-negative strains, often detecting high MIC values. The hybrid dimers **12** and **13**, characterized by monomers with different length of carbon chains, exhibited an intermediate activity profile between the eight-membered (**2**) and six-membered (**11**) dimers.

The presence of two (**14**) or four (**15**) benzyl groups in lieu of the cyclopropylmethyl substituents caused a moderate decrease in terms of activity, especially on Gram-positive strains. Observing the biological data, it emerged that the increasing of the number and the lipophilicity of substituents did not lead to any improvement of the antibacterial profile. Compound **16**, with no substituent on the guanidines, showed moderate activity, highlighting the importance of alkyl moieties on the guanidine terminals. Despite our expectations, the comparison between the antimicrobial profile of **2** and its isomer **17** revealed that the symmetry inversion compromised the activity of the molecule, although their superficial charge was the same. Dimer **18** with three, instead of two, substituted guanidines was also evaluated and, despite maintaining a significant antibacterial activity on Gram-positive pathogens, it showed a very poor activity on Gram-negative organisms.

MIC values of the hydrochloride salt (**19**) were found to be almost identical to the correspondent trifluoroacetate derivative **2**

The *N*-methyl amidinoureas derivative **20** showed to be active against almost all tested strains, even if a decrease of the antibacterial potency was found in particular against the Gram-negative *P. aeruginosa*.

The isolation of **74** and **75** led us to investigate these derivatives belonging to the asymmetric series. In particular, the determination of MICs revealed a very good antibacterial profile for dimer **22** (**Asy 8HHs**). In fact, it showed a strong activity on almost all the tested strains and its MICs are always lower than those of the correspondent symmetric derivative (**16**, **8HH/8HHs**). This observation is in contrast with data for dimers **2** and **3** (**Asy8CHs**) or **18** (**6CH/6CHs**) and **21** (**Asy6CHs**), leading us to hypothesize a different kind of interaction with bacterial cells.

Little chemical modifications were performed, furnishing a library of compounds active against almost all the tested bacterial strains, showing in some cases a broad-spectrum activity. Eventually, we did not observe a significant change of the antibacterial profile and the hit compound **2** still remains the most potent compound. Hence, more different derivatives **23-29** were tested to gain a better understanding of the pharmacophores. In particular, we found that all the *N*-substitutions (*arms*) on the central urea are essential for the activity. In fact, the progressive removal of the moieties implied an increasing reduction of the antibacterial potency. Observing the data, compound **23** retained a certain activity on all the tested strains, while **24** and **25**, without two arms, were almost inactive. The investigation on the number of guanidino functions turned up the need for at least three guanidines in the molecule. When three (**28**) or two (**27** and **29**) of them were removed from the dimer, a significant worsening or loss of activity emerged. However, compound **26**, characterized by only one *turned off*-guanidine was found to retain an interesting activity profile, especially on Gram-negative strains. Thus, we prepared homologous derivatives of **26** by changing the length of the alkyl chain in the linker of two carbons. The biological evaluation of the three derivatives **30-32** did not highlight any substantial differences in terms of activity.

Evaluation of antibacterial activity in presence of plasma proteins.

Several antibiotics are characterized by a high affinity for plasma proteins, resulting in a strong binding that can cause a decrease in their concentration in the target site and a consequent decrease of potency. However, a lot of drugs that are highly bound are used to treat infectious diseases: for example, telavancin is a semi-synthetic derivative of vancomycin and, even though characterized by PPB major than 90%, it has received the approval by FDA in 2009. It was found to have clinical efficacy, also if it showed a 10-fold reduction of *in vitro* activity in presence of plasma proteins. Another example is the one of daptomycin: its *in vitro* antibacterial activity is inhibited by the presence of plasma but not so much as expected by its high PPB.[181] A high PPB can be sometimes used as an advantageous strategy for the treatment of infections in close proximity to the bloodstream, restricting the antibiotic distribution into the body tissues and allowing the increase of its concentration in the bloodstream. This useful strategy is used for cetirizine, a cephalosporine with very high PPB.[182] In the end, we should not forget that high PPB can be also used as a system of drug reservoir that allows its slow release.
However, in order to estimate the possible effect of plasma protein binding (PPB) on antibacterial activity of alkyl-guanidine derivatives, compound **2** was tested on *E. coli* MG 1655 in Mueller Hinton Broth (MHB) supplemented with 10% complement-free human serum (HS). *E. coli* MG 1655 was completely inhibited by 2 µg/mL of compound **2** (MIC; **Figure 41**) while 1 µg/mL did not affect the growth profile (½ MIC; **Figure 41**). The addition of 10% HS to MHB moderately increased its MIC value (2-fold), although growth *E. coli* MG 1655 was clearly retarded in MHB-HS supplemented with 2 µg/mL of compound **2** (MIC; **Figure 41A**). This result indicates that HS component(s) can reduce, but not abrogate, the efficacy of compound **2**. An obvious candidate is human serum albumin (HSA), the most abundant plasma protein (concentration 35–50 g/L),[183] which acts as a trap for a variety of ligands. Since bovine serum albumin (BSA) shares 76% sequence identity with HSA,[184] the susceptibility of *E. coli* MG 1655 to compound **2** was tested in MHB supplemented with increasing concentrations of BSA. Remarkably, the addition of BSA (5 to 40 mg/mL) to MHB did not alter the MIC value of compound **2** (**Figure 41B**), suggesting that other serum proteins (e.g. alpha-1-acid glycoprotein, AGP) could eventually affect the activity of compound **2** in HS. In fact, the binding assays performed on HSA and AGP (reported in ADME paragraph) confirmed a high affinity to this latter, without compromising the antibacterial activity. These results are compatible with a future development of the alkylguanidine derivatives as antibacterial agents. In fact, also if incremented, the observed MIC value for compound **2** on the tested *E. coli* strain in presence of plasma proteins can be considered a reliable prediction of a maintained antibacterial activity *in vivo* studies.

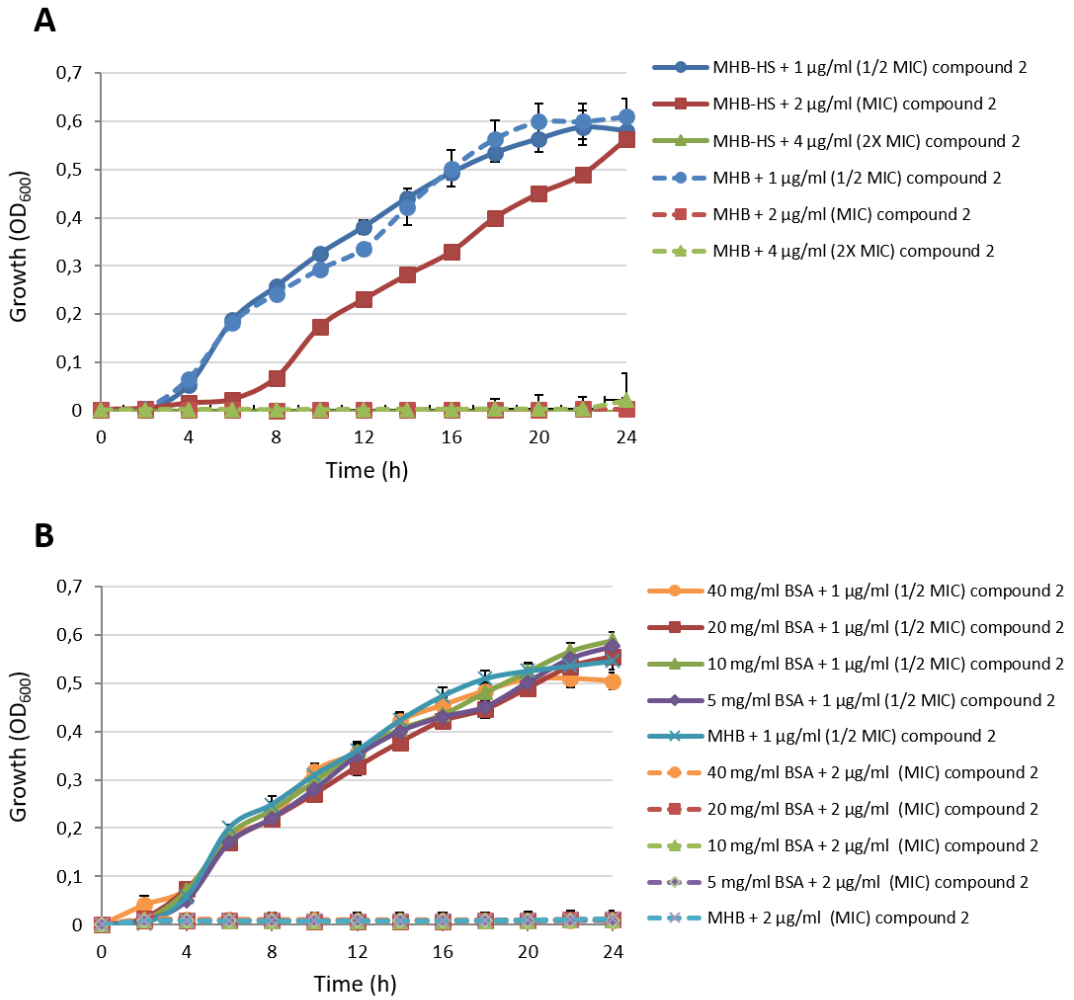


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Figure 41. Effect of HS and BSA on the inhibitory activity of dimer **2**. A, Growth of *E. coli* MG1655 in MHB (solid lines) and MHB supplemented with 10% of HS (dotted lines) in presence of 1 µg/mL compound 2 (½ MIC, blue lines), 2 µg/mL (MIC, red lines) and 4 µg/mL (2×MIC, green lines). B, Growth of *E. coli* MG1655 in MHB supplemented with increasing concentrations of BSA (from 0 to 40 mg/L) and supplied with 1 µg/mL (½ MIC, solid lines) and 2 µg/mL (MIC, dotted lines) of compound 2. Bacterial growth, expressed as OD600, was monitored for up to 24 h. Data are the mean ± standard deviation of triplicate experiments.

Evaluation of biological activity against antibiotic-resistant strains.

The antibacterial properties of selected analogs of compound **2** were further investigated on recent antibiotic-resistant clinical isolates, including pan-drug-resistant *K. pneumoniae*. Most interestingly, all compounds retained much of their activity on specific pathogens regardless of the resistance phenotype, and particularly colistin-resistant *K. pneumoniae*. This indicates that, despite the polycationic nature of both colistin (a polymyxin antibacterial peptide) and our alkyl-guanidines, the latter likely show a different mechanism of action and are not susceptible to alterations of the LPS layer determining a decrease of the negative net charge on the bacterial cell surface.[186]

Table 6. MICs of selected compounds on Gram-negative antibiotic-resistant clinical isolates.

Cpd	Cpd Code	MIC (µg/mL)[a]				
		A. baumannii AC-54/97	*B. cepacia* SI-R2	*E. cloacae* VA-417/02	*K. pneumonia* SI-081*	*S. maltophilia* 634/08
2	**8CH*/8CHs**	2	16	1	2	16
15	**8BH*/8BHs**	-	16	4	8	8
16	**8HH*/8HHs**	8	64	8	>64	32

[a]-: not determined; *: clinical isolate with pan-drug-resistant phenotype. MICs (µg/mL) are expressed as median values calculated from experiments performed at least in triplicate.

4. Mode of action investigation

Bactericidal mode of action

The biological profile of the synthesized library was further investigated through MBC assay to distinguish whether it is bactericidal or bacteriostatic. Herein, we reported data for dimer **2** on three bacterial strains (**Table 7**). The found MBC values resulted identical to those of the MICs, indicating a strong bactericidal activity According to the CLSI standard,[187] the MBC/MIC ratio is essential to understand how the antibiotic acts. In particular, when the ratio is equal or minor than 2, it is considered indicative of bactericidal action, whereas antibiotics with a ratio higher than 8 are bacteriostatic. Since MBC and MIC values found for compound **2** were perfectly matching (MIC ratio = 1), we could confirm its bactericidal behavior.

Table 7. Comparison of MIC and MBC values for compound **2**.

Bacterial strain	2, 8CH*/8CHs	
	MIC (µg/mL)	MBC (µg/mL)
E. coli CCUG[T]	2	2
A. baumannii ATCC 17978	4	4
S. aureus ATCC 25923	2	2

MICs and MBCs are expressed as the average values calculated from experiments performed at least in triplicate.

This was further confirmed by time-kill curve experiments on a Gram-negative (*E. coli*) and a Gram-positive (*S. aureus*) microorganisms. In the first case, the bactericidal activity of dimer **2** (4 µg /mL, 2 x MIC) on *E. coli* was found to be ≥4 $\log_{10}$ in one hour and thus superior to that of colistin, a polymyxin polycationic antibacterial peptide. Furthermore, the bacterial load measured after 24 hours of exposure to 2 x MIC of these antibiotics was comparable (**Figure 42**, left). Also towards *S. aureus*, dimer **2** was found to be a fast bactericidal agent, with a reduction of viable microbial load of >3 $\log_{10}$ after only 1 hour of exposure to the compound (final concentration, 10 × MIC). A further reduction of the viable count was progressively observed (>5 $\log_{10}$ after 4 hours) and no viable cells could be detected after 24 hours (**Figure 42**, right).

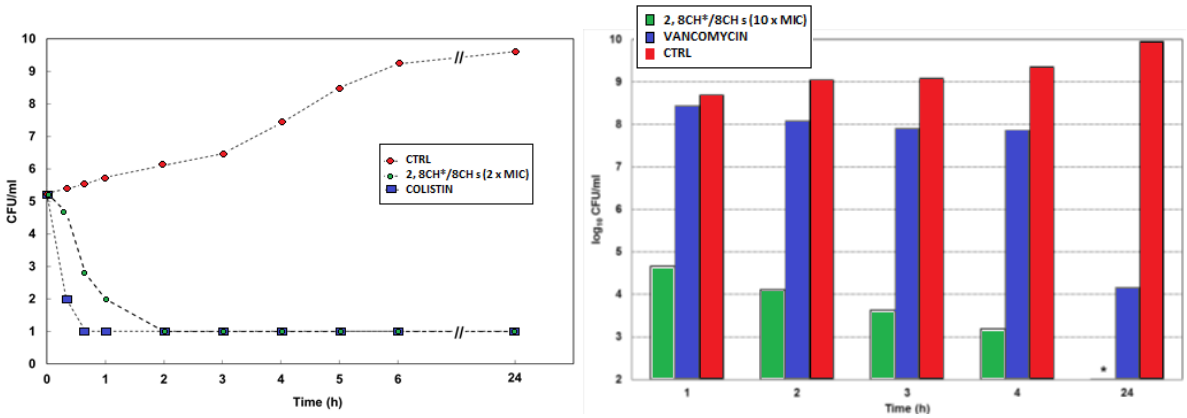

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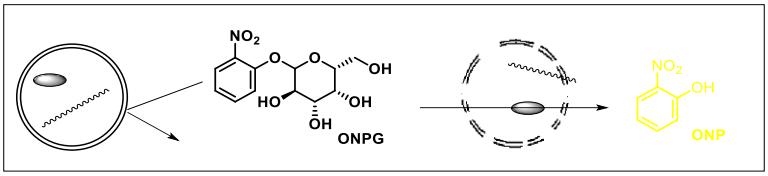
Figure 42. Time-kill curve experiments performed with (left) *E. coli* CCUG^T and (right) *S. aureus* ATCC 25923. The experiments on *E. coli* were performed in the absence (growth control, red circles) or presence of 2 x MIC of compound 2 (green circles) or colistin (2 x MIC, blue squares), used as a comparator. The experiments on *S. aureus* were performed in the absence (growth control, red bars) or presence of 10 × MIC of compound 2 (green bars) or vancomycin (10 × MIC, blue bars), used as a comparator. *: viable count ≤ 10 CFU/mL.

Impairment of the structural integrity of the Gram-negative cytoplasmic (inner) membrane.

Considering the polycationic nature of our compounds, a potential mechanism of action could be represented by the impairment of the structural integrity of the bacterial cytoplasmic (inner) membrane or the outer membrane of Gram-negative organisms. Thus, the first hypothesis was tested using a simple enzyme-based whole assay, in which the activity of a cytoplasmic enzyme on a poorly permeable chromogenic substrate was evaluated. Indeed, the chromogenic substrate *o*-nitrophenyl-*β*-D-galactoside (ONPG), colorless, releases the yellow *o*-nitrophenol (ONP) upon enzymatic hydrolysis but only reaches the cytoplasm following permeability defects, such as those acting on membrane integrity, such as colistin and other antimicrobial peptides (AMP).[188,189] The assay was conducted for some rationally selected compounds (**1, 2, 6, 10** and **16**) in three bacterial strains characterized by physiological or induced high-level of intracellular production of β-galactosidase. Interestingly, none of the tested compounds, besides sodium dodecyl sulfate (SDS) which was used as a positive control, apparently induced significant levels of OPNG conversion, indicating that they did not cause a macroscopic alteration of the bacterial membrane (**Figure 43**). However, this assay does not allow to exclude the possibility that the tested compounds could generate very small, microstructural, damages in the inner membrane.



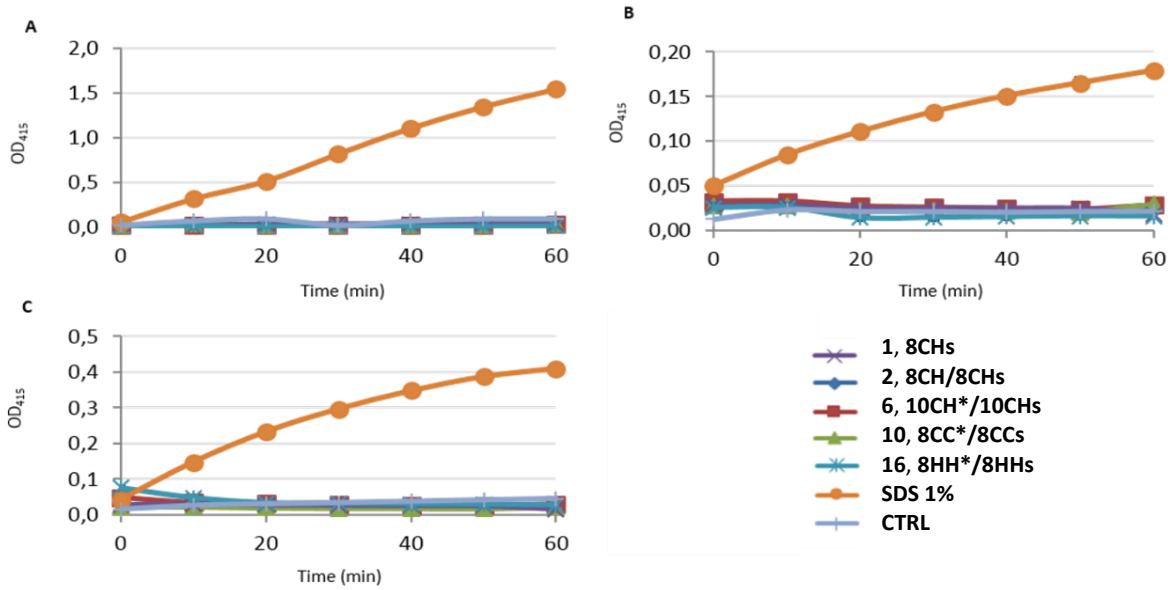


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Figure 43. Effect of selected compounds on bacterial permeability. Time-depending production of *o*-nitrophenol from ONPG by A) *E. coli* MG1655; B) *A. baumannii* ATCC 19606^T harboring plasmid pMP220::PrrnB; C) *P. aeruginosa* ATCC 15692 harboring plasmid pMP220::PrrnB, untreated (negative control) and upon exposure to 256 µg/mL of each compound or 1% of SDS (positive control), as indicated.

Perturbation of the Gram-negative outer membrane

The possibility to affect the outer membrane integrity of Gram-negative species was investigated by an additional specific fluorescence assay. Three Gram-negative bacteria were exposed to increasing concentrations of the selected compounds, colistin or DMSO, as positive and negative controls, respectively. Then, the addition of 1-*N*-phenyl naphthylamine (NPN) followed. This lipophilic dye weakly fluoresces in aqueous environments but it emits strong fluorescence in the hydrophobic ones, thus enabling to detect potential outer membrane damage. In fact, the LPS external layer of the outer membrane of Gram-negative bacteria acts as a permeability barrier to lipophilic substances like NPN. Disruption of outer membrane integrity makes phospholipids of the internal outer membrane layer accessible to NPN, allowing fluorescence emission by the probe.[190] Notably, all the tested compounds caused a dose-dependent increase in the NPN fluorescence emission for all Gram-negative species tested (**Figure 44**). Interestingly, compounds **2, 6** and **10** caused higher fluorescence emission than colistin, whereas any fluorescence was detected in presence of DMSO. In contrast, compound **1** showed the lowest activity on the outer membrane. These results indicate that all compounds perturb the Gram-negative outer membrane, although to a different extent.

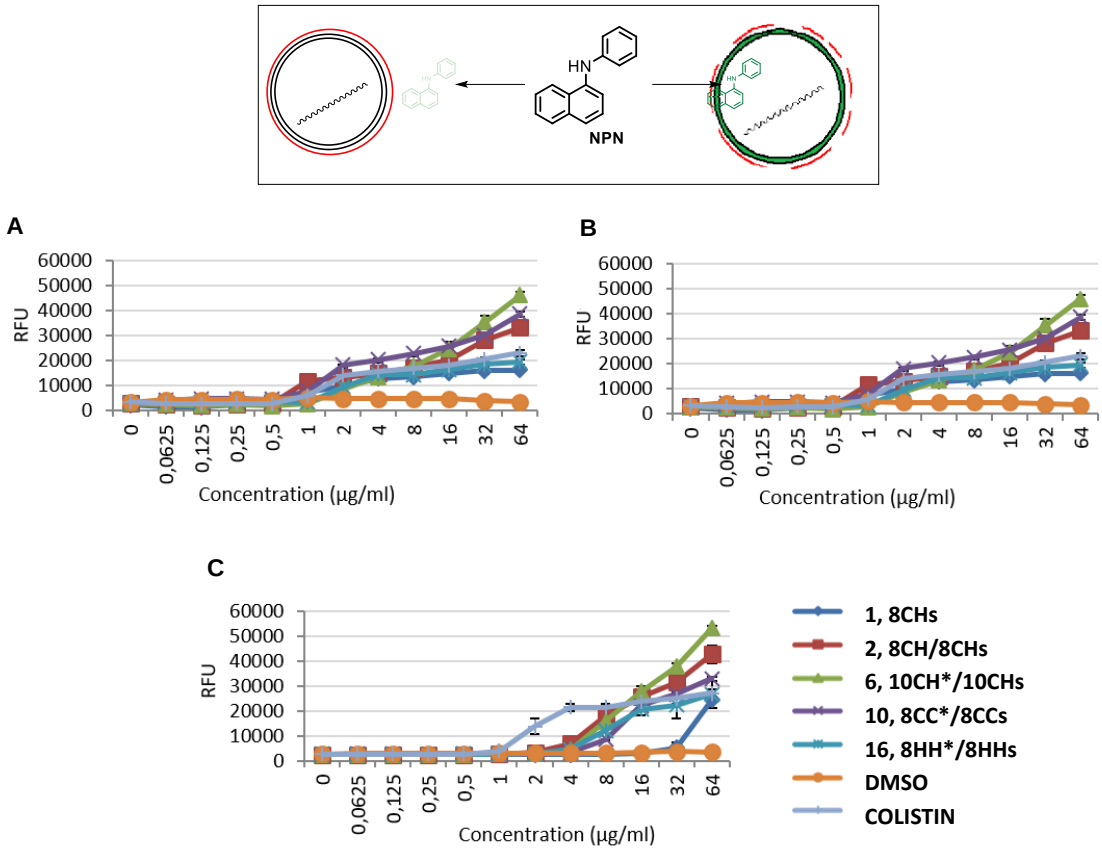

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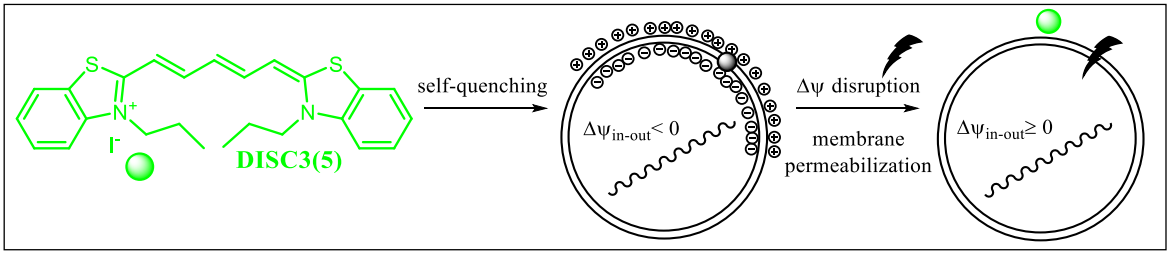
Figure 44. Effect of selected compounds on Gram-negative outer membrane integrity. The uptake of NPN (measured as relative fluorescence unit, RFU) by A) *E. coli* MG1655, B) *A. baumannii* ATCC 19606^T and C) *P. aeruginosa* ATCC 15692 in presence of increasing concentrations of each selected compound. Colistin or DMSO was used as positive and negative controls, respectively. Data are the mean ± standard deviation of triplicate experiments.

Depolarization of bacterial cytoplasmatic membranes.

As another potential mechanism of action, the depolarization of the cytoplasmic membrane (thus affecting the proton motive force) was also investigated. Thus, compound **2** was tested for its ability to depolarize *E. coli* membranes using the fluorescent dye 3,3'-dipropylthiacarbocyanine ($DiSC_3$-5), essentially as described by Breeuwer and Wand.[191,192] This probe usually accumulates in the cytoplasmatic membrane in physiological conditions. When the electric potential of the cell is disrupted or the membrane is permeabilized, the dye is released in the medium, resulting in an increase in fluorescence. Compound **2** was unable to restore any fluorescence after incubation of bacterial cells at a concentration 2 x MIC (4 µg/mL), thus suggesting that membrane depolarization was likely not the prevalent mechanism accounting for its fast bactericidal activity.



Inhibition of biofilm formation.

Biofilm is a very complex microbial architecture that allows bacteria to attach to surfaces and to form aggregates, encasing them into a matrix composed of self-produced extracellular polymers. Biofilm makes microorganisms more resistant to antibacterial treatments, harsh environments, and human immunity. One of the current strategies to prevent the formation of biofilms is the treatment with AMPs like colistin, polymixins, and gramicidin. [193,194] These molecules are able to bind electrostatically the negative charged LPS through their cationic amino acids (lysine and arginine) and to stabilize this binding through hydrophobic interactions between the hydrophobic amino acids of the peptide and fatty acyl chains of LPS.[195] Our compounds, being polycationic and amphiphilic, bear both the polar and nonpolar functions and have a polyamine-based skeleton known to attribute to the disruption of biofilm.[196] All these considerations let us hypothesizing a similar trend. For these reasons, in-depth analyses were performed to evaluate their behavior toward the biofilm, revealing that they are able neither to inhibit the formation of biofilm (assessed through the crystal violet assay) nor to alter the three-dimensional structure of biofilm (**Table 8** and **Figures 45**).

Many pathogens, including *A. baumannii*, *P. aeruginosa*, *E. coli,* and *S. aureus* can grow as matrix-encased communities called biofilms. Cells growing as biofilms are characterized by low metabolic activity and are less susceptible to antimicrobial agents, [S197] so we wondered whether guanidine derivatives **1**, **2**, **6**, **10** and **16** are able to prevent biofilm formation. To this aim, *A. baumannii* ATCC 19606^{T}, *P. aeruginosa* ATCC 15692, *E. coli* MG 1655 and *S. aureus* ATCC 35556 were used to test the effect of guanidine compounds on biofilm formation. In order to define the highest concentration of compounds able to prevent the biofilm formation without affecting the planktonic growth of bacteria, all strains were cultivated for 24 hours in presence of increased concentrations of the selected compounds and the MICs (**Table 8**) of each compound were determined.

Table 8. MICs of selected compounds.

Cpd	Cpd Code	MIC (µg/mL)[a]			
		E. coli MG 1655	*A. baumannii*^{T} ATCC 19606	*P. aeruginosa* ATCC 15692	*S. aureus* ATCC 35556
1	8CHs	32	>64	>64	32
2	8CH*/8CHs	2	8	8	2
6	10CH*/10CHs	16	16	32	4
10	8CC*/8CCs	4	8	8	4
16	8HH*/8HHs	8	16	16	4

MICs are expressed as the average values calculated from experiments performed at least in triplicate.

It was also noticed that ½ MIC did not affect the planktonic growth profile for all compounds tested (**Figure 45**). Biofilm inhibition was quantitatively investigated after 24 hours-exposure to compounds **1**, **2**, **6**, **10** and **16** (½ MIC) in MHB, by using the crystal violet (CV) assay in 96-well microtiter plates. None of the guanidine derivatives tested considerably affects the biofilm formation.

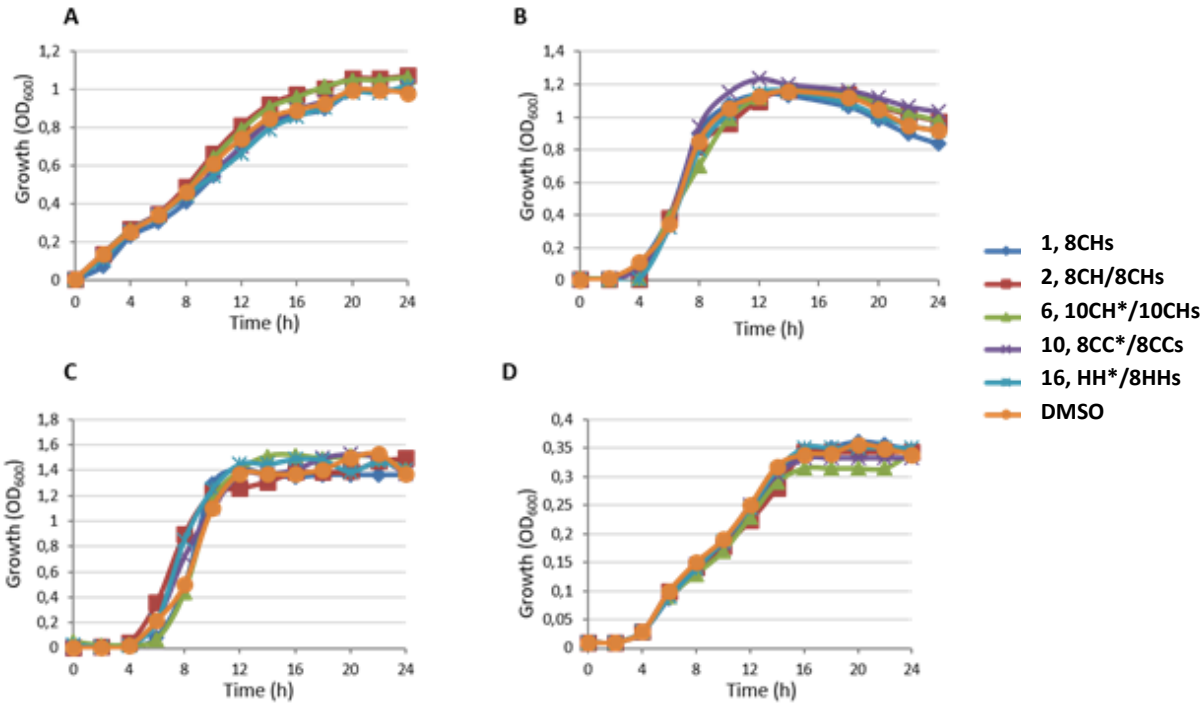


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Figure 45. Growth profile of different bacterial species in MHB supplemented with selected compound at ½ MIC. (A) *E. coli* MG 1655, (B) *A. baumannii* ATCC 19606^T, (C) *P. aeruginosa* ATCC 15692 (D) *S. aureus* ATCC 35556 growth in presence of compound 1 (16 µg/mL), 2 (1 µg/mL), 50 (2 µg/mL), 55 (2 µg/mL) and 58 (2 µg/mL) or DMSO. Bacteria were grown in 96-well microtiter plate and the OD_{600} was periodically measured for up to 24 h.

Alteration of the 3D structure of biofilm

The CV assay results do not exclude that the compounds may alter the biofilm tridimensional structure. To test this hypothesis, the effect of the selected compound was also investigated by confocal microscopy. Bacteria were grown in 8-well chamber slides for 24 hours at 37 °C in MHB in presence of compounds at ½ MIC. Confocal microscopy analysis of AO-stained biofilms confirmed that none of the selected compounds causes appreciable changes in the biofilm structures (**Figure 46**). Overall these results demonstrate that compounds **1**, **2**, **6**, **10** and **16** do not affect the ability of the *A. baumannii*, *P. aeruginosa*, *E. coli,* and *S. aureus* to form the biofilm.

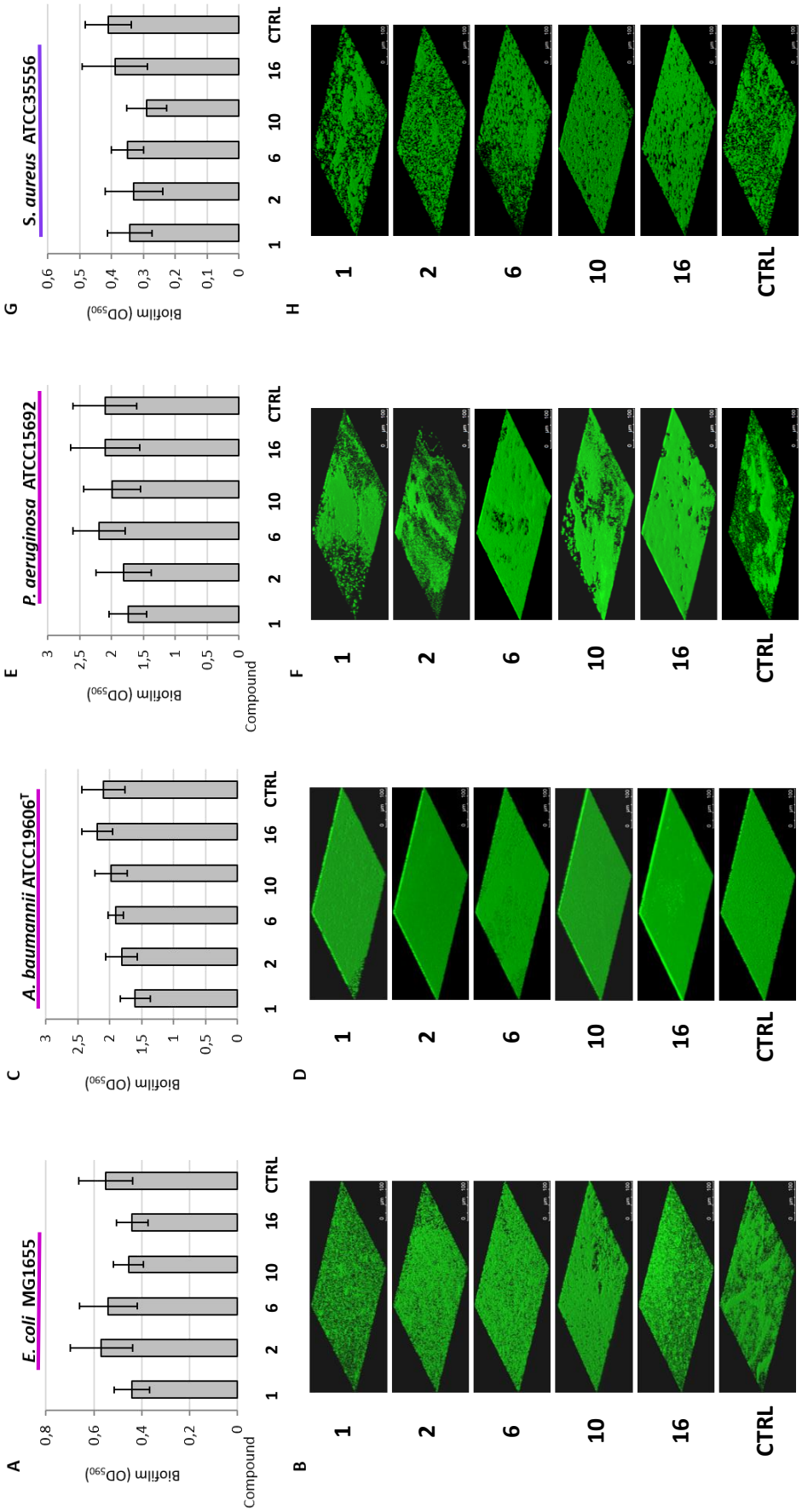

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Figure 46. Effect of selected compounds on biofilm formation by different bacterial species. Biofilm levels were determined by the CV assay (A, C, E, G) and confocal microscopy analysis (B, D, F, H) after 24-h growth in MHB at 37°C in presence of each compound at ½ MIC or DMSO (CTRL). The CV assay values represent the means (± standard deviations) of two

independent assay. The confocal microscope images are representative bacterial biofilms developed after 24 h in 8-well chamber slide in MHB in presence of each compound at ½ MIC. Bacterial species are indicated on top of each panel: *E. coli* MG1655 (A, B), *A. baumannii* ATCC 19606^T (C, D), *P. aeruginosa* ATCC 15692 (E, F) and *S. aureus* ATCC 35556 (G, H).

Interference with Polyamines pathways.

Considering that our monomer derivatives are endowed with the inhibition of Maize Poly-Amino Oxidase (mPAO) with a micromolar activity, as already published,[125] we strongly hypothesize a possible involvement of the dimeric derivatives in the bacterial polyamine pathways. Polyamines in bacteria have crucial roles in the cell pathways, such as the increase of protein synthesis by binding to nucleic acid and the response to oxidative stress. Moreover, physiological polyamines are involved in the pathogenesis of infection: for example, they are effector molecules of carcinoma caused by *H. pylori* infection, they are implicated in the host cell apoptosis and the control of biofilm and they are crucial to virulence phenotypes, causing the escape from phagolysosomes and the bacteriocin production, regulating the toxin activity, producing or modifying porins, with consequent decreasing of membrane permeabilization, and protecting the bacterial cell from oxidative and acid stress.[116,198] In fact, recent studies confirmed the influence of polyamines when co-administrated with antibiotics, resulting in increased efficacy of the antibiotics on bacterial strains that are no longer susceptible to their action.[199,200] However, different effects are detected towards bacterial strains, for example, polyamines were found to develop Colistin-resistant genes. [199,201]

As a preliminary investigation, we supposed that our dimers could be a possible substrate of some poly-amino transporters, present in all kind of cells, also in bacteria ones,[202,203] since they show an amphiphilic structure like the polyamines. Polyamine uptake in *E. coli* occurs through two different ATP binding cassette (ABC) transporters: a spermidine-preferential system and a putrescine-specific one. These carriers consist of a substrate-binding site in the periplasm (D or F), two channel-forming proteins (B, C or H, I) and a membrane-associated ATPase (A or G) that is involved in energy supply (**Figure 47**).[204,205] The operons that encode the carriers are PotA, PotB, PotC and PotD for spermidine system and PotF, PotG, PotH and PotI for the uptake of putrescine. Moreover, another transporter is the protein E, encoded by PotE, which catalyze both uptake and excretion of putrescine (**Figure 47**).[206] Thus, compound **2** was already tested on *E. coli* mutants in which the genes encoding the main components of polyamine transport were inactivated (PotA$^-$, PotB$^-$, PotC$^-$, PotD$^-$, PotE$^-$, PotF$^-$, PotG$^-$, PotH$^-$ and PotI$^-$ mutants from the Keio library).[207] Comparing to the activity on *E. coli* CCUG^T, the MIC values on the mutant strains were unaffected. This does not completely invalidate our hypothesis because the assay results could be explained in two ways, being the polyamine transport systems different for spermidine (potABCD) and putrescine (potE and potGHIF): dimer **2** could interact with neither potABCD nor potGHIF or could interact with both the systems so when one is abolished by a single mutation, it can bind the other one. Unfortunately, limited literature in the field of bacterial polyamines is available so far, but ours is just a preliminary assumption and further studies are certainly needed to better investigate this issue.

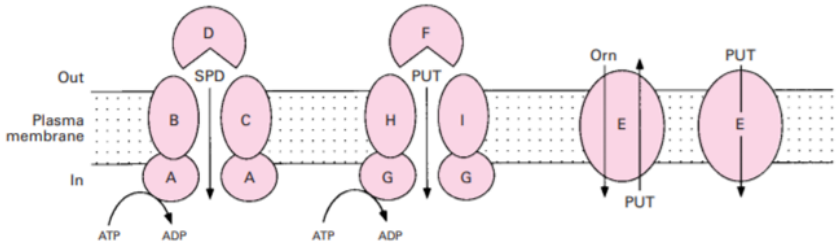

Figure 47. Polyamine transporters in *E. coli*. Spermidine (SPD), putrescine (PUT), ornithine (ORN) and putrescine (PUT).[204]

Selection for resistant strains.

In order to evaluate the propensity of dimer **2** to select for resistant mutants, and potentially investigate the resistance mechanism(s), *E. coli* CCUG^T (starting inoculum, 10^6–10^7 CFU/mL) was subcultured sequentially in MHB containing increasing concentrations of compound **2** (4-32 µg/mL corresponding to 2-16 x MIC). No growth could be observed at concentrations higher than 32 µg/mL, after up to 48 hours. Cells recovered from the various cultures were plated on agar plates containing the same concentration of compound **2**. Surprisingly, the phenotypic analysis of these isolates did not show the acquisition of a stable resistant phenotype, as the 2 MIC values measured for 25 randomly-selected isolates was essentially identical to that of the parental strain. This would indicate that **2** could induce the formation of persisters in the bacterial population, rather than selecting stable mutants with an acquired mutation, but that this tolerance phenomenon could only promote survival at concentrations equal or below to 32 µg/mL.
Persisters are bacteria with wild-type phenotypes endowed with metabolic particularities that allow them to become spontaneously and transiently tolerant to antibiotics, achieving a dormant, non-dividing state, without undergoing genetic changes or mutations. However, persisters represent only a small subpopulation of cells that survive to high doses of a bactericidal agent and there is no close correlation between antibiotic resistance and persistence,[208,209] even if the phenotypic plasticity of bacteria can involve an adaptation to harsh conditions, like resistance does, making bacteria not susceptible to the specific antibiotic, as it occurs in chronical infections.[210,211]

5. Evaluation of Toxicity

Hemolysis

A preliminary investigation of the hemolytic activity of compound **2** was performed by spotting the test molecule on the surface of a blood agar plate at high concentration (up to 50 µg). Any detectable hemolysis emerged.
Furthermore, rationally selected compounds (**6**, **10** and **16**), in addition to dimer **2**, have been investigated about their behavior towards human membranes. Hemolysis of red blood cells is often used as a tool to study the effect of compounds on mammalian cell integrity. Each compound was tested at 64 µg/mL on human erythrocytes recovered from healthy donors with different blood groups; 0.2% Triton X-100 and 1.6 % DMSO were used as positive and negative controls, respectively. As shown in **Table 9**, the assay revealed that there was no evidence of hemolysis, with the exception of compound **10**, that showed a significant percentage of hemolysis ranging from 41 to 54%, depending on the blood group. This is interesting considering that the only compound with hemolytic activity showed a lower antibacterial activity, indicating that the length of the alkyl chain could be very important for selectivity. Only minor blood group-dependent hemolytic differences were observed for all compounds.

Table 9. Hemolytic activity of selected compounds for each blood group.

Cpd[a]	Haemolysis (%)			
	0 Rh+	A Rh+	B Rh+	A Rh-
2	3	4	5	5
50	54	41	44	44
55	3	2	2	3
58	3	4	6	7
CTRL +	100	100	100	100
CTRL –	0	0	0	0

[a]Compounds in DMSO were tested at 64 µg/mL, 0.2% TRITON X-100 and 1.6 % DMSO were used as positive and negative controls respectively.

Cytotoxicity on mammalian cells

The potential cytotoxicity of compound **2** was further evaluated on the commercially available HeLa cells. When tested at 16 µg/mL, it showed only minimal cytotoxicity after 24 hours of incubation. Cytotoxicity was more evident at 256 µg/mL, although it did not exceed 60% after 24 hours (**Figure 48**), indicating that the selectivity towards bacterial cells should be improved.

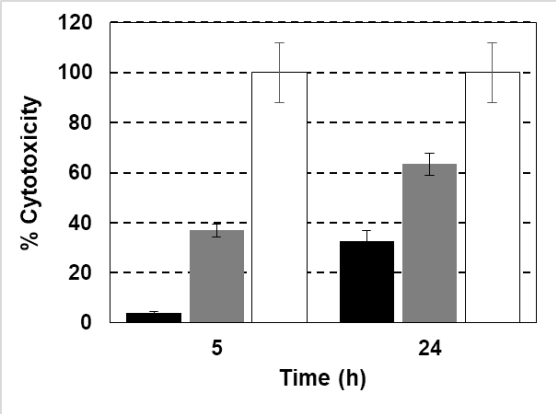

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Figure 48. Cytotoxicity of compound **2** on HeLa cells. Cytotoxicity (%) after 5 and 24 hours of incubation when **2** was tested at 16 µg/mL (black bars) or 256 µg/mL (grey bar) is reported. The error bars are referred to SD. The positive control is shown in white. % cytotoxicity = (100 x LDH release of cells treated with cpd – spontaneous LDH)/(maximum LDH – spontaneous LDH), spontaneous LDH: LDH released by untreated cells; maximum LDH: total LDH released from cells treated with a lysis agent.

Cytotoxicity data for compound **19**, the hydrochloride salt, were identical to compound **2**.
The toxicity of the whole synthesized library has been evaluated on HeLa cell line through a colorimetric quantification of intracellular lactate dehydrogenase (LDH) released upon cell lysis caused by exposure at the test compound at 24 hours or different times. Unfortunately, the calculated selectivity index for the library is very narrow, even if some compounds emerged to have lower cytotoxicity (data not shown).

6. Preliminary ADME characterization of hit compound 2.

Initially, some chemo-physical properties, such as lipophilicity and solubility were predicted through *in silico* studies. The lipophilicity of a molecule can be determined through the calculation of the partition constant, logP, or the distribution constant, logD. Accordingly, both these values are parameters that could help to predict how much a compound will be absorbed in the cell, how much it will permeate the amphiphilic biological membranes. It is known that for optimal gastrointestinal absorption, values should be moderate, ranging from 0 to 3, while values major than 3 are characteristic of hydrophobic compounds with aqueous solubility issues. Finally, compounds with values minor than 0 usually have poor lipid bilayer permeability and good solubility in water. LogP and logD are interchangeable for non-polar compounds. In fact, logP calculation considers a molecule as a *single entity* (neutral form) in equilibrium between aqueous and organic environments but it is actually true only for no-ionizable compounds. Our compounds are characterized by long alkyl chains that make them hydrophobic but they also bear guanidino moieties that at physiological pH should be positively charged, generating a basic-acid equilibrium between the protonated and deprotonated species, driven by the own pKa. This suggests relevant changes in partition in respect of the pH of the environment. In fact, pH has a non-negligible influence in the partition generates a more complex system in which each species is in equilibrium with the others but also between the organic and aqueous phases. Thus, for polar molecules, the logD calculation is more appropriate and it can be used as an index for the behavior of compounds in plasma when measured at physiological pH (7.4).[212] Both clogP and clogD were quickly calculated by a lot of software, thus we challenged some available ones, obtaining different values for compound **2**, as representative of the whole library. As visible in **Table 10**, the predicted values of logP and logD are very distant from each other. Hence, we used more specific programs for clogP and we found a value of around 6.4 by QikProp from Schrodinger (this value is at the boundary of the *range 95% of drugs* -2–6.5) while ALOGPS and ChemAxon (that are reference logP calculators in the DrugBank www.drugbank.ca) estimated a value of 4.14 and 1.73, respectively, highlighting again a big discrepancy. This large variation of results might be due to the approach used to estimate the clogP, even though this parameter is generally computed as the sum of conformational-independent fragments contributions and it is not credible for large and flexible compounds like ours. Furthermore, the lipophilic properties are closely related to water solubility. Thus, with the aim to escape from any possible source of error in the calculation of logP or logD and to obtain an unbiased estimation of the water solubility and lipophilicity of our derivatives, the logD (octanol/Tris buffer pH 7.4) was determined experimentally on compound **2**. In summary, we have performed a measurement of the partition between octanol and water in presence of TRIS as buffer. The observed logD (pH 7.4) was -0.79, a value that is comparable to the ones of daptomycin and vancomycin and it is in accordance with the predictions (< 0).

Table 10. Calculation for lipophilicity and solubility.

Software	Calculations		
	clogP	clogD (pH 7.4)	clogS
FafDrugs4	7.4	-3.11	
Marvin Sketch 15.6	6.55	-3.11	
QikProp (Schrodinger)	6.4		-3.2
ALOGPS	4.14		
ChemAxon	1.73		

Then, we calculated logS for aqueous solubility, being a more effective descriptor of lipophilicity and solubility of compound **2**. LogS is the intrinsic solubility, the equilibrium of a free acid\base of an ionizable compound at a pH where it is fully unionized. According to the QikProp manual, optimized structures should be used for the input. Thus, compound **2** was relaxed in explicit water solvent by means of 200 ns of unrestrained molecular dynamics simulation (AMBER). By cluster analysis, we extrapolated the most representative frame (**Figure 49**) and we used it as input for QikProp calculation. The software returned a logP value of 6.4, while the logS was estimated to be -3.2. This value, clearly indicates that the molecule is soluble in water at a concentration within or near the millimolar range, which in our opinion is definitely reasonable and acceptable for a lead compound. Finally, the molecule in water adopts an extended conformation, which gives QikProp results closer to the experimental data than compact conformations (QikProp manual).

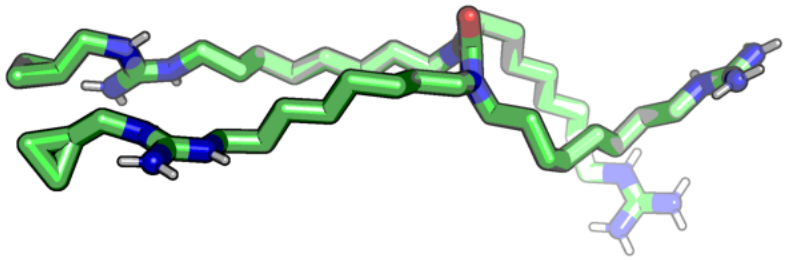


Figure 49. Most representative frame of a cluster of compound **2** relaxed in explicit water solvent after 200 ns of unrestrained molecular dynamics simulation. The figure was prepared by means of PyMOL 2.0.7. Carbon atoms are shown in green, nitrogens in blue, polar hydrogens in grey and oxygen in red.

In the end, preliminary absorption, distribution, metabolism, excretion (ADME) properties were determined *in vitro* for compound **2** (**Table 10**). In particular, **2** was found to be metabolically stable, as measured by means of human liver microsomal proteins and very soluble in pure water. This feature is in agreement with the calculation of logS and allows a future intravenous administration development.

Moreover, through a validated indirect fluorescence protocol, [136] we evaluated the PPB for compound **2**. The main goal of drug binding assays is the determination of plasma-bounded drug fraction at therapeutic concentrations. During the discovery stage of a research project, this information could help to classify drugs as high, intermediate or poorly bound to plasmatic proteins. The drugs that are highly bound, such as warfarin and diazepam, show dissociation rate constant (K_d) of less than 100 µM, while the poorly bound drugs, as paracetamol, are characterized by a K_d value higher than 1 mM. Compound **2** shows a good affinity to the AGP, as expected for molecules that bear positive charges at physiological pH but it has a low percentage of bond (B_{max}) values, as reported in **Table 11.**

Thus, a low maximal percentage of bond and a slow dissociation rate of compound **2** are considered as permissive conditions to the predicted *in vivo* ADME.[182] Moreover, as above-reported, kill curve experiments in presence of albumin or serum, showed that the PPB does not impair restrictively the antibacterial activity.

Table 11.Preliminary ADME assay results for compound **2**

Metabolic stability (%)[a]	Solubility [g/L][b]	logD (pH 7.4)[c]	HSA		AGP	
			Kd [μM][d]	Bmax (%)[d]	Kd [μM][d]	Bmax (%) [d]
> 99	0.292 ± 0.022	-0.79	23.27 ± 14.6	14.0	0.11 ± 0.06	30.2

[a]The human liver microsome stability is expressed as a percentage of the unmodified parent drug. [b]Aqueous solubility was determined by means of the LC-MS method. [c]Distribution coefficient 1-octanol/ TRIS buffer pH 7.4 . [d]K_d and B_{max} values are measured by means of the indirect fluorescence method.

The parallel artificial membrane permeability assay (PAMPA), validated with known drugs (rifamixin and chloramphenicol)[134] revealed a low apparent permeability (P_{app}) at physiological pH, as expected since ionic compounds cannot pass across the membrane (**Table 11**): dimer **2** is, in fact, a trifluoroacetic salt with four net positive charges on the guanidine moieties and no pH adjustments have been performed during the assay. Since this experiment, from Kansy et al,[213] replaces cellular membranes (phospholipids, enzymes, proteins, sterols) with a barrier made of only phospholipids (phosphatidylcholine in our case), it correlates only with passive diffusion and can give false results for compounds that are substrate of membrane enzymes or transporters (active permeability). In fact, it is known from the literature that organic cations, such as endogenous substances bearing guanidine and amine functions, can cross biological membranes through apical transport mechanisms, like H^+ antiports and P-glycoprotein G (P-gp), and the less studied basolateral ones. In general, a family of organic cation transporters has been cloned and studied to give more information about absorption and secretion of cationic compounds.[214] To evaluate if compound **2** is a substrate of this kind of transporters, in-depth permeability experiments were conducted. In this frame, we opted for the Caco-2 permeability assays that are based on the use of a polarized cell monolayer. It is the gold standard *in vitro* test system, since it is quicker to use, convenient, and it produces more reproducible data than animal studies, giving an effective assessment of the permeability (P_{app}) of new compounds. Caco-2 is the best known immortal human colon carcinoma cell line as a model of the epithelial cell layer permeability barrier that compounds encounter in the small intestine. This cell line is characterized by a particular morphology that allows multiple permeability mechanisms and it provides the opportunity to investigate various permeability pathways. This model resembles the morphology of gastrointestinal epithelial cells for the presence of microvilli on the apical surface and for the expression of certain enzymes and membrane transporters, such as P-gp, breast cancer resistance protein (BCRP), and multidrug resistance protein 2 (MRP2). These characteristics make Caco-2 cells the most suitable and frequent used line to conduct permeability experiments and to rank compounds, according to the Biopharmaceutics Classification System (BCS) by Food and Drug Administration (FDA), as low, medium or high permeability compounds.[215–217] We calculated the P_{app} apical-to-basolateral and basolateral-to-apical by means of two different experiments, placing the test compound in the apical or in the basolateral side buffers. The first value provides information about the permeability in the absorptive direction, furnishing a quite reliable model of the gastrointestinal absorption. Compound **2** showed a very low permeability, in accordance with PAMPA results. In fact, the low

$P_{app(A>B)}$ found (**Table 12**) indicates that it is not absorbed through passive diffusion (as already showed by PAMPA experiment), but also paracellular permeation and active transport are not involved in its absorption. This corresponds to a low oral bioavailability, that can be overcome by intravenous administration, as it happens for the majority part of natural drugs, as example those antibiotics active against chemo-resistant organisms, such as colistimethate (prodrug of colistin, indicated for treatment of infection by multi-drug resistant Gram-negative strains, often used as a last resort), daptomycin (for treatment of nosocomial Gram-positive infections including MRSA and VRE strains) and vancomycin (for treatment of Gram-positive bacterial infections). The opposite experiment was performed to study the permeability of compound **2** by cell membrane transporters in the efflux direction. Also in this case, we obtained a very low P_{app}. In **Table 12**, we reported the ratio between P_{app} in the B>A and the A>B directions: this value, known as efflux ratio, is 0.65, because the permeability values are approximatively the same (in the same range), highlighting that compound **2** could permeate only by passive or paracellular diffusions, not being substrates of active transporters for the cellular uptake or efflux. In fact, in the first case, it should have been A>B/B>A) ≥ 2 and in the second one B>A/A>B) ≥ 2.[182,218] Considering that the efflux is a serious issue for an antibiotic, not allowing it to reach the appropriate concentration in the cell (MIC) to have antibacterial efficacy and provoking the rise of resistant strains, a low efflux is a relevant finding.

Table 12. Apparent Permeability Coefficients obtained by PAMPA and Caco-2 cell line experiments and efflux ratio.

Cpd	Apparent Permeability (PAMPA) P_{app} [10^{-6} cm/s]	Caco-2 Permeability[a] $P_{app(A>B)}$ [10^{-6} cm/s]	Efflux ratio[b] $P_{app(A>B)}$) / $P_{app(B>A)}$
2	1.60 ± 0.43	0.19 ± 0.09	0.65
Rifamixin	0.06 ± 0.01	-	
Chloramphenicol	0.30 ± 0.50	-	
Atenolol	-	0.13 ± 0.04	0.37
Propranolol	-	22.4 ± 5.4	0.40

Atenolol (characterized by a low permeability) and propranolol (classified as a highly permeable compound) were tested as reference compounds. [a]A, apical; B, basolateral; $P_{app(A>B)}$ is the Papp in the apical-to-basolateral direction. [b]B>A/A>B is the ratio of the basolateral-to-apical and the apical-to-basolateral permeation rate. Results are obtained from the average values of P_{app} (A>B) and (B>A).

Conclusions

Recent findings in our laboratories led us to the development of alkyl-guanidino oligomers, serendipitously originated from a monomeric compound (**1**), as antibacterial agents [135] In particular, a symmetric dimer (**2**) was identified as a potent broad-spectrum bactericidal. Thus, during the period I have spent in Botta's research group, we have in-depth analyzed the original mixture containing the oligomers by means of MS procedures and we synthesized a small chemical library of derivatives (**11-18**) which have been tested allowing us to perform some preliminary SAR considerations.

It clearly appears that the optimal length of the aliphatic linker should include 8 carbon atoms, even if interesting antibacterial profile emerged for hybrid derivatives, whereas the nature and the number of the substituents on the guanidine moieties did not considerably affect the antibacterial properties of these chemical series. Biological data indicate that almost all the synthesized derivatives are endowed with good bactericidal activity against a panel of Gram-positive and in some cases against Gram-negative pathogens. Despite the structural changes performed in compounds **11-18**, dimer **2** still remains the best compound in antibacterial activity terms.

Dimer **2** and some rationally selected derivatives turn out to be active also against drug-resistant clinical isolates, acting through a bactericidal behavior similar to that of colistin, i.e. by not causing macroscopic permeabilization of bacterial membranes or cell lysis. They were also investigated about their behavior toward human cell membranes, through hemolytic assays, revealing that there is no evidence of hemolysis. Also, SI for all the synthesized compounds was calculated by performing cytotoxicity assays on HeLa cells. Unfortunately, results seem to highlight a toxicity trend for most of them (data not shown), even if, in some cases, the SI is broader, especially when only Gram-positive pathogens are taken into account. Any significant change in the toxic behavior was detected when the anionic counterpart of dimer **2** was replaced by the chloride (**19**) or two amidinourea functions were inserted in the structure in lieu of guanidino moieties (**20**). Moreover, the isolation of two side products allowed us to make some general comments on the asymmetrics' series (**21** and **22**): observing the MIC values, a discrepancy between symmetric and asymmetric isomers emerged, highlighting a significant antibacterial activity for compound **22**.

In the end, extended biological investigations of dimer **2** were performed in order to gain an understanding of its mode(s) of action and also to design different derivatives by more rational structural changes. Preliminary investigation on the pharmacophores led us to synthesize compounds characterized by the removal of whole *N*-substitution on the central urea moiety, furnishing the *arms*-removed derivatives **23-25** and by the gradual deletion of guanidino functions, obtaining the *turned off*-guanidine compounds **26-32**. By means of MICs comparison with *traditional* dimers, we found that the molecule should be a tetrakis urea, endowed with all the four *arms*, and should bear at last three guanidines on the linker terminals to be active as antibacterial agent. However, even if improved, the SI of these compounds is still narrow.

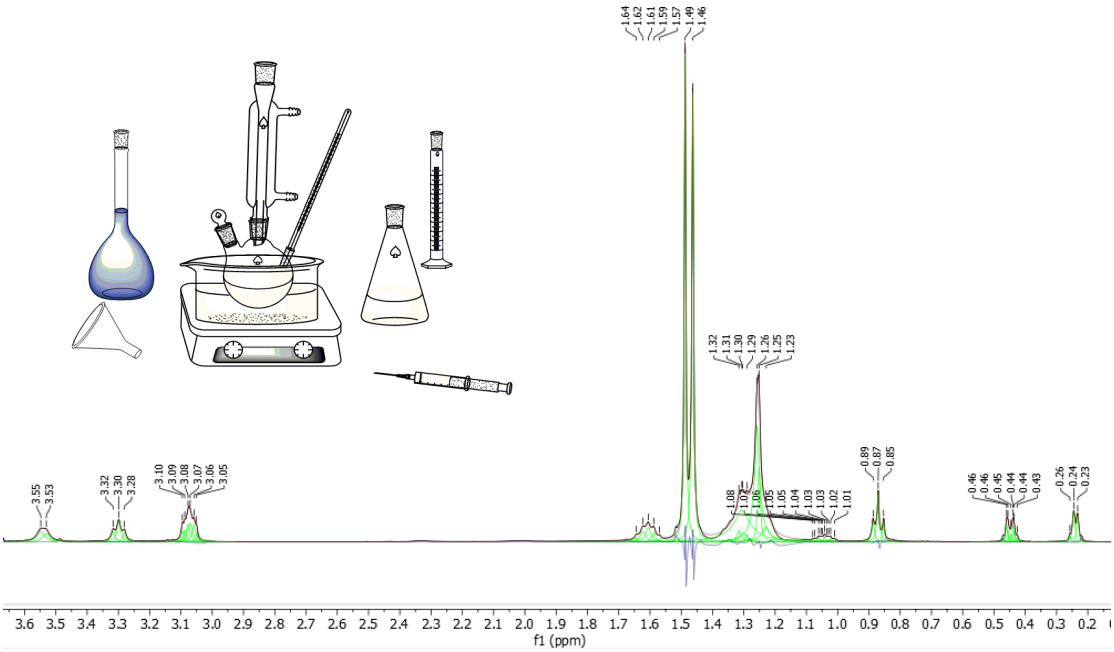
With the aim to understand the possible involvement of our compounds in the physiological polyamines pathways, we investigated the interaction with polyamine transport systems but the performed experiments were inconclusive. The nature of these molecules strongly suggests that they could act on the membranes by electrostatic interactions, even if macroscopic alterations of the bacterial membrane were not observed. However, we cannot exclude the generation of microstructural damages through the conducted experiments. They were found to perturb the Gram-negative outer membrane even if they seemed to affect neither the proton motive force nor the ability of bacteria to form the biofilm. Furthermore, considering the short timeline of the antibiotic life, the

propensity of compound **2** to select for resistant mutants was studied, revealing the ability to induce the formation of persisters without generating stable mutants.

Finally, the *in vitro* ADME properties of dimer **2** was investigated: it was found to be metabolically stable, very soluble in pure water and it showed low binding to plasma proteins that does not impair the activity and low passive and active permeability.

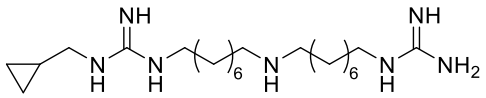
In summary, we synthesized a small library of alkyl-guanidine symmetric dimers as bactericidal agents, in some cases characterized by a broad-spectrum activity, including on drug-resistant clinical isolates. These compounds are generally not hemolytic, moderately toxic and do not apparently select for stable resistant mutants, as found for the hit compound **2**. Notably, ADMET properties of **2** make it suitable to proceed with further optimization, including the improvement of selectivity.

Material and Methods

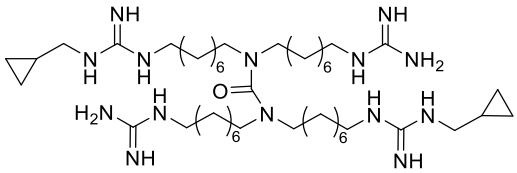


Structures of compounds 1-10 mentioned in the ***State of the Art*** Chapter.

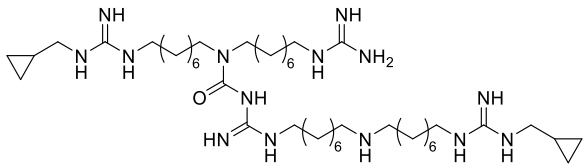
N-{8-[(8-carbamimidamidooctyl)amino]octyl}-N'-(cyclopropylmethyl)guanidine trifluoroacetate salt (1, 8CHs)



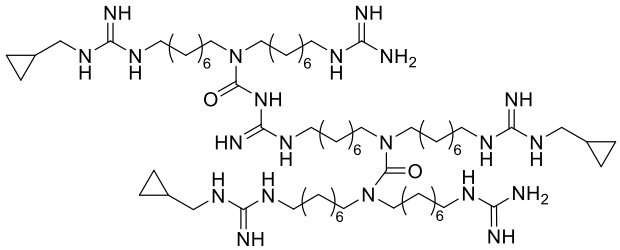
1,3-bis(8-carbamimidamidooctyl)-1,3-bis({8-[N'-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (2, 8CH*/8CHs)



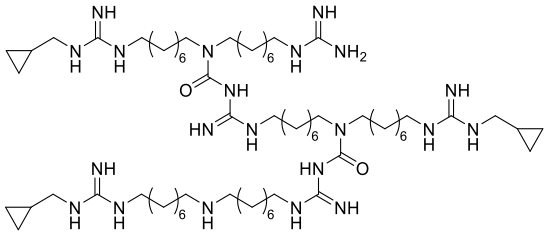
1-(8-carbamimidamidooctyl)-1-[8-[[N-(cyclopropylmethyl)carbamimidoyl]amino]octyl]-3-[N-[8-[8-[[N-(cyclopropylmethyl) carbamimidoyl]amino]octylamino]octyl]carbamimidoyl]urea trifluoroacetate salt (3, Asy8CHs)



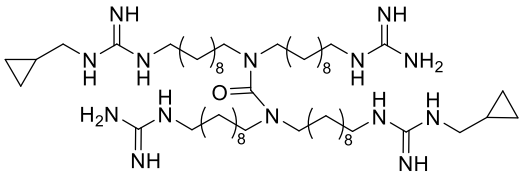
3-(8-carbamimidamidooctyl)-1-{8-[({[(8-carbamimidamidooctyl)({8-[N'-(cyclopropylmethyl)carbamimidamido]octyl})carbamoyl]amino}methanimidoyl)amino]octyl}-1,3-bis({8-[N'-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (4, Trimer8CHs)



1-(8-carbamimidamidooctyl)-1-[8-[[N-(cyclopropylmethyl)carbamimidoyl]amino]octyl]-3-[N-[8-[8-[[N-(cyclopropylmethyl)carbamimidoyl]amino]octyl-[[N-[8-[8-[[N-(cyclopropylmethyl)carbamimidoyl]amino]octylamino]octyl]carbamimidoyl]carbamoyl]amino]octyl]carbamimidoyl]urea trifluoroacetate salt (5, TrimerAsy8CHs)



1,3-bis(10-carbamimidamidodecyl)-1,3-bis({10-[N'-(cyclopropylmethyl)carbamimidamido]decyl})urea trifluoroacetate salt (6, 10CH*/10CHs)



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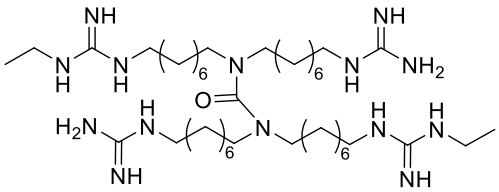

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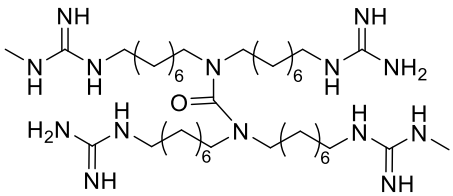
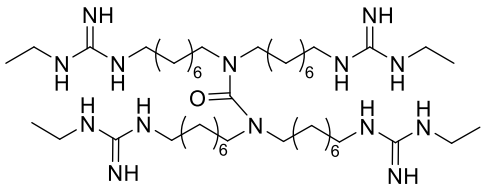
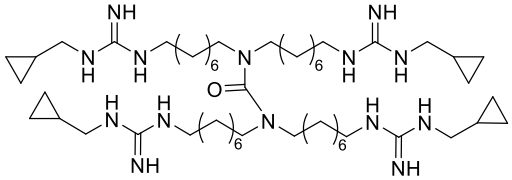


1,3-bis({8-[*N'*-(ethyl)carbamimidamido]octyl})-1,3-bis({8-[*N'*-(ethyl)carbamimidamido]octyl})urea trifluoroacetate salt (9, 8EE*/8EEs)



1,3-bis({8-[*N'*-(cyclopropylmethyl)carbamimidamido]octyl})-1,3-bis({8-[*N'*-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (10, 8CC*/8CCs)



General Chemistry. All commercially available chemicals and solvents were used as purchased. DCM was dried over calcium hydride and THF was dried over sodium and benzophenone prior to use. Anhydrous reactions were run under a positive pressure of dry nitrogen. Chromatographic separations were performed on columns packed with silica gel (230-400 mesh, for flash technique). TLCs were visualized under UV light and stained with Ninhydrin or basic permanganate stains. 1H NMR and ^{13}C NMR were recorded at 400 and 100 MHz respectively on a Bruker AC200F spectrometer and are reported in parts per million (δ scale) and internally referenced to the $CDCl_3$, CD_3OD, and DMSO-D_6 signal, respectively at δ 7.24, 3.31, and 2.50 ppm. Chemical shifts for carbon are reported in parts per million (δ scale) and referenced to the carbon resonances of the solvent ($CDCl_3$ at δ 77.00, CD_3OD at δ 49.00 ppm, and DMSO-D_6 at δ 39.00). Data are shown as following: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qi = quintet, m = multiplet and/or multiplet resonances, br = broad signal), coupling constant (*J*) in Hertz (Hz) and integration. Mass spectra (LC-MS) were acquired using an Agilent 1100 LC-MSD VL system (G1946C) by direct injection with a 0.4 mL/min flow rate using a binary solvent system of 95/5 MeOH/H_2O. UV detection was monitored at 221 or 254 nm. Mass spectra were acquired in positive mode scanning over the mass range 105-1500 *m/z*, using a variable fragmentor voltage of 10-70 mV.

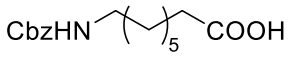
Determination of purity. The purity of final products (**2, 11-32**) was 95% or higher and it was assessed by HPLC-UV-MS, using an Equivalence 3 C18 column (ACE EQV-8977: 150 x 4.6 mm, 5 µm particle size) at a flow rate of 0.6 mL/min with a linear gradient elution from 100/0 to 50/50 v/v CH_3CN (formic acid 0.1% v/v)/H_2O (formic acid 0.1% v/v). UV detection was monitored at 210 nm. Mass spectra were acquired in positive mode scanning over the mass range 105-1500 *m/z*, using a fragmentor voltage of 70 mV.

The preparation of all the compounds is reported in the order they appear in the *Results and Discussion* Chapter.

Procedures for synthesis and characterization of compounds 11-113.

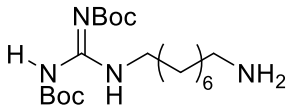
Preparation of *Traditional* Monomers

8-{[(benzyloxy)carbonyl]amino}octanoic acid (42)



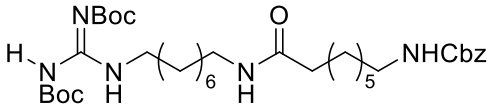
8-Aminooctanoic acid (500 mg, 3,14 mmol) was dissolved in a solution of THF/H_2O 1/1 and $NaHCO_3$ (290.1 mg, 3.45 mmol) was added. Then, to the stirring mixture, benzyl chloroformate (0,67 mL, 4,71 mmol) was added dropwise at -20 °C. The resulting mixture was stirred at r.t. for 16 h. The reaction was quenched with H_2O and AcOEt was added. Aqueous phase was separated and the pH of aqueous phase was adjusted to 2.0 by addition of HCl 4 N and extracted twice with AcOEt. The combined organic layers were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was used in the next step without any further purification. ^1H NMR ($CDCl_3$) δ (ppm): 1.29 (m, 6H), 1.46 (m, 2H), 1.59 (m, 2H), 2.30 (t, 2H, *J*= 7.2 Hz), 3.14 (m, 2H), 5.12 (s, 2H), 7.32 (m, 5H)10.70 (s, 1H). ^{13}C NMR (CDCl3) δ (ppm): 25.4, 28.9, 29.2, 30.7, 40.6, 34.5, 128.3, 66.7, 156.5, 136.6, 178.6. LCMS *m/z* (ES+) = 294.1 $[M+H]^+$, 316.0 $[M+Na]^+$, 609.0 $[2M+Na]^+$. Yield: 97%.

tert-butyl _N_-[[(8-aminooctyl)amino]({[(_tert_-butoxy)carbonyl]amino})methylidene]carbamate (43)



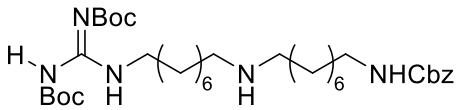
To a solution of 1,8-diaminooctane (1.0 g, 6.94 mmol) and DIPEA (0.58 mL, 3.34 mmol) in CH_3CN/MeOH 9/1 (12.0 mL), a solution of *N,N*-diBoc-pyrazole-1-carboxamidine in CH_3CN/MeOH 9/1 (12.0 mL) was added and the reaction mixture was stirred at r.t for 16 h. Then the reaction mixture was concentrated under reduced pressure and the crude product was purified by flash column chromatography (silica gel) (DCM/MeOH 9/1), affording the product as a pale-yellow oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.31 (s, 12H), 1.49 (s, 18H), 2.33 (br, 2H), 2.71 (t, 2H, *J*= 7.2 Hz), 3.37-3.42 (m, 2H), 8.29 (br, 1H), 11.49 (br, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 26.6, 26.7, 26.9, 27.3, 27.5, 27.8, 28.0, 28.3, 28.6, 29.3, 30.1,30.4, 40.8, 83.2, 153.4, 156.3,163.7. LC-MS *m/z* (ES+) = 387.0 $[M + H]^+$. Yield: 67%.

benzyl _N_-{7-[(8-{[{[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl]amino}octyl)carbamoyl]heptyl}carbamate (44)



To a stirred solution of **44** (143.0 mg, 0.37 mmol) in dry DMF (0.6 mL), **43** (152.4 mg, 0.52 mmol) in dry DMF (0.6 mL) was added under nitrogen atmosphere. Then, EDC (57.4 mg, 0.37 mmol), HOBt (50.0 mg, 0.37 mmol) and dry DIPEA (56.8 mg, 0.44 mmol) were added at 0 °C. The mixture was stirred at r.t. for 16 h. Then, H_2O and AcOEt were added and the aqueous layer was extracted with AcOEt. The combined organic layers were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by purified by flash column chromatography (silica gel) (AcOEt /Hexane 8/2), yielding **44** as a white solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.13-1.02 (m, 18H), 1.26 (s, 9H), 1.27 (s, 9H), 1.44-1.34 (m, 4H), 1.92-1.90 (m, 2H), 2.91-2.88 (m, 2H), 2.97-2.96 (m, 2H), 3.20-3.09 (m, 2H), 4.84 (s, 2H), 5.37 (bs, 1H), 6.36 (bs, 1H), 7.14-7.00 (m, 5H), 8.09 (bs, 1H), 11.31 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 25.4, 26.3, 26.5, 26.6, 27.5, 27.8, 28.0, 28.1, 28.7, 28.9, 29.3, 29.6, 36.2, 39.2, 40.7, 40.8, 66.0, 78.9, 82.8, 127.6, 128.0, 128.2, 136.6, 153.0, 155.9, 156.4, 163.3, 173.1. LCMS *m/z* (ES+) = 662.4 $[M + H]^+$, 684.4 $[M + Na]^+$. Yield:70%.

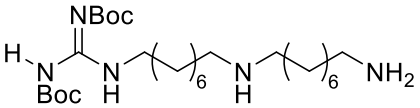
benzyl _N_-{8-[(8-{_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl]amino}octyl)amino]octyl}carbamate (45)



To a stirred solution of **44** (1.03 g, 1.56 mmol) in dry DCM (29 mL) cooled at -78 °C a solution of DIBAL(H) in DCM (1M, 3.12 mL, 3.12 mmol) was added dropwise under nitrogen atmosphere and the mixture was stirred for 1 h. Then it was allowed to reach r.t. and stirred for 16 h. Then, the mixture was treated with Rochelle salt saturated solution. The it was extracted with AcOEt and the combined organic layers were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was used in the next step without any further purification. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.28-1.35 (m, 24H), 1.49 (s, 18H), 2.60 (t, 4H, *J*= 7.2 Hz), 3.18 (q, 2H, *J*= 6.5 Hz), 3.39 (q, 2H, *J*= 6.6 Hz), 5.09 (s, 2H), 7.28-7.34 (m,

5H), 8.28 (br, 1H), 11.49 (br, 1H). ^{13}C NMR $CDCl_3$) δ (ppm): 26.4, 26.7, 28.4, 29.3, 30.3, 30.5, 40.3, 41.9, 49.9, 66.8, 79.9, 84.6, 127.1, 127.6, 128.9, 136.1, 153.7, 155.9, 158.0, 160.4. LC-MS *m/z* (ES+) = 648.2 $[M + H]^+$. LCMS *m/z* (ESI) = 670.6 $[M+Na]^+$. Yield: 30%.

tert-butyl _N_-[({8-[(8-aminooctyl)amino]octyl}amino)({[(_tert_-butoxy)carbonyl]amino})methylidene]carbamate (41a)



^{1}H NMR (CD_3OD) δ (ppm):1.30-1.40 (m, 24H), 1.52 (s, 18H), 2.62 (t, *J*= 7.6 Hz, 4H), 3.28-3.32 (m, 2H), 3.32-3.38 (m, 2H). ^{13}C NMR (CD_3OD) δ (ppm): 26.3, 26.4, 26.8, 27.1, 28.6, 28.7, 29.0, 29.0, 31.6, 40.3, 40.8, 49.1, 78.8, 83.0, 152.8, 156.1, 163.1. LC-MS *m/z* (ES+) = 514.0 $[M + H]^+$.

Via Amide Reduction

To a solution of **44** (290.3 mg, 0.45 mmol) in *i*-PrOH (30.0 mL), Pd/C 10% (64.0 mg, 0.06 mmol) was added. The reaction mixture was subjected to three cycles of vacuum followed by a flash of H_2, then, it was stirred under H_2 atmosphere for 16 h. The reaction mixture was diluted with MeOH and filtered through a plug of celite. The filtrate was evaporated under reduced pressure and the compound was obtained without any further purification as a yellowish oil. Yield: quantitative.

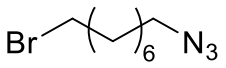
Via Cesium hydroxide

To a solution of **47** (431.2 mg, 0.8 mmol) in THF (6.0 mL), triphenylphosphine (262.0, 1mmol) was added and the mixture was stirred for 1 h at r.t. Then, H_2O (0.30 mL, 16 mmol) was added and the reaction mixture was stirred at r.t. for 16 h. The reaction mixture was diluted with AcOEt and extracted with HCl 1 N. The aqueous phase was carefully basified with NaOH 1 N and back-extracted with AcOEt. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. No further purification was necessary. Compound **41a** was obtained as colorless oils. Yield: quantitative.

Via Triamine

The triamine derivative **40a** (607.0 mg, 2.24 mmol) was dissolved in THF/MeOH 5/3 (11.0 mL) and the temperature was increased to 45 °C. When the compound was completely solubilized, a solution of 1,3-Bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (217.5 mg, 0.75 mmol) in THF (11.0 mL) was added dropwise over 2 h through the syringe pump and the reaction mixture was stirred at 45 °C for 16 h. The solvent was evaporated and the crude product was purified through flash chromatography (DCM/MeOH/TEA 8/2/1), affording the desired compound as a whitish solid. Yield: 85%.

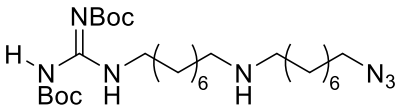
1-azido-8-bromooctane (46a)



To a solution of 1,8-dibromooctane (6.0 mL, 32.4 mmol) in DMF (60.0 mL), sodium azide (1.2 g, 19.4 mmol) was added and the reaction mixture was stirred at 50 °C for 24 h. After cooling, the reaction mixture was diluted with AcOEt. and H_2O. Then, it was extracted with AcOEt and the combined organic

layers were washed with brine, dried over Na_2SO_4, filtered and, then, evaporated under reduced pressure. The crude product was purified through flash chromatography (Hexane) to afford the corresponding bromoazide derivative as oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.33 (m, 6H), 1.43 (m, 2H), 1.60 (m, 2H), 1.85 (m, 2H), 3.25 (m, 2H), 3.40 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.5, 27.9, 28.5, 28.7, 28.8, 32.6, 33.8, 51.3. Yield: 68%.

1-azido-8-bromooctane; *tert*-butyl *N*-[({8-[(8-azidooctyl)amino]octyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]carbamate (47)



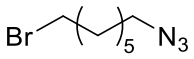
To cesium hydroxide monohydrate (266.0 mg, 1.59 mmol) and molecular sieves (600.0 mg) dry DMF (5.0 mL) was added under nitrogen atmosphere. The mixture was stirred for 10 min, then, a solution of **43** (612.0 mg, 1.59 mmol) in dry DMF (5.0 mL) was added dropwise and the mixture was stirred for further 30 min. Then **42** (297.0 mg, 1.27 mmol) was added and the reaction mixture was stirred at r.t. for 24 h. The mixture was diluted with AcOEt, filtered over cotton, washed with AcOEt and concentrated under reduced pressure. The residue was treated with $NaHCO_3$ and extracted with AcOEt. The combined organic layers were washed with H_2O, an aqueous solution of LiCl 5% and brine. The crude product was purified by flash column chromatography (silica gel) (DCM/MeOH 9/1), affording the product as a yellowish oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.25-1.35 (m, 24H), 1.44 (s, 18H), 2.52 (t, *J*= 7.0 Hz, 4H), 3.19 (t, *J*= 6.8 Hz, 2H), 3.34 (q, 2H, *J*= 5.6 Hz), 8.23 (br, 1H), 11.50 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.7, 27.0, 28.4, 29.3, 30.1, 30.3, 30.5, 41.9, 49.9, 50.0, 79.9, 84.6, 153.7, 158.0, 160.4. LC-MS *m/z* (ES+) = 540.1 $[M + H]^+$. Yield: 30%.

General procedure for the synthesis of bromoazide derivatives 46a-c.

To a solution of 1,n-dibromoalcane (32.4 mmol) in DMF (60.0 mL), sodium azide (19.4 mmol) was added and the reaction mixture was stirred at 50 °C for 24 h. After cooling, the reaction mixture was diluted with AcOEt. and H_2O. Then, it was extracted with AcOEt and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered and, then, evaporated under reduced pressure. The crude product was purified through flash chromatography (eluent: Hexane) to afford the corresponding bromoazide derivatives as oils.

46a has been described above.

1-azido-7-bromheptane (46b)



^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.32-1.39 (m, 4H), 1.41-1.46 (m, 2H), 1.54-1.60 (m, 2H), 1.82-1.87 (m, 2H), 3.24 (t, 2H, *J*=6.8 Hz), 3.38 (t, 2H, *J*= 7.2 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.5, 27.9, 28.5, 28.6, 32.4, 33.5, 51.0.Yield: 50%.

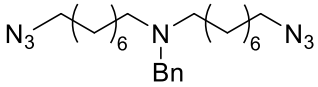
1-azido-10-bromodecane (46c)

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.25-1.30 (m, 10H), 1.40-1.44 (m, 2H), 1.56-1.60 (m, 2H), 1.81-1.84 (m, 2H), 3.21-3.25 (m, 2H), 3.36-3.39 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 26.6, 28.0, 28.6, 28.7, 29.0, 29.2, 32.7, 33.8, 51.4. Yield: 82%.

General procedure for the synthesis of diazido-benzylamine derivatives 48a-c.

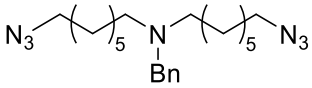
Benzylamine (3.67 mmol), potassium carbonate (11.01 mmol) and potassium iodide (1.28 mmol) were dissolved in *N*-butanol (8.4 mL). The mixture was heated at 115 °C and a solution of **46a-c** (9.18 mmol) in *N*-butanol (21.0 mL) was added. The reaction mixture was stirred at 115°C for 24 h. After cooling, the reaction mixture was filtered and the white solid was washed with AcOEt. The organic phase was washed with brine, dried over Na_2SO_4 and then evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 95/5), affording the compounds as yellowish oils.

bis(8-azidooctyl)(benzyl)amine (48a)



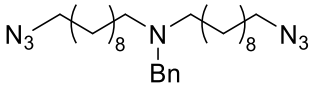
^{1}H NMR ($CDCl_3$) δ (ppm): 1.26 (s, 12H), 1.33 (m, 4H), 1.46 (m, 4H), 1.58 (m, 4H), 2.40 (t, 4H, *J*= 6.8 Hz), 3.24 (t, 4H, *J*= 6.8 Hz), 3.55 (s, 2H), 7.24 (m, 1H), 7.29 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.6, 26.9, 27.2, 28.7, 29.0, 29.3, 51.4, 53.7, 58.6, 126.5, 128.0, 128.7, 140.3. LCMS *m/z* (ES+) = 414.0 $[M + H]^+$. Yield: 96%.

bis(7-azidoheptyl)(benzyl)amine (48b)



^{1}H NMR ($CDCl_3$) δ (ppm): 1.27-1.28 (m, 12H), 1.50-1.59 (m, 8H), 2.44 (m, 4H), 3.23 (t, 4H, *J*= 6.8 Hz), 3.6 (s, 2H), 7.24-7.34 (m, 5H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.4, 27.2, 28.5, 29.1, 29.3, 51.4, 53.7, 58. 7, 126.54, 128.6, 129.0, 140.2. LC-MS *m/z* (ES+) = 386.0 $[M + H]^+$ 408 $[M+Na]^+$. Yield: 69%.

10-azido-*N*-(10-azidodecyl)-*N*-benzyldeca*N*-1-amine (48c)



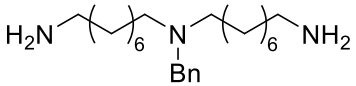
^{1}H NMR ($CDCl_3$) δ (ppm): 1.26 (s, 24H), 1.45-1.47 (m, 4H), 1.57-1.59 (m, 4H), 2.38-2.42 (m, 4H), 3.23-3.25 (m, 4H), 3.55 (s, 2H), 7.22-7.24 (m, 1H), 7.30-7.32 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.6, 26.9, 27.3, 28.8, 29.1, 29.4, 29.5, 51.4, 53.7, 58.6, 126.5, 127.9, 128.7, 140.1. LC-MS *m/z* (ES+) = 470.1 $[M + H]^+$. Yield: 82%.

General procedure for the synthesis of benzyltriamine derivatives 49a-c.

To a solution of **48a-c** (5.06 mmol) in THF (40.0 mL), triphenylphosphine (15.18 mmol) was added and the mixture was stirred for 1 h at r.t. Then H_2O (202.4 mmol) was added and the reaction mixture was

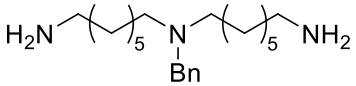
stirred at r.t. for 16 h. The reaction mixture was diluted with AcOEt and extracted with HCl 2 N. The aqueous phase was carefully basified with NaOH 2 N and back-extracted with Et_2O. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The products were obtained without any further purification as colourless oils.

bis(8-aminooctyl)(benzyl)amine (49a)



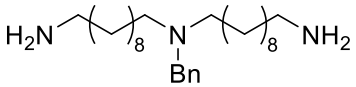
^{1}H NMR (CD_3OD) δ (ppm): 1.26 (s, 12H), 1.43 (s, 12H), 2.37 (t, 4H, *J*= 7.2 Hz), 2.67 (t, 4H, *J*= 7.0 Hz), 3.53 (s, 2H), 7.21 (m, 1H), 7.29 (m, 4H). ^{13}C NMR (CD_3OD) δ (ppm): 26.8, 26.9, 27.3, 29.4, 29,5, 33,8, 42.2, 53.7, 58.6, 126.5, 127.9, 128.7, 140.3. LCMS *m/z* (ES+) = 362.0 $[M + H]^+$. Yield: quantitative.

bis(7-aminoheptyl)(benzyl)amine (49b)



^{1}H NMR (CD_3OD) δ (ppm): 1.21-1.27 (m, 12H), 1.41-1.47 (m, 8H), 2.39 (t, 4H, *J*= 7.2 Hz), 2.58 (t, 4H, *J*= 7.2 Hz), 3.53 (s, 2H), 7.20-7.22 (m, 1H), 7.28-7.30 (m, 4H). ^{13}C NMR (CD_3OD) δ (ppm): 26.2, 26.6, 27.1, 29.0, 32.3, 41.1, 53.3, 58.2, 126.5, 127.7, 128.9, 138.9. LC-MS *m/z* (ES+) = 334.0 $[M + H]^+$. Yield: quantitative.

bis(10-aminododecyl)(benzyl)amine (49c)

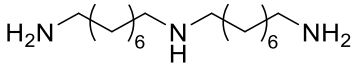


^{1}H NMR ($CDCl_3$) δ (ppm): 1.20-1-23 (m, 24H), 1.37-1.39 (m, 8H), 2.34 (t, 4H, *J*= 7.4 Hz), 2.63 (t, 4H, *J*= 7.0 Hz), 3.49 (s, 2H), 7.14-7.16 (m, 1H), 7.24-7.26 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.8, 26.9, 27.3, 29.4, 29.5, 33.7, 42.1, 53.7, 58.5, 126.4, 127.9, 128.7, 140.2. LC-MS *m/z* (ES+) = 418.0 $[M + H]^+$. Yield: quantitative.

General procedure for the synthesis of triamine derivatives 40a-c.

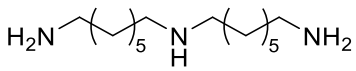
To a solution of **49a-c** (1.18 mmol) in *i*-PrOH (19.0 mL), Pd/C (10%, 0.06 mmol) and AcOH (6.1 mL) were added. The reaction mixture was subjected to three cycles of vacuum followed by a flux of H_2, and it was stirred under a strong flux of H_2 for 16 h. Then, the reaction mixture was diluted with MeOH and filtered through a plug of celite. The filtrate was concentrated and NaOH 2 N was added until the formation of a precipitate. The aqueous phase was extracted three times with AcOEt and the combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The triamine derivatives **40a-c** were obtained as white solids without any further purification.

bis(8-aminooctyl)amine (40a)



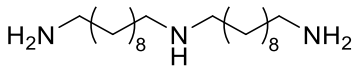
^{1}H NMR (CD_3OD) δ (ppm): 1.33 (s, 16H), 1.48 (m, 8H), 2.54 (t, 4H, *J*= 7.4 Hz), 2.61 (t, 4 H, *J*= 7.2 Hz). ^{13}C NMR (CD_3OD) δ (ppm): 26.5, 27.0, 29.1, 29.4, 32.4, 41.1, 46.97, 47.1, 47.3, 47.5, 47.7, 47.9, 48.2, 49.3. LCMS *m/z* (ES+) = 272.0 $[M + H]^+$. Yield: quantitative.

bis(7-aminoheptyl)amine (40b)



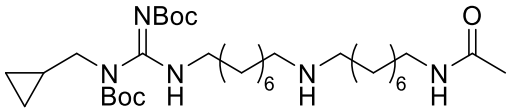
^{1}H NMR (CD_3OD) δ (ppm): 1.33 (s, 12H), 1.49-1.51 (m, 8H), 2.57 (t, 4H, *J*= 7.2 Hz), 2.64 (t, 4H, *J*= 7.2 Hz). ^{13}C NMR (CD_3OD) δ (ppm): 26.5, 26.9, 28.9, 29.3, 32.2, 41.1, 49.3. LC-MS *m/z* (ES+) = 244.0 $[M + H]^+$. Yield: quantitative.

bis(10-aminododecyl)amine (40c)



^{1}H NMR (CD_3OD) δ (ppm): 1.32 (s, 24H), 1.47-1.49 (m, 8H), 2.54 (t, 4H, *J*= 7.6 Hz), 2.61 (t, 4H, *J*= 7.2 Hz). ^{13}C NMR (CD_3OD) δ (ppm): 26.6, 27.0, 29.0, 29.2, 32.4, 41.1, 46.9, 47.1, 47.3, 47.5, 47.7, 47.9, 49.3. LC-MS *m/z* (ES+) = 328.0 $[M + H]^+$, 164.5 $[M + 2H]^{2+}$, 350.0 $[M + Na]^+$. Yield: quantitative.

***tert*-butyl *N*-[{[(*tert*-butoxy)carbonyl]imino}({8-[(8-acetamidooctyl)amino]octyl}amino)methyl]-*N*-(cyclopropylmethyl)carbamate (53)**

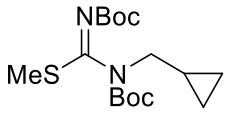


^{1}H NMR ($CDCl_3$) δ (ppm): 0.21 (m, 2H), 0.451 (m, 2H), 1.02 (m, 1H), 1.34 (m, 16H), 1.44 (s, 9H), 1.48 (s, 9H), 1.60 (m, 4H), 1.78 (m, 2H), 1.96 (m, 2H), 2.16 (s, 3H), 2.69 (m, 2H), 2.84 (t, 2H, *J*= 7.6 Hz), 3.20 (t, 2H, *J*= 6.4 Hz), 3.25 (t, 2H, *J*= 11.2 Hz) 3.52 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 23.3, 26.3, 26.6, 26.8, 27.2, 28.2, 28.8, 29.2, 31.0, 39.2, 43.7, 48.2, 52.2, 149.0, 166.3, 170.4. LCMS *m/z* (ES+) = 610.0 $[M + H]^+$, 305.0 $[M + 2H]^{2+}$**.**

General procedure for the synthesis of guanylating agents 39 and 54-57.

N,N-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (for **39**, 0.93 g, 3.22 mmol) or *N,N*-Di-Boc-1*H*-pyrazole-1-carboxamidine (for **54-57**, 1.00 g, 3.22 mmol) was dissolved in dry THF (12.4 mL). Then triphenylphosphine (1.27 g, 4.83 mmol) and the suitable alcohol (4.19 mmol) were added. The reaction mixture was cooled at 0 °C and Diisopropyl azodicarboxylate (0.95 mL, 4.83 mmol) was added dropwise. The temperature was increased to 66 °C and the reaction mixture was stirred at reflux overnight. After cooling, the reaction mixture was concentrated and then diluted with DCM (20.0 mL) and H_2O (20 mL). The aqueous phase was extracted for three times with DCM (20.0 mL), the combined organic phases were washed with brine (60.0 mL) twice and dried over Na_2SO_4. The solvent was removed under reduced pressure. The crude product was purified through flash chromatography (eluent: Hexane/AcOEt 9/1) to afford the desired compounds as colourless oils.

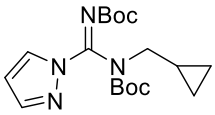
***tert*-butyl N-[({[(*tert*-butoxy)carbonyl]imino}(methylsulfanyl)methyl]-N (cyclopropylmethyl)carbamate (39)**



^{1}H NMR ($CDCl_3$) δ (ppm): 0.30 (q, 2H, *J*= 4.8 Hz), 0.51 (q, 2H, *J*= 5.0 Hz), 1.13-1.18 (m, 1H), 1.49 (s, 9H), 1.50 (s, 9H), 1.55 (s, 1H), 2.40 (s, 3H, *J*= 3 Hz), 3.43 (d, 2H, *J*= 7.2 Hz. **^{13}C NMR** ($CDCl_3$) δ (ppm): 7.2, 10.8,

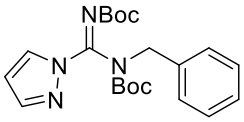
14.7, 27.7, 28.7, 44.1, 80.0, 81.3, 155.7, 156.9. **LCMS** *m/z* (ES+) = 367.0 [M + Na]$^+$, 711.0 [2M + Na]$^+$. **Yield:** 85%.

***tert*-butyl *N*-cyclopropropylmethyl-*N*-[{[(*tert*-butoxy)carbonyl]imino}(1H-pyrazol-1-yl)methyl]carbamate (54)**



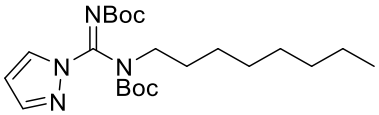
^{1}H NMR ($CDCl_3$) δ (ppm): 0.45 (m, 2H), 0.49 (m, 2H), 1.27 (s, 9H), 1.49 (s, 9H), 1.54 (s, 1H), 3.60 (d, 2H, *J*= 6.8 Hz), 6.41 (t, 1H, *J*= 2.2 Hz), 7.69 (d, 1H, *J*= 1.2 Hz), 7.95 (s, 1H). **^{13}C NMR** ($CDCl_3$) δ (ppm): 9.8, 27.7, 27.8, 28.0, 53.2, 82.2, 82.4, 108.7, 129.7, 142.9, 152.5, 157.5. **LCMS** *m/z* (ES+) = 387.0 [M + Na]$^+$, 751.0 [2M + Na]$^+$. **Yield:** 92%.

***tert*-butyl *N*-benzyl-*N*-[{[(*tert*-butoxy)carbonyl]imino}(1H-pyrazol-1-yl)methyl]carbamate (55)**



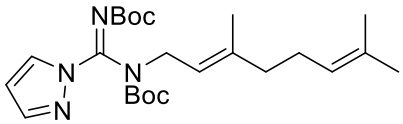
^{1}H NMR ($CDCl_3$) δ (ppm): 1.23 (s, 6H), 1.41 (s, 3H), 1.45 (s, 6H), 1.50 (s, 3H), 4.91 (s, 2H), 6.32 (s, 1H), 7.28 (m, 5H), 7.43 (d, 1H, *J*= 7.6 Hz), 7.65(s, 1H), 11.00 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 27.5, 27.6, 27.7, 27. 9, 52.13, 82.1, 83.0, 127.2, 127.6, 128.2, 129.7, 129.9, 142.7, 152.4, 157.4. LCMS *m/z* (ES+) = 400.9 [M + H]$^+$,422.9 [M + Na]$^+$, 822.9 [2M + Na]$^+$. Yield: 85%.

***tert*-butyl *N*-octyl-*N*-[{[(*tert*-butoxy)carbonyl]imino}(1H-pyrazol-1-yl)methyl]carbamate (56)**



^{1}H NMR ($CDCl_3$) δ (ppm): 0.86 (t, 3H, *J*= 6.8 Hz), 1.26 (m, 10H), 1.50 (s, 18H), 1.71 (m, 2H), 3.65 (t, 2H, *J*= 8 Hz), 6.40 (s, 1H), 7.68 (m, 1H), 7.93 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.2, 22.9, 26.8, 27.8, 28.2, 28.3, 28.6, 28.7, 31.7, 43.8, 80.2, 82.4, 108.7, 129.7, 142.9, 152.5, 157.5. LCMS *m/z* (ES+) = 423.0 [M + H]$^+$,445.0 [M + Na]$^+$, 890.9 [M + Na]$^+$. Yield: 89%.

***tert*-butyl *N*-[{[(*tert*-butoxy)carbonyl]imino}(1H-pyrazol-1-yl)methyl]-*N*-[(2E)-3,7-dimethylocta-2,6-die*N*-1-yl]carbamate (57)**



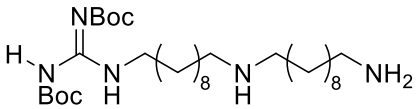
^{1}H NMR ($CDCl_3$) δ (ppm): 1.27 (s, 9H), 1.48 (s, 9H), 1.54 (s, 3H), 1.58 (s, 3H), 1.62 (s, 3H), 1.96-2.00 (m, 4H), 4.29 (d, 2H, *J*= 6.8 Hz), 5.02 (m, 1H), 5.36 (t, 1H, *J*= 6.8 Hz), 6.36 (s, 1H), 7.64 (s, 1H), 7.87 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.3, 27.3, 27.5, 27.7, 28.0, 39.5, 46.4, 82.0, 82.4, 108.0, 108.7, 109.3, 118.3, 123.7, 124.0, 129.6, 131.4, 140.7, 142.8, 143.0, 152.3, 157.3. LCMS *m/z* (ES+) = 447.0 [M + H]$^+$, 468.9 [M + Na]$^+$, 915.0 [2M + Na]$^+$. Yield: 93%.

General procedure for the synthesis of monoguanylated monomer derivatives 41a-e.

The triamine derivative **40a-b** or **c-e** (commercially available) (2.24 mmol) was dissolved in THF/MeOH 5/3 (11.0 mL) and the temperature was increased to 45 °C. When the compound was completely solubilized, a solution of 1,3-Bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (0.75 mmol) in THF (11.0 mL) was added dropwise over 2 h through the syringe pump and the reaction mixture was stirred at 45 °C for 16 h. The solvent was evaporated and the crude product was purified through flash chromatography (silica gel) (DCM/MeOH/TEA 8/2/1), affording the desired compound as a yellowish oil.

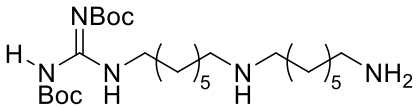
41a has been described above.

***tert*-butyl *N*-[({10-[(10-aminodecyll)amino]decyl}amino)({[(*tert*-butoxy)carbonyl]amino})methylidene]carbamate (41b)**



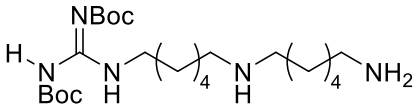
^{1}H NMR (CD_3OD) δ (ppm): 1.31-1.33 (m, 32H), 1.47 (s, 9H), 1.52 (s, 9H), 2.55-2.59 (m, 4H), 2.62-2.64 (m, 2H), 3.38 (t, 2H, *J*= 7.0 Hz). ^{13}C NMR (CD_3OD) δ (ppm): 26.5, 26.8, 27.0, 27.2, 28.6, 28.8, 28.9, 29.1, 32.2, 40.3, 41.0, 49.3, 78.8, 82.9, 152.8, 156.0, 163.1. LC-MS *m/z* (ES+) = 570.0 $[M + H]^+$, 285.5 $[M + 2H]^{2+}$, 592.0 $[M + Na]^+$. Yield: 82%.

***tert*-butyl *N*-[({7-[(7-aminoheptyl)amino] heptyl}amino)({[(*tert*-butoxy)carbonyl]amino})methylidene]carbamate (41c)**



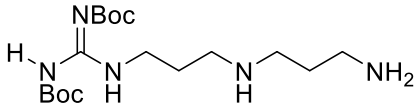
^{1}H NMR (CD_3OD) δ (ppm): 1.30-1.32 (m, 20H), 1.47 (s, 9H), 1.50 (s, 9H), 2.53-2.56 (m, 4H), 2.62-2.64 (m, 2H), 3.38 (m, 2H). ^{13}C NMR (CD_3OD) δ (ppm): 25.1, 26.0, 26.2, 27.0, 27.5, 28.0, 28.8, 28.9, 29.0, 32.2, 40.3, 41.0, 48.0, 79.0, 82.5, 152.8, 156.3, 161.1. LC-MS *m/z* (ES+) = 486.0 $[M+H]^+$. Yield: 73%.

***tert*-butyl *N*-[({6-[(6-aminohexyl)amino]hexyl}amino)({[(*tert*-butoxy)carbonyl]amino})methylidene]carbamate (41d)**



^{1}H NMR ($CDCl_3$) δ (ppm): 1.31 (m, 16H), 1.45 (s, 18H), 1.53 (m, 4H), 2.57 (t, 4H, *J*= 7.2 Hz), 2.65 (t, 2H, *J*= 6.8 Hz), 3.39 (q, 2H, *J*= 6.5 Hz), 8.28 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm):26.6, 26.7, 26.8, 26.9, 27.9, 28.2, 28.8, 29.2, 29.3, 32.8, 40.7, 41.7, 49.4, 50.1, 79.1, 82.9, 153.2, 156.0, 163.5. LCMS *m/z* (ES+) = 480.2 $[M + Na]^+$, 458.3 $[M + H]^+$. Yield: 74%.

tert-butyl _N_-[({3-[(3-aminopropyl)amino]propyl}amino)({[(_tert_-butoxy)carbonyl]amino})methylidene]carbamate (41e)

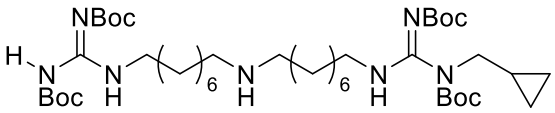


^{1}H NMR ($CDCl_3$) δ (ppm): 1.46 (s, 9H), 1.52 (s, 9H), 1.69 (quint, 2H, *J*= 7.2 Hz), 1.77 (quint, 2H, *J*= 6.8 Hz), 2.62-2.67 (m, 4H), 2.72 (t, 2H, *J*= 6.8 Hz), 3.43 (t, 2H, *J*= 6.8 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 27.9, 28.2, 20.6, 38.7, 46.3, 79.8, 80.3, 153.8, 154.1, 163.0. LC-MS *m/z* (ES+) = 374.0 $[M+H]^+$. Yield: 85%.

General procedure for the synthesis of asymmetric monomers 33, 59-65.

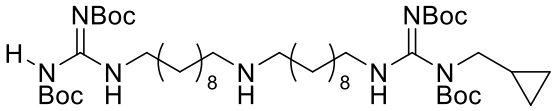
A solution of the appropriate guanylating agent **54-57** (1.32 mmol) in THF (10.8 mL) was added to monoguanylated triamine **40a-e** (1.10 mmol). DIPEA (0.19 mL, 1.10 mmol) was added and the reaction mixture was stirred at r.t. for 16 h. Then the mixture was diluted with DCM and washed with $NaHCO_3$ s.s. and brine. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 9/1), affording the monomers as yellowish oils.

tert-butyl _N_-({[8-({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}amino)octyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate (8CH, 33)



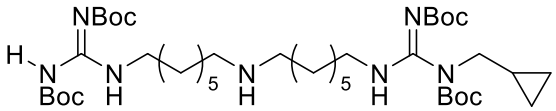
^{1}H NMR ($CDCl_3$) δ (ppm): δ 0.24 (d, 2H, *J*= 4.8 Hz,), 0.45 (d, 2H, *J*= 7.6 Hz), 1.00-1.10 (m, 1H), 1.25-1.41 (m, 16H), 1.49 (s, 36H), 1.50-1.60 (m, 8H), 2.58 (t, 4H, *J*= 7.2 Hz), 3.25-3.35 (m, 2H), 3.39 (q, 2H, *J*= 6.5 Hz), 3.51-3.58 (m, 2H), 8.28 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 10.4, 26.1, 26.6, 26.7, 27.9, 28.1, 28.2, 28.8, 29.0, 29.1, 40.7, 44.0, 47.6, 52.0, 79.0, 81.8, 82.9, 153.2, 156.0, 163.5. LC-MS *m/z* (ES+) = 809.9 $[M + H]^+$, 405.5 $[M + 2H]^{2+}$. Yield: 70%.

tert-butyl _N_-({[10-({10-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]decyl}amino)decyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate (10CH, 59)



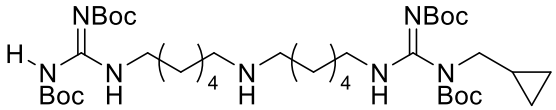
^{1}H NMR ($CDCl_3$) δ (ppm): 0.09-1.01 (m, 2H), 0.29-0.31 (m, 2H), 0.91-0.93 (m, 1H), 1.12-1.14 (m, 32H), 1.35 (s, 36H), 2.49 (t, 4H, *J*= 7.2 Hz), 3.15-3.17 (m, 2H), 3.23-3.26 (m, 2H), 3.38-3.40 (m, 2H), 8.15 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.3, 10.4, 26.6, 26.7, 27.1, 27.9, 28.0, 28.1, 28.7, 29.0, 29.2, 40.7, 43.7, 49.5, 51.9, 78.9, 81.6, 82.7, 153.1, 155.9, 163.4. LC-MS *m/z* (ES+) = 866.1 $[M + H]^+$, 433.5 $[M + 2H]^{2+}$. Yield: 60%.

***tert*-butyl *N*-({[7-({7-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]heptyl}amino)heptyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)-*N*-(cyclopropylmethyl)carbamate (7CH, 60)**



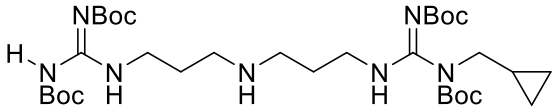
^{1}H NMR ($CDCl_3$) δ (ppm): 0.21 (m, 2H), 0.43 (m, 2H), 1.01 (m, 1H), 1.42 (m, 12H), 1.46-1.49 (m, 36H), 1.57 (m, 4H), 1.83 (m, 4H), 2.87 (m, 4H), 3.27 (m, 2H), 3.35-3.36 (m, 2H), 3.59 (m, 2H), 8.25 (br, 1H), 11.46 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 10.5, 26.0, 26.6, 26.7, 27.7, 28.0, 28.1, 28.5, 29.0, 40.6, 44.8, 47.8, 52.0, 79.1, 82.0, 82.5, 153.0, 156.1, 163.0. **LC-MS** *m/z* (ES+) = 782.0 $[M+H]^+$ 804.0 $[M+Na]^+$. Yield: 52%.

***tert*-butyl *N*-({[6-({6-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]hexyl}amino)hexyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)-*N*-(cyclopropylmethyl)carbamate (6CH, 61)**



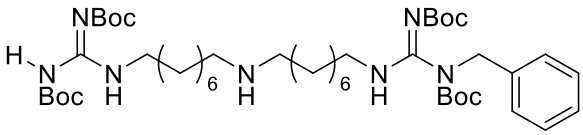
^{1}H NMR ($CDCl_3$) δ (ppm): 0.18 (m, 2H), 0.39 (m, 2H), 0.98 (m, 1H), 1.31 (m, 8H), 1.41 (m, 36H), 1.52 (m, 8H), 2.61 (m, 4H), 3.25 (m, 2H), 3.34 (m, 2H), 3.48 (m, 2H), 8.23 (br, 1H), 11.48 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 10.44 26.5, 26.6, 26.7, 27.9, 28.1, 28.2, 28.8, 29.0, 40.7, 43.6, 49.1, 52.0, 53.3, 79.0, 81.8, 82.8, 153.2, 156.0, 163.5. LCMS *m/z* (ES+) = 754.5 $[M + H]^+$, 377.7 $[M + 2H]^{2+}$. Yield: 68%.

***tert*-butyl *N*-({[3-({3-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]propyl}amino)propyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)-*N*-(cyclopropylmethyl)carbamate (3CH, 62)**



^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.25 (m, 2H), 0.49 (m, 2H), 1.06 (m, 1H), 1.45 (s, 9H), 1.46(s, 9H), 1.47 (s, 9H), 1.49 (s, 9H), 2.16-2.17 (m, 2H), 2.24 (m, 2H), 3.00 (m, 2H), 3.06 (m, 2H), 3.32-3.62 (m, 6H), 8.66 (br, 1H), 10.09 (br, 2H), 11.43 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.50, 10.2, 22.9, 26.6, 28.0, 27.9, 28.1, 28.2, 28.3, 28.6, 28.7, 30.7, 43.8, 80.3, 81.3, 135.4, 140.0, 153.5, 164.0. LC-MS *m/z* (ES+) = 670.0 $[M+H]^+$. Yield: 20%.

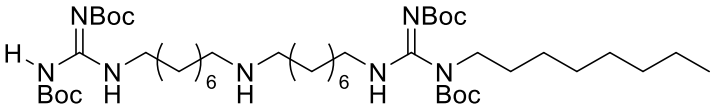
***tert*-butyl *N*-({[8-({8-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]octyl}amino)octyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)-*N*-benzylcarbamate (8BH, 63)**



^{1}H NMR ($CDCl_3$) δ (ppm): 1.24 (s, 6H), 1.30 (s, 14H), 1.42 (s, 9H), 1.48 (s, 9H), 1.49 (s, 27H), 1.70 (m, 4H), 2.78 (t, 4H, *J*= 7.6 Hz), 3.99 (m, 2H), 3.38 (q, 2H, *J*= 6.8 Hz), 4.84 (s, 2H), 7.26 (m, 5H), 8.27 (br, 1H),

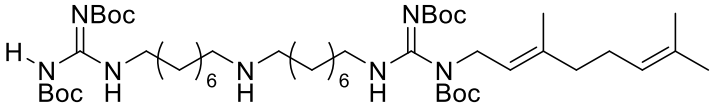
11.48 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.4, 26.6, 27.0, 28.1, 28.25, 28.6, 28.8, 28.9, 29.0, 29.1, 29.5, 40.8, 43.7, 49.1, 50.1, 50.7, 79.0, 82.8, 127.4, 128.3, 137.4, 153.2, 156.0, 163.5. LCMS *m/z* (ES+) = 846.0 $[M + H]^+$, 423.5 $[M + 2H]^{2+}$ Yield: 62%.

***tert*-butyl *N*-({[8-({8-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]octyl}amino)octyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)-*N*-octylcarbamate (8OH, 64)**



^{1}H NMR ($CDCl_3$) δ (ppm): 0.85 (t, 3H, *J*= 6.6 Hz), 1.24 (26H, dd, *J*= 7.0 Hz), 1.31 (9H, s), 1.41-1.50 (27H, m), 1.53 (m, 6H), 1.86 (m, 4H), 2.89 (m, 4H), 3.20 (t, 2H, *J*= 7.1 Hz), 3.37 (q, 1H, *J*= 6.8 Hz), 3.59 (br, 1H), 3.60 (t, 2H, *J*= 7.6 Hz), 9.44 (br, 1H), 11.48 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.1, 22.6, 25.8, 26.7, 26.8, 28.1, 28.2, 28.2, 28.3, 28.9, 28.9, 29.1, 29.2, 29.2, 31.8, 40.9, 47.4, 53.4, 79.2, 83.0, 153.3, 156.1, 163.6. LCMS *m/z* (ES+) = 770.0 $[M + H]^+$, 793.0 $[M + Na]^+$, 385.5 $[M + 2H]^{2+}$. Yield: 40%.

***tert*-butyl *N*-[({8-[(8-{[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)amino]octyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-[(2E)-3,7-dimethylocta-2,6-die*N*-1-yl]carbamate (8GH, 65)**

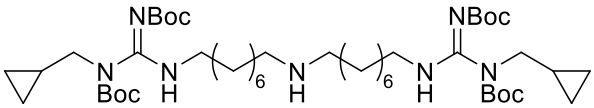


^{1}H NMR ($CDCl_3$) δ (ppm): 1.32 (s, 18H), 1.44-1.57 (m, 36H), 1.59 (s, 3H), 1.67 (s, 6H), 1.88 (m, 4H), 2.00 (m, 2H), 2.04 (m, 2H), 2.91 (m, 24H), 3.22 (m, 2H), 3.39 (m, 20H), 4.25 (m, 2H), 5.00 (t, *J*= 7.2, 1H), 5.06 (t, *J*= 7.2, 1H), 8.29 (br, 1H), 9.42 (br, 1H), 11.49 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 6.4, 26.4, 28.1, 29.0, 29.1, 30.8, 39.5, 58.1, 68.5, 78.8, 78.8, 114.6, 120.2, 123.8, 130.6, 146.1, 151.7. LCMS *m/z* (ES+) = 892.1 $[M + H]^+$, 914.1 $[M + Na]^+$, 446.8 $[M + 2H]^{2+}$. Yield: 40%.

General procedure for the synthesis of symmetrical monomers 52-67.

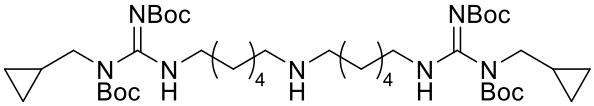
A solution of the appropriate guanylating agent **54** or **55** (0.45 mmol) in THF (2.5 mL) was added to the triamine **40a** or **40d** (0.18 mmol), solubilized in the minimum MeOH required. DIPEA (0.06 mL, 0.36 mmol) was added and the reaction mixture was stirred at r.t. for 16 h. Then the reaction mixture was diluted with AcOEt and washed with $NaHCO_3$ s.s. and brine. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 9/1), affording the compounds as yellowish oils.

***tert*-butyl *N*-({[8-({8-[({[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]octyl}amino)octyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)-*N*-(cyclopropylmethyl)carbamate (8CC, 52)**



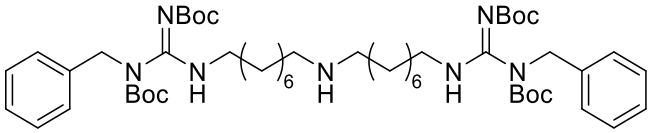
^{1}H NMR ($CDCl_3$) δ (ppm): 0.21 (m, 4H), 0.41 (m, 4H), 1.00 (m, 2H), 1.28 (m, 16H), 1.43 (s, 18H), 1.45 (s, 18H), 1.56 (m, 8H), 2.64 (t, 4H, *J*= 7.4 Hz), 3.26 (t, 4H, *J*= 6.8 Hz), 3.50 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 10.45, 26.8, 27.0, 28.1, 28.6, 29.0, 29.1, 43.8, 49.2, 50.4, 50.5, 79.1, 81.8, 152.7, 158.0, 165.6. LCMS *m/z* (ES+) = 864.6 $[M + H]^+$, 432.9 $[M + 2H]^{2+}$. Yield: 83%.

tert-butyl _N_-({[6-({6-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]hexyl}amino)hexyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate (6CC, 66)



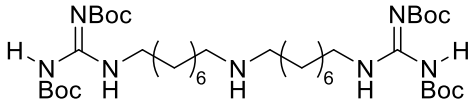
^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (m, 4H), 0.45 (m, 4H), 1.05 (m, 2H), 1.38 (m, 12H), 1.46 (s, 18H), 1.48 (s, 18H), 1.62 (m, 4H), 2.67 (t, 4H, *J*= 7.4 Hz), 3.30 (t, 4H, *J*= 6.8 Hz), 3.54 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.6, 10.6, 25.6, 26.5, 26.6, 27.0, 28.0, 28.2, 29.1, 43.6, 48.0, 51.0, 52.1, 53.4, 67.9, 79.2, 82.0, 152.7, 158.0, 165.5. LCMS *m/z* (ES+) = 808.0 $[M + H]^+$, 404.5 $[M + 2H]^{2+}$ .Yield: 69%.

tert-butyl _N_-benzyl-_N_-({[8-({8-[({benzyl[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}amino)octyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)carbamate (8BB, 67)



^{1}H NMR ($CDCl_3$) δ (ppm): 1.23 (s, 24H), 1.40 (s, 18H), 1.49 (s, 18H), 2.49 (t, 4H, *J*= 7.2 Hz), 2.98 (m, 4H), 4.83 (s, 4H), 7.24 (m, 10H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.6, 27.2, 28.1, 28.2, 29.0, 29.2, 29.8, 49.9, 50.8, 79.0, 126.0, 127.4, 137.8, 163.4. LCMS *m/z* (ES+) = 936.6 $[M + H]^+$, 468.9 $[M + 2H]^{2+}$. Yield: 71%.

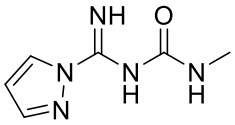
tert-butyl _N_-({[(_tert_-butoxy)carbonyl]amino}({[8-({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}amino)octyl]amino})methylidene)carbamate (8HH, 68)



To a solution of **6a** (220.0 mg, 0.81 mmol) in MeOH (1.5 mL) DIPEA (0.03 mL, 1.62 mmol) and a solution of *N,N*-Di-Boc-1*H*-pyrazole-1-carboxamidine (630.0 mg, 2.03 mmol) in THF (6.4 mL) were and the reaction mixture was stirred at r.t. for 16 h. Then the reaction mixture was concentrated under reduced pressure and treated with DCM and $NaHCO_3$ s.s. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 95/5), affording the compound as a yellowish oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.29 (s, 16H), 1.46 (s, 36H), 1.47 (m, 8H), 2.57 (t, 4H, *J*= 7.2 Hz), 3.38 (q, 4H, *J*= 6.8 Hz), 8.25 (bs, 2H), 11.45 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.7, 27.1, 28.0, 28.2, 28.8, 29.1, 29.2, 29.5, 49.7, 79.1, 82.9, 153.2, 156.0, 163.5. LCMS *m/z* (ES+) = 755.9 $[M + H]^+$, 378.5 $[M + 2H]^{2+}$ YIELD: 65%.

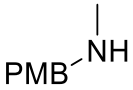
Preparation of *N*-methyl amidinourea Monomer.

3-methyl-1-(1*H*-pyrazole-1-carboximidoyl)urea (69)



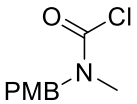
To a solution of 1*H*-pyrazole-1-carboxamidine hydrochloride (200 mg, 1,32 mmol) in dry DCM (10 mL), *N*-succinimidyl-*N*-methyl carbamate (200 mg, 1,2 mmol) and DIPEA (0,46 mL, 2,64 mmol) were added under nitrogen atmosphere. The mixture was stirred at r.t. for 16 h. The solvent was evaporated and the crude was purified by flash chromatography (silica gel) (AcOEt/ Hexane 6/4) to afford 69 as white solid. ^{1}H NMR (CD_3OD) δ (ppm): 2.74 (s, 3H), 6.41 (t, 1H, *J*= 2.0 Hz), 7.68 (s, 1H), 8.36 (d, 1H, *J*= 2.8 Hz).^{13}C NMR (CD_3OD) δ (ppm): 25.4, 107.2, 108.8, 128.0, 142.7, 152.2, 165.5. LCMS *m/z* (ES+) =168.3 $[M + H]^+$, 190.4 $[M + Na]^+$. Yield: 50%.

[(4-methoxyphenyl)methyl](methyl)amine (70)



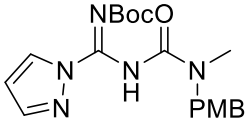
To a stirred solution of *p*-anisaldehyde (1.0 mL, 8.2 mmol) in MeOH (3.5 mL), a 40% aqueous solution of methylamine (1.05 mL, 12.33 mmol) was added dropwise and the mixture was stirred at r.t. for 1 h. Then the mixture was cooled to 0 °C and sodium borohydride (311 mg, 8.22 mmol) was added portio*N*-wise and the mixture was stirred at 0 °C for 2 h. The reaction was quenched with HCl 1 N and partially evaporated under reduced pressure. The residue was basified with NaOH 1 N and extracted with DCM. The combined organic phases were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The oil obtained was used in the next step without further purification. ^{1}H-NMR (CDCl3) δ (ppm): 1.89 (d, 1H, J= 80 Hz), 2.55 (s, 3H), 3.70 (s, 2H), 3.80 (s, 3H), 6.86 (d, 2H, *J*= 6.6 Hz), 7.26 (d, 2H, *J*= 6.6 Hz). ^{13}C-NMR (CDCl3) δ (ppm): 35.7, 55.3, 55.4, 113.8, 129.5, 158.8,187.9. LCMS *m/z* (ES+) = 152.1 $[M + H]^+$. Yield: 77%

***N*-[(4-methoxyphenyl)methyl]-*N*-methylcarbamoyl chloride (71)**



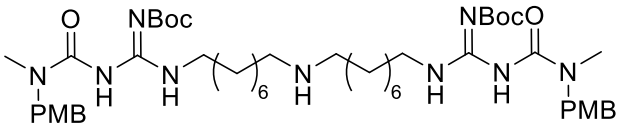
To a stirred solution of **70** (1.00 g, 6.62 mmol) in dry DCM (5 mL) under N2 atmosphere at 0 °C, DIPEA (1.15 mL, 6.62 mmol) and triphosgene (0.69 g, 2.31 mmol) were added. The mixture was stirred at 0 °C for 30 min and then at room temperature for 1 h. The reaction was treated with $NaHCO_3$ s.s. and extracted with DCM. The combined organic phases were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The oil obtained was purified through flash flash chromatography (silica gel) (Hexane/AcOEt 9/1). ^{1}H-NMR (CDCl3) δ (ppm): 2.93 (s, 1H), 3.00 (s, 2H), 3.76 (s, 3H), 4.47 (s, 1H), 4.60 (s, 1H), 6.86 (m, 2H), 7.17 (m, 2H). ^{13}C-NMR (CDCl3) δ (ppm): 36.1, 37.6, 53.9, 55.3, 55.8, 114.2, 168.7, 129.6, 159.5. LCMS *m/z* (ES+) = 235.8 $[M + Na]^+$. Yield: 63%.

***tert*-butyl *N*-[({[(4-methoxyphenyl)methyl](methyl)carbamoyl}amino)(*1H*-pyrazol-1-yl)methylidene]carbamate (72)**



To a stirred solution of *N*-Boc-1*H*-pyrazole-1-carboxamidine (327.0 mg, 1.56 mmol) in dry THF (5.0 mL) under nitrogen atmosphere at 0 °C, a 60% mineral oil dispersion of sodium hydride (94.0 mg, 2.35 mmol) was added at 0 °C and the mixture was stirred for 10 min. Then a solution of **71** (1.0 g, 4.70 mmol) in dry THF (5.0 mL) was added dropwise and the mixture was stirred at reflux for 16 h. Then, the reaction was treated with $NaHCO_3$ s.s. and extracted with AcOEt. The combined organic phases were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The oil obtained was purified through flash flash chromatography (silica gel) (Hexane/AcOEt 7/3). Compound 72 was obtained as yiellowish oil. ^{1}H-NMR (CDCl3) δ (ppm): (mixture of rotamers) 1.49 (s, 9H), 2.88 (s, 3H), 3.83 (s, 3H), 4.56 (s, 2H), 6.39 (d, 1H, *J*= 8.4 Hz), 6.87 (m, 2H), 7.31 (m, 2H), 7.63 (s, 1H), 8.24 (d, 1H, *J*= 17.8 Hz). ^{13}C-NMR (CDCl3) δ (ppm): (mixture of rotamers) 27.8, 32.3, 53.0, 55.1, 82.8, 109.4, 113.8, 129.7, 128.9, 129.6, 139.8, 142.6, 149.3, 158.7, 160.5. LCMS *m/z* (ES+) = 388.0 $[M+H]^+$, 410.0 $[M + Na]^+$, 796.9 $[2M+Na]^+$.Yield: 43%.

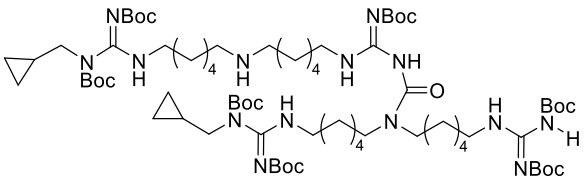
***tert*-butyl *N*-({[8-({8-[({[(*tert*-butoxy)carbonyl]imino}({[(4-methoxyphenyl)methyl](methyl)carbamoyl}amino)methyl)amino]octyl}amino)octyl]amino}({[(4-methoxyphenyl)methyl](methyl)carbamoyl}amino)methylidene)carbamate (8UU(PMB), 73)**



To a solution of **40a** (50.0 mg, 0.081 mmol) in MeOH (0.4 mL), DIPEA (0.07 mL, 0.41 mmol) and a solution of the guanylating agent **72** (157.0 mg, 0.41 mmol) in THF (1.2 mL) were and the reaction mixture was stirred at r.t. for 16 h. Then, the reaction mixture was concentrated under reduced pressure and treated with DCM and $NaHCO_3$ s.s. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried on Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 9/1), affording the compound as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.25 (m, 20H), 1.46 (s, 18H), 1.83 (m, 4H), 2.84 (s, 3H), 2.83 (m, 4H), 3.00 (m, 3H), 3.28 (m, 4H), 3.78 (s, 6H), 4.49 (s, 3H), 4.70 (s, 3H), 6.84 (m, 4H), 7.20 (m, 4H), 8.02 (br, 2H), 12.32 (br, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.7, 27.0, 28.4, 29.4, 30.4, 30.6, 35.1, 41.6, 49.9, 54.1, 55.8, 79.8, 114.1, 128.7, 131.5, 149.2, 155.9, 158.0, 159.0. LCMS *m/z* (ES+) = 910.5 $[M + H]^+$. Yield: 21%.

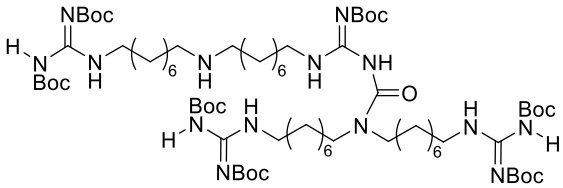
Isolation of asymmetric dimers.

***tert*-butyl *N*-[{[6-({[({6-[(6-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}hexyl)amino]hexyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]carbamoyl}(6-{[{[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}hexyl)amino)hexyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (Asy6CH, 74)**



^{1}H NMR ($CDCl_3$) δ (ppm): 0.20-0.22 (m, 4H), 0.42-0.44 (m, 4H), 1.03 (m, 2H), 1.38 (m, 16H), 1.44-1.48 (m, 63H), 1.54-1.61 (m, 8H), 1.77 (m, 8H), 2.90 (m, 4H), 3.23 (m, 2H), 3.29 (m, 6H), 3.38 (m, 4H), 3.53 (m, 4H), 7.99 (br, 1H), 8.27 (br, 1H), 11.47 (br, 1H), 12.30 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 26.3, 26.5, 26.8, 28.0, 28.0, 28.1, 28.1, 28.2, 28.3, 28.7, 28.9, 29.2, 29.6, 30.8, 30.8, 40.4, 43.8, 46.3, 47.9, 52.1, 52.1, 79.2, 79.5, 82.0, 83.1, 104.9, 127.7, 153.2, 153.7, 163.7. LCMS *m/z* (ES+) = 717.5 $[M + 2H]^{2+}$, 478.6 $[M + 3H]^{3+}$. Yield: 1.1%.

tert-butyl _N_-[{[(8-{[{[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl]amino}octyl)(8-{[(1Z)-{[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl]amino}octyl)carbamoyl]amino}({8-[(8-{[{[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl]amino}octyl)amino]octyl}amino)methylidene]carbamate (8HH Asy, 75)

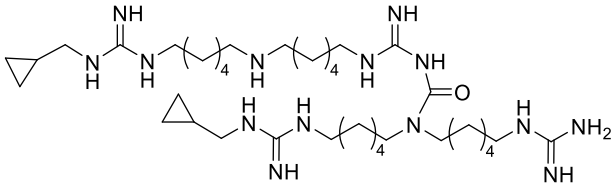


^{1}H NMR ($CDCl_3$) δ (ppm): 1.30 (m, 32H), 1.48 (m, 63H), 1.50 (m, 12H), 1.74 (m, 4H), 2.93 (m, 4H), 3.24 (m, 2H), 3.29 (m, 6H), 3.28 (m, 2H), 3.48 (m, 8H), 7.97 (br, 1H), 8.28 (br, 1H), 11.50 (br, 1H), 12.31 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 319.0, 26.0, 26.6, 26.7, 26.9, 27.1, 28.1, 28.1, 28.3, 28.3, 29.0, 29.1, 29.3, 29.5, 29.7, 40.7, 40.9, 41.0, 47.9, 53.4, 76.7, 77.0, 77.3, 77.4, 79.2, 79.3, 81.9, 83.0, 83.1, 153.3, 153.6, 153.7, 156.1, 163.7, 163.8. LCMS *m/z* (ES+) = 1437.3 $[M + H]^{+}$, 719.2 $[M + 2H]^{2+}$, 479.9 $[M + 3H]^{3+}$. Yield: 0.8%.

General procedure for Boc deprotection, asymmetric dimers salts 21 and 22.

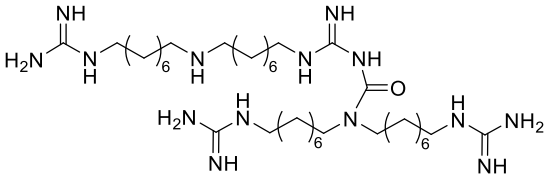
Protected dimeric compound **74** and **75** (0.05 mmol) was dissolved in dry DCM (3.2 mL) and freshly distilled TFA (final concentration 20%, 0.8 mL) was added. The reaction mixture was stirred at r.t. for 5 h. Then the solvent was evaporated and the crude was dissolved and evaporated several times first with MeOH to remove TFA residue and then with Et_2O to precipitate the desired compound. No further purification followed and the product was obtained a colorless oil.

1-(6-carbamimidamidohexyl)-1-{6-[_N_-(cyclopropylmethyl)carbamimidamido]hexyl}-3-{_N_-[6-({6-[_N_-(cyclopropylmethyl)carbamimidamido]hexyl}amino)hexyl]carbamimidoyl}urea (Asy6CHs, 21)



^{1}H NMR (CD_3OD) δ (ppm):0.26 (d, 4H, *J*= 4.4 Hz), 0.56 (d, 4H, *J*= 4.4 Hz), 1.04 (m, 2H), 1.27-1.42 (m, 16H), 1.58-1.67 (m, 16H), 2.98 (t, 4H, *J*= 8.0 Hz), 3.04 (d, 4H, *J*= 8.0 Hz), 3.14-3.17 (m, 8H), 3.28 (m, 4H). ^{13}C NMR (CD_3OD, 100 MHz): δ 3.5, 11.0, 26.0, 27.2, 28.1, 29.0, 30.4, 41.4, 42.0, 42.2, 44.5, 50.0, 155.1, 155.0, 157.5, 160.0. LCMS *m/z* (ES+) = 734.0 $[M + H]^+$, 367.5 $[M + 2H]^{2+}$, 245.1 $[M + 3H]^{3+}$. Yield: quantitative.

3,3-bis(8-carbamimidamidooctyl)-1-(*N*-{8-[(8-carbamimidamidooctyl)amino]octyl}carbamimidoyl)urea trifluoroacetate salt (Asy8HHs, 22)

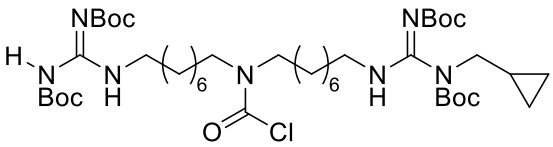


^{1}H NMR (CD_3OD) δ (ppm): 1.36-1.39 (m, 32H), 1.58 (m, 10H), 1.67 (m, 6H), 2.97 (t, 4, *J*= 8.0 Hz), 3.16 (t, 6H, *J*= 7.2 Hz), 3.28 (t, 2H, *J*= 7.2 Hz), 3.30 (m, 4H). ^{13}C NMR (CD_3OD) δ (ppm): 22.7, 25.8, 26.0, 26.1, 26.2, 27.8, 28.4, 28.6, 28.8, 28.9, 29.2, 40.9, 45.2, 445.8, 155.1, 155.0, 157.5, 162.2. LC-MS *m/z* (ES+) = 738.0 $[M + H]^+$, 369.5 $[M + 2H]^{2+}$, 246.7 $[M + 3H]^{3+}$. Yield: quantitative.

Preparation of carbamoyl derivatives 76-82.

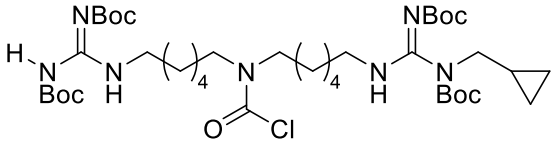
To a solution of monomer (**33**, **52, 61**, **63**, **67**or **68**; 0.08 mmol) in dry DCM (1.7 mL), DIPEA (15 µL, 0.08 mmol) was added under nitrogen atmosphere. Then, a solution of triphosgene (13.0 mg, 0.04 mmol) in dry DCM (1.5 mL) was added dropwise at 0 °C. The mixture was stirred 0.5 h at 0 °C and then it was allowed to reach r.t. for 3 h. Then $NaHCO_3$ s.s. was added to the reaction mixture and it was stirred for 10 min. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried on Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 98/2), affording the compounds as oils.

***N*-{8-[*N'*-(cyclopropylmethyl)-*N',N''*-di-Boc-carbamimidamido]octyl}-*N*-[8-(*N',N''*-di-Boc-carbamimidamido)octyl]carbamoyl chloride (8CH*, 76)**



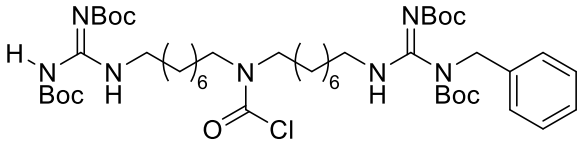
^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (d, 2H, *J*= 4.8 Hz), 0.44 (d, 2H, *J*= 8.0 Hz), 0.81-0.91 (m, 2H), 1.25-1.40 (m, 16H), 1.49 (s, 36H), 1.53-1.68 (m, 8H), 3.28-3.32 (m, 4H), 3.33-3.41 (m, 4H), 3.49-3.60 (m, 2H), 8.27 (br, 1H), 11.49 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 14.0, 22.6, 24.7, 26.5, 26.6, 26.8, 27.4, 28.0, 28.1, 28.2, 28.3, 28.8, 29.0, 29.6, 31.8, 33.7, 40.8, 43.8, 49.8, 51.1, 52.1, 79.1, 81.9, 82.9, 85.2, 131.6, 148.9, 153.2, 156.0, 163.5. LC-MS *m/z* (ES+) = 872.2 $[M + H]^+$, 436.5 $[M + 2H]^{2+}$. Yield: 60%.

***tert*-butyl *N*-({[6-({6-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]hexyl}(chlorocarbonyl)amino)hexyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)-*N*-(cyclopropylmethyl)carbamate (6CH*, 77)**



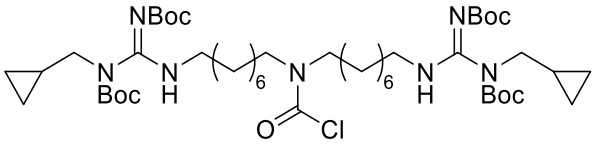
^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (m, 2H), 0.45 (m, 2H), 0.87 (m, 1H), 1.34 (m, 8H), 1.47 (m, 36H), 1.61 (m, 8H), 3.30 (m, 4H), 3.37 (m, 4H), 3.52 (m, 2H), 8.28 (br, 1H), 11.48 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.4, 14.1, 22.6, 24.7, 26.5, 26.6, 26.8, 27.4, 27.9, 28.2, 28.3, 28.4, 28.9, 29.0, 29.6, 31.8, 33.7, 40.9, 43.8, 49.8, 51.1, 52.1, 79.1, 81.9, 82.9, 85.3, 131.6, 148.9, 153.2, 156.1, 163.6. LCMS *m/z* (ES+) = 816.2 $[M + H]^+$, 408.5 $[M + 2H]^{2+}$. Yield: 26%.

tert-butyl _N_-benzyl-_N_-({[8-({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}(chlorocarbonyl)amino)octyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)carbamate (8BH*, 78)



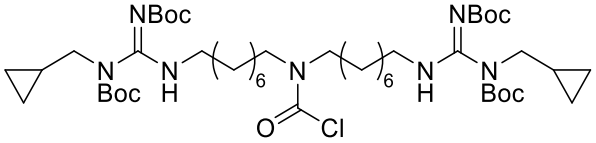
^{1}H NMR ($CDCl_3$) δ (ppm): 1.21 (m, 8H), 1.30 (s, 8H), 1.41 (s, 9H), 1.47 (s, 9H), 1.48 (s, 18H), 1.56 (m, 8H), 2.98 (m, 2H), 3.29 (t, 2H, *J*= 7.2 Hz), 3.37 (m, 4H), 4.83 (s, 2H), 7.27 (m, 5H), 8.27 (br, 1H), 11.48 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm):14.1, 26.5, 26.6, 27.4, 27.9, 28.0, 28.2, 28.2, 28.3, 28.8, 28.9, 29.0, 29.6, 40.8, 43.7, 49.8, 51.1, 60.3, 79.1, 82.9, 127.4, 128.3, 137.8, 153.2, 156.0, 163.6. LCMS *m/z* (ES+) = 454.5 $[M + 2H]^{2+}$. Yield: 57%.

tert-butyl _N_-({[8-({8-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}(chlorocarbonyl)amino)octyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate (8CC*, 79)



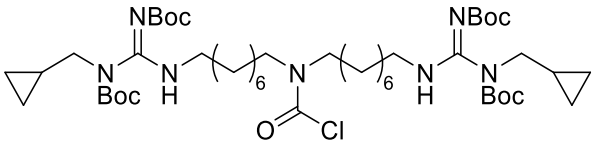
^{1}H NMR ($CDCl_3$) δ (ppm): 0.22 (m, 4H), 0.43 (m, 4H), 1.02 (m, 2H), 1.31 (m, 16H), 1.44 (s, 18H), 1.47 (s, 18H), 1.59 (m, 8H), 3.28 (m, 6H), 3.35 (m, 2H), 3.52 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 8.3, 26.8, 27.0, 28.5, 29.4, 30.4, 42.2, 50.3, 51.2, 79.8, 82.5, 148.7, 154.3, 158.0, 158.5. LCMS *m/z* (ES+) = 926.0 $[M + H]^+$, 463.7 $[M + 2H]^{2+}$. Yield: 40%.

tert-butyl _N_-({[6-({6-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]hexyl}(chlorocarbonyl)amino)hexyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate (6CC*, 80)



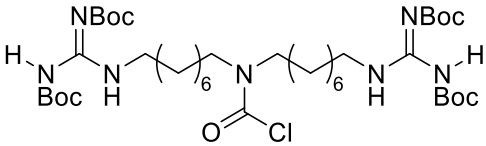
^{1}H NMR ($CDCl_3$) δ (ppm): 0.28 (m, 4H), 0.46 (m, 4H), 1.03 (m, 2H), 1.35 (m, 8H), 1.47 (s, 18H), 1.49 (s, 18H), 1.63 (m, 8H), 3.32 (m, 6H), 3.38 (t, 2H, *J*= 7.6 Hz), 3.55 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.6, 10.6, 26.4, 26.4, 26.5, 26.7, 26.7, 27.0, 28.2, 29.0, 29.9, 42.3, 46.4, 49.0, 53.4, 79.7, 82.5, 148.7, 154.3, 158.0, 158.6. LCMS *m/z* (ES+) = 870.0 $[M + H]^+$, 892.0 $[M + Na]^+$, 435.5 $[M + 2H]^{2+}$. Yield: 51%.

***tert*-butyl *N*-benzyl-*N*-({[8-({8-[({benzyl[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]octyl}(chlorocarbonyl)amino)octyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)carbamate (8BB*, 81)**



^{1}H NMR ($CDCl_3$) δ (ppm): 1.22 (m, 18H), 1.42 (s, 18H), 1.49 (s, 18H), 1.58 (m, 6H), 2.99 (m, 4H), 3.30 (t, 2H, *J*= 7.6 Hz), 3.34 (t, 2H, *J*= 7.6 Hz), 4.84 (s, 4H), 7.26 (m, 10H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.5, 27.4, 28.0, 28.2, 28.3, 28.9, 29.6, 43.7, 49.8, 51.1, 81.9, 82.8, 127.4, 128.3, 148.9, 153.3, 156.2, 162.1. LCMS *m/z* (ES+) = 500.4 $[M + 2H]^{2+}$. Yield: 44%.

***tert*-butyl *N*-({[(*tert*-butoxy)carbonyl]amino}({[8-({8-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]octyl}(chlorocarbonyl)amino)octyl]amino})methylidene)carbamate (8HH*, 82)**



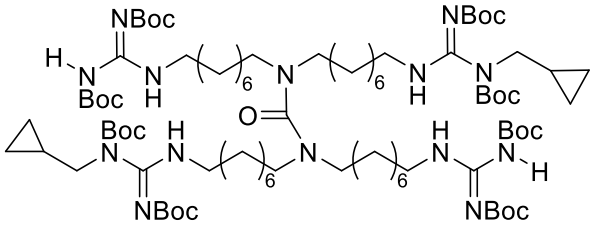
^{1}H NMR ($CDCl_3$) δ (ppm): 1.26 (m, 16H), 1.48 (s, 18H), 1.49 (m, 18H), 1.55 (m, 8H), 3.30 (m, 2H, *J*= 7.6 Hz), 3.38 (m, 6H), 8.29 (br, 2H), 11.49 (br, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 22.6, 26.5, 26.6, 27.4, 28.0, 28.2, 28.8, 29.0, 29.6, 40.9, 79.2, 83.0, 153.2, 156.0, 163.7. LCMS *m/z* (ES+) = 818.4 $[M + H]^+$, 409.7 $[M + 2H]^{2+}$. Yield: 17%.

Preparation of *Traditional Dimers* 83-92.

To a solution of the suitable **carbamoyl derivative** (**nXX***, 0.06 mmol) in dry DCM (0.7 mL) and sodium iodide (0.006 mmol) in a tube, a solution of the suitable **monomer** (**mYY**, 0.07 mmol) in dry DCM (1.3 mL) and dry DIPEA (12 µL, 0.07 mmol) were added under nitrogen atmosphere. Then, the tube was sealed and the reaction mixture was stirred at 40 °C for 48-72 h. After cooling, DCM and $NaHCO_3$ s.s. were added to the mixture and it was stirred for 10 min. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried on Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 98/2), affording the compounds as oils.

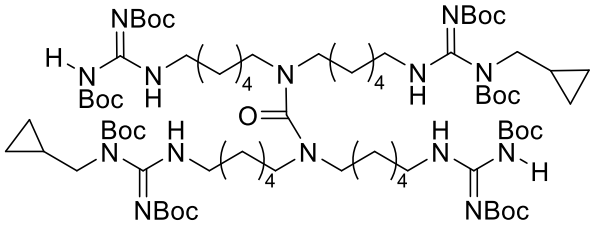
***tert*-butyl *N*-{[(8-{[(8-{[(*tert*-butoxy)carbonyl]({[(*tert*-butoxy)carbonyl]imino}[(cyclopropylmethyl)amino]methyl)amino}octyl)({7-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]heptyl})carbamoyl]({8-[({[(*tert*-butoxy)carbonyl]amino}({[(*tert*-**

butoxy)carbonyl]imino})methyl)amino]octyl})amino}octyl)amino]({[(*tert*-butoxy)carbonyl]imino})methyl}-*N*-(cyclopropylmethyl)carbamate (8CH*/8CH, 83)



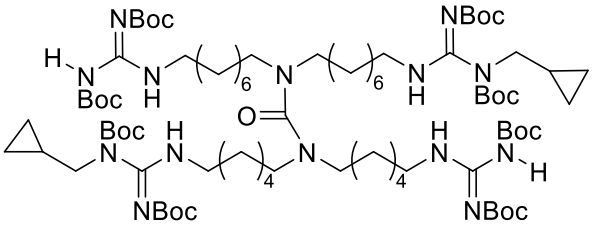
^{1}H NMR ($CDCl_3$): δ 0.26 (d, *J*= 4.8 Hz, 4H), 0.43 (d, *J*= 7.6 Hz, 4H), 0.99-1.09 (m, 2H), 1.18-1.39 (m, 48H), 1.48 (s, 72H), 3.01-3.09 (m, 8H), 3.28-3.32 (m, 4H), 3.35-3.43 (m, 4H), 3.49-3.58 (m, 4H), 8.26 (br, 2H), 11.49 (br, 2H). ^{13}C NMR ($CDCl_3$): δ 3.4, 10.5, 26.7, 26.9, 27.0, 28.0, 28.1, 28.2, 28.9, 29.2, 29.5, 40.8, 43.8, 48.2, 48.3, 52.1, 79.0, 81.8, 82.9, 153.2, 156.0, 163.5, 165.2. LCMS *m/z* (ES+) = 823.5 $[M + 2H]^{2+}$, 549.4 $[M + 3H]^{3+}$. Yield: 60%.

***tert*-butyl *N*-[({10-[(10-{[amino({[(*tert*-butoxy)carbonyl]imino})methyl][(*tert*-butoxy)carbonyl]amino}decyl)[(10-{[amino({[(*tert*-butoxy)carbonyl]imino})methyl][(*tert*-butoxy)carbonyl]amino}decyl)({10-[({[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl)amino]decyl})carbamoyl]amino]decyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (6CH*/6CH, 84)**



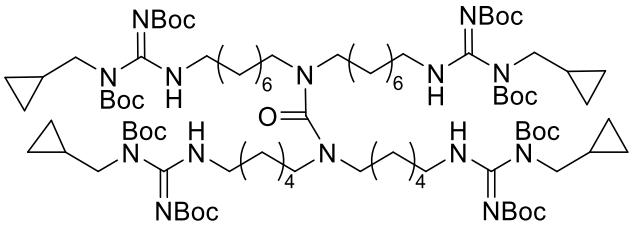
^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (m, 4H), 0.44 (m, 4H), 1.03 (m, 2H), 1.24 (m, 20H), 1.47 (m, 72H), 1.49 (m, 12H), 3.05 (m, 8H), 3.29 (m, 4H), 3.39 (m, 4H), 3.53 (m, 4H), 8.27 (br, 2H), 11.49 (br, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 10.5, 26.8, 26.9, 27.0, 27.9, 28.0, 28.3, 28.9, 29.1, 29.6, 40.9, 43.8, 48.1, 48.4, 52.1, 79.3, 81.8, 83.0, 153.2, 156.1, 163.9, 165.0. LCMS *m/z* (ES+) = 767.5 $[M + 2H]^{2+}$, 512.4 $[M + 3H]^{3+}$. Yield: 75%.

***tert*-butyl *N*-[[(6-{[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)(8-{[{[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)carbamoyl](6-{{[(*tert*-butoxy)carbonyl]amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}hexyl)amino}hexyl)amino]({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (8CH*/6CH, 85)**



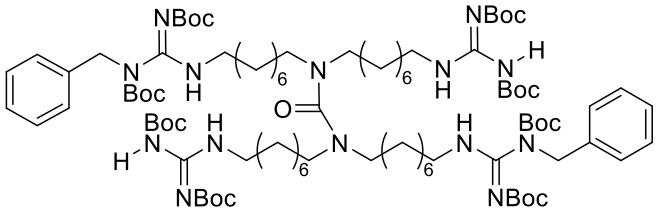
^{1}H NMR ($CDCl_3$) δ (ppm): 0.20 (d, 4H, *J*= 4.4 Hz), 0.42 (d, 4H, *J*= 7.2 Hz), 1.01 (m, 2H), 1.20-1.34 (m, 24H), 1.43-1.46 (m, 72H), 1.51-1.58 (m, 16H), 3.03 (m, 8H), 3.27 (m, 4H), 3.35-3.36 (m, 4H), 3.51 (m, 4H), 8.25 (br, 2H), 11.46 (br, 2H) ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 26.8, 27.0, 28.0, 28.1, 28.2, 28.9, 29.2, 29.6, 40.8, 43.8, 48.2, 52.1, 79.1, 81.8, 82.9, 153.2, 156.0, 163.5, 165.2. LCMS *m/z* (ES+) = 795.2 $[M+2H]^{2+}$, 530.5 $[M + 3H]^{3+}$. Yield: 50%.

tert-butyl _N_-{[(6-{[bis({8-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})carbamoyl]({6-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]hexyl})amino}hexyl)amino]({[(_tert_-butoxy)carbonyl]imino})methyl}-_N_-(cyclopropylmethyl)carbamate (8CC*/6CC, 86)



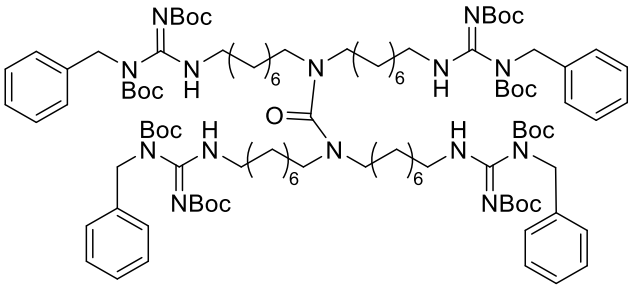
^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (m, 8H), 0.46 (m, 8H), 1.05 (m, 4H), 1.31 (m, 24H), 1.47 (s, 36H), 1.49 (s, 36H), 1.62 (m, 16H), 3.07 (m, 8H), 3.31 (m, 8H), 3.55 (m, 8H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 12.0, 18.7, 25.7, 27.1, 28.2, 29.5, 78.9, 81.7, 105.0, 141.2, 151.8. LCMS *m/z* (ES+) = 871.2 $[M + 2Na]^{2+}$, 860.2 $[M + H + Na]^{2+}$, 849.2 $[M + 2H]^{2+}$, 566.4 $[M + 3H]^{3+}$. Yield: 21%.

tert-butyl _N_-benzyl-_N_-[({8-[({8-[({benzyl[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})carbamoyl)({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})amino]octyl}amino)({[(_tert_-butoxy)carbonyl]imino})methyl]carbamate (8BH*/8BH, 87)



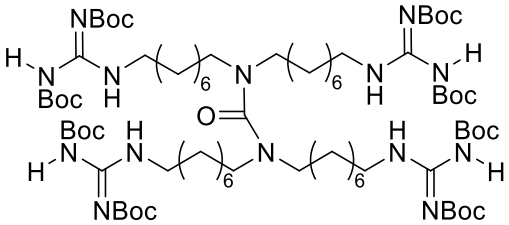
^{1}H NMR ($CDCl_3$) δ (ppm): 1.24 (m, 32H), 1.41 (m, 16H), 1.48 (m, 72H), 2.98 (m, 4H), 3.05 (t, 8H, *J*= 8.0 Hz), 3.37 (q, 4H, *J*= 6.8 Hz), 4.83 (s, 4H), 7.28 (m, 10H), 8.26 (br, 1H), 11.49 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.6, 27.0, 27.0, 27.9, 28.0, 28.2, 28.9, 29.1, 29.2, 29.2, 40.9, 43.8, 48.4, 51.2, 79.1, 82.9, 127.4, 128.3, 137.9, 153.4, 156.0, 163.6, 165.3. LCMS *m/z* (ES+) = 859.5 $[M + 2H]^{2+}$, 573.3 $[M + 3H]^{3+}$, 429.1 $[M + 4H]^{4+}$. Yield: 54%.

tert-butyl _N_-benzyl-_N_-({[8-({8-[({benzyl[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}[bis({8-[({benzyl[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})carbamoyl]amino)octyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)carbamate (8BB*/8BB, 88)



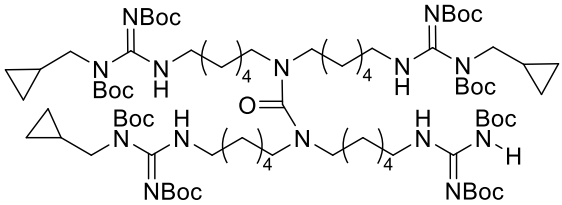
^{1}H NMR ($CDCl_3$) δ (ppm): 1.22 (m, 32H), 1.41 (m, 16H), 1.49 (s, 72H), 2.98 (m, 6H), 3.05 (m, 10H), 4.84 (s, 8H), 7.28 (m, 20H), 8.26 (br, 1H), 11.49 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.6, 27.0, 28.0, 28.2, 29.0, 29.1, 29.2, 29.6, 30.8, 31.8, 43.8, 48.3, 50.8, 79.1, 82.4, 127.4, 128.3, 137.9, 153.3, 163.6. LCMS *m/z* (ES+) = 949.7 $[M + 2H]^{2+}$, 960.6 $[M + H + Na]^{2+}$, 971.7 $[M + 2Na]^{2+}$, 633.3 $[M + 3H]^{3+}$. Yield: 24%.

tert-butyl _N_-{[(8-{[bis({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})carbamoyl]({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})amino}octyl)amino]({[(_tert_-butoxy)carbonyl]amino})methylidene}carbamate (8HH*/8HH, 89)



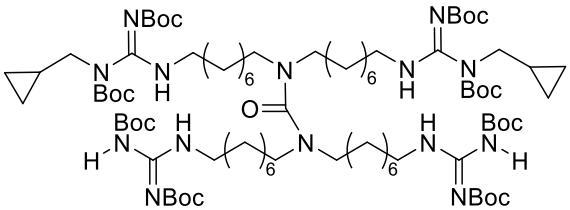
^{1}H NMR ($CDCl_3$) δ (ppm): 1.27 (m, 32H), 1.48 (s, 36H), 1.49 (s, 36H), 1.54 (m, 16H), 3.06 (t, 8H, *J*= 7.2 Hz), 3.39 (q, 8H, *J*= 6.8 Hz), 8.27 (br, 4H), 11.49 (br, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.6, 26.8, 26.9, 27.5, 27.8, 28.0, 28.1, 28.3, 28.6, 28.8, 28.9, 40.8, 48.2, 79.0, 82.8, 153.2, 155.9, 163.5. LCMS *m/z* (ES+) = 769.7 $[M + 2H]^{2+}$, 513.3 $[M + 3H]^{3+}$. Yield: 28%.

tert-butyl _N_-({[6-({6-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]hexyl}({6-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]hexyl}({6-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]hexyl})carbamoyl)amino)hexyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate (6CC*/6CH, 90)



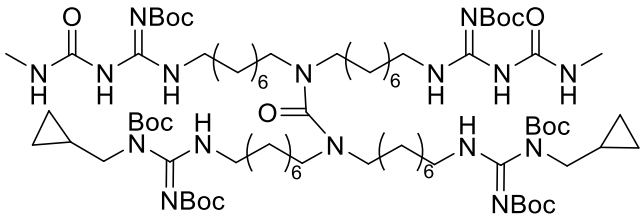
^{1}H NMR ($CDCl_3$) δ (ppm): 0.22 (m, 6H), 0.24 (m, 6H), 1.03 (m, 3H), 1.27 (m, 8H), 1.34 (m, 8H), 1.47 (m, 72H), 1.60 (m, 16H), 3.05 (m, 8H), 3.29 (m, 6H), 3.37 (m, 2H), 3.53 (m, 6H), 8.26 (br, 1H), 11.49 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 26.7, 26.9 28.0, 28.2, 28.9, 29.9, 40.8, 49.9, 82.9, 156.0, 163.6. LCMS *m/z* (ES+) = 794.2 $[M + 2H]^{2+}$, 805.5 $[M + H + Na]^{2+}$, 816.5 $[M + 2Na]^{2+}$. Yield: 35%.

tert-butyl _N_-{[(8-{[bis({8-[({[(_tert_-butoxy)carbonyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})carbamoyl]({8-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})amino}octyl)amino]({[(_tert_-butoxy)carbonyl]imino})methyl}-_N_-(cyclopropylmethyl)carbamate (8CC*/8HH, 91)



^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (m, 4), 0.44 (m, 4H), 1.08 (m, 2H), 1.30 (m, 32H), 1.48 (m, 72H), 1.60 (m, 16H), 3.06 (m, 8H), 3.30 (m, 4H), 3.39 (m, 4H), 3.55 (m, 4H), 8.28 (br, 2H), 11.50 (br, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 14.1, 22.7, 26.9, 27.0, 27.1, 28.1, 28.3, 28.3, 29.0, 29.4, 29.7, 32.0, 41.0, 44.0, 48.4, 79.2, 83.0, 118.1, 142.0, 153.4. LCMS *m/z* (ES+) = 823.2 $[M + 2H]^{2+}$, 549.3 $[M + 3H]^{3+}$, 412.3 $[M + 4H]^{4+}$. Yield: 25%.

tert-butyl _N_-{[(8-{[bis({8-[({[(_tert_-butoxy)carbonyl]imino}({[(4-methoxyphenyl)methyl](methyl)carbamoyl}amino)methyl)amino]octyl})carbamoyl]({8-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl})amino}octyl)amino]({[(_tert_-butoxy)carbonyl]imino})methyl}-_N_-(cyclopropylmethyl)carbamate (8CC*/8UU (PMB), 92)

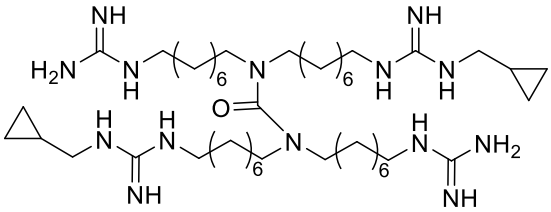


^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (m, 4H), 0.46 (m, 4H), 1.04 (m, 2H), 1.25 (m, 32H), 1.46 (m, 54H), 1.59 (m, 16H), 2.84 (s, 3H), 3.00 (m, 3H), 3.06 (m, H), 3.39 (m, 8H), 3.54 (m, 4H), 3.78 (s, 6H), 4.49 (s, 3H), 4.70 (s, 3H), 6.83 (m, 4H), 7.18 (m, 4H), 8.02 (br, 2H), 12.34 (br, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 8.3, 26.7, 26.8, 29.3, 28.5, 29.4, 30.4, 35.1, 41.5, 42.2, 50.3, 50.5, 54.1, 55.8, 79.7, 82.5, 114.1, 128.7, 131.6, 149.1, 154.2, 155.8, 158.1, 158.5, 159.0, 164.6. LCMS *m/z* (ES+) = 922.3 $[M + 2Na]^{2+}$, 912.3 $[M + H + Na]^{2+}$, 900.7 $[M + 2H]^{2+}$. Yield: 29%.

General procedure for the synthesis of dimer salts 2, 11-18 and 20.

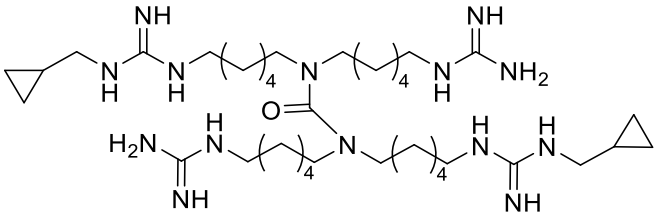
Same procedure for compounds **21** and **22**. All the products were obtained as colourless oils.

1,3-bis(8-carbamimidamidooctyl)-1,3-bis({8-[*N'*-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (2)



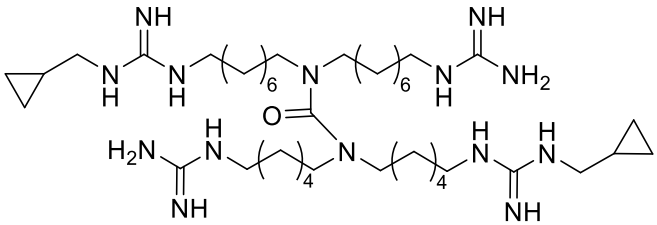
^{1}H NMR (CD_3OD) δ (ppm): 0.23-0.30 (m, 4H), 0.53-0.61 (m, 4H), 1.01-1.10 (m, 2H), 1.31-1.45 (m, 32H), 1.48-1.55 (m, 8H), 1.55-1.63 (m, 8H), 3.05 (d, 4H, *J*= 6.8 Hz), 3.08-3.21 (m, 12H), 3.28-3.32 (m, 4H). ^{13}C NMR (CD_3OD) δ (ppm): 2.4, 9.5, 26.2, 26.5, 27.5, 28.4, 28.4, 28.8, 28.9, 29.2, 41.0, 41.1, 45.8, 46.9, 47.1, 47.3, 47.5, 47.7, 47.9, 48.1, 155.6, 165.4. LCMS *m/z* (ES+) = 845.8 $[M + H]^+$, 423.1 $[M + 2H]^{2+}$, 282.5 $[M + 3H]^{3+}$, 212.1 $[M + 4H]^{4+}$. Yield: quantitative.

1,3-bis(6-carbamimidamidohexyl)-1,3-bis({6-[*N'*-(cyclopropylmethyl)carbamimidamido]hexyl})urea trifluoroacetate salt (6CH*/6CHs, 11)



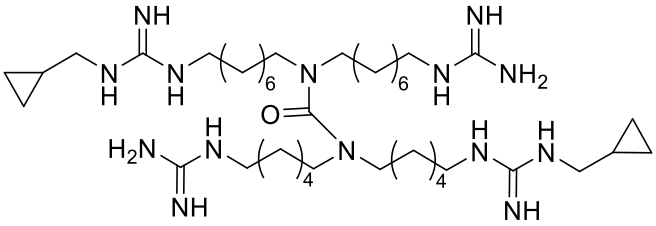
^{1}H NMR (CD_3OD) δ (ppm): 0.27 (q, 4H, *J*= 4.8 Hz), 0.58 (q, 4H, *J*= 5.6 Hz), 1.06 (m, 2H), 1.34 (m, 16H), 1.55 (m, 16H), 3.05 (d, 4H, *J*= 7.2 Hz), 3.16 (m, 16H). ^{13}C NMR (CD_3OD) δ (ppm): 4.4, 6.1, 9.5, 22.7, 26.1, 26.3, 27.5, 28.4, 29.3, 41.0, 42.2, 45.8, 155.6, 165.4. LCMS *m/z* (ES+) = 367.1 $[M + 2H]^{2+}$, 245.1 $[M + 3H]^{3+}$. Yield: quantitative.

1-(6-carbamimidamidohexyl)-3-(8-carbamimidamidooctyl)-1-{6-[*N*-(cyclopropylmethyl)carbamimidamido]hexyl}-3-{8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl}urea (8CH*/6CHs, 12)



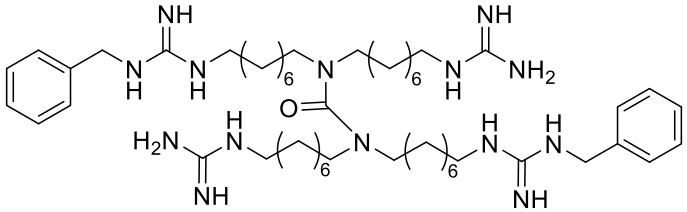
^{1}H NMR (CD_3OD) δ (ppm): 0.25 (d, 4H, *J*= 4.8 Hz), 0.56 (d, 4H, *J*= 7.6 Hz), 1.04 (m, 2H), 1.30 (m, 24H), 1.50-1.56 (m, 16H), 2.88 (d, 4H, *J*= 7.6 Hz), 3.03-3.18 (m, 16H) ^{13}C NMR (CD_3OD) δ (ppm): 2.4, 9.5, 26.2, 28.4, 28.9, 39.3, 41.0, 45.8, 52.4, 155.9, 157.2, 165.8. LCMS *m/z* (ES+) = 789.0 $[M+H]^+$, 395.1 $[M+2H]^{2+}$, 263.6 $[M+3H]^{3+}$. Yield: quantitative.

3,3-bis({6-[*N'*-(cyclopropylmethyl)carbamimidamido]hexyl})-1,1-bis({8-[*N'*-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (8CC*/6CCs, 13)



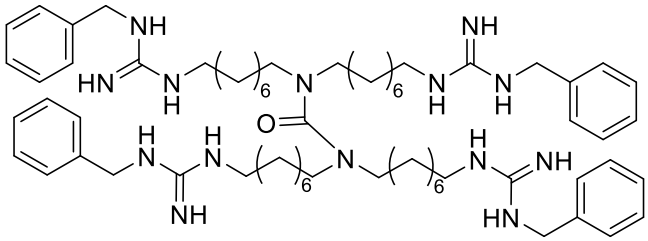
^{1}H NMR (CD_3OD) δ (ppm): 0.26 (q, 8H, *J*= 4.8 Hz), 0.58 (q, 8H, *J*= 8.0 Hz), 1.03 (m, 4H), 1.34 (m, 24H), 1.55 (m, 16H), 3.05 (d, 8H, *J*= 7.2 Hz), 3.17 (m, 16H). ^{13}C NMR (CD_3OD) δ (ppm): 3.4, 11.2, 26.3, 26.4, 26.7, 28.9, 29.4, 30.4, 42.3, 44.7, 50.5, 157.8, 164.5. LCMS *m/z* (ES+) 449.0 $[M + 2H]^{2+}$, 299.0 $[M + 3H]^{3+}$.Yield: quantitative.

1,3-bis[8-(*N'*-benzylcarbamimidamido)octyl]-1,3-bis(8-carbamimidamidooctyl)urea trifluoroacetate salt (8BH*/8BHs, 14)



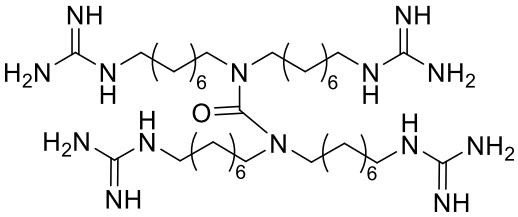
^{1}H NMR (CD_3OD) δ (ppm): 1.33 (m, 32H), 1.53 (m, 16H), 3.13 (q, 8H, *J*= 7.2 Hz), 3.19 (t, 8H, *J*= 6.8 Hz), 4.41 (s, 4H), 7.30 (m, 6H), 7.36 (m, 4H). ^{13}C NMR (CD_3OD) δ (ppm): 22.3, 26.3, 26.6, 26.9, 27.6, 28.5, 28.9, 29.0, 29.3, 29.5, 41.2, 44.4, 126.6, 127.4, 127.9, 128.4, 136.6, 139.1, 155.9, 157.2, 162.0. LCMS *m/z* (ES+) = 459.0 $[M + 2H]^{2+}$, 306.4 $[M + 3H]^{3+}$, 230.1 $[M + 4H]^{4+}$. Yield: quantitative.

1,1,3,3-tetrakis[8-(*N'*-benzylcarbamimidamido)octyl]urea trifluoroacetate salt (8BB*/8BBs, 15)



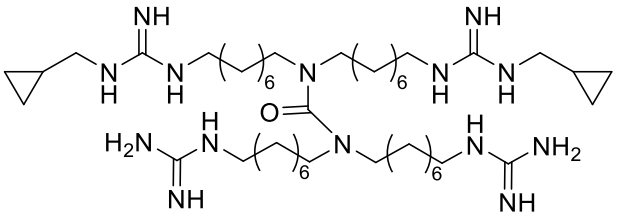
^{1}H NMR (CD_3OD) δ (ppm): 1.29 (m, 32H), 1.51 (m, 8H), 1.56 (m, 8H), 3.13 (t, 8H, *J*= 7.2 Hz), 3.19 (t, 8H, *J*= 7.2 Hz), 4.41 (s, 8H), 7.30 (m, 12H), 7.37 (m, 8H). ^{13}C NMR (CD_3OD) δ (ppm): 22.7, 26.1, 26.6, 26.9, 27.5, 28.4, 28.8, 28.9, 29.3, 29.4, 41.2, 44.4, 126.7, 127.5, 128.0, 128.4, 136.4, 139.1, 155.9, 158.2, 161.1, 165.1. LCMS *m/z* (ES+) = 549.1 $[M + 2H]^{2+}$, 366.5 $[M + 3H]^{3+}$, 275.0 $[M + 4H]^{4+}$. Yield: quantitative.

1,1,3,3-tetrakis(8-carbamimidamidooctyl)urea trifluoroacetate salt (8HH*/8HHs, 16)



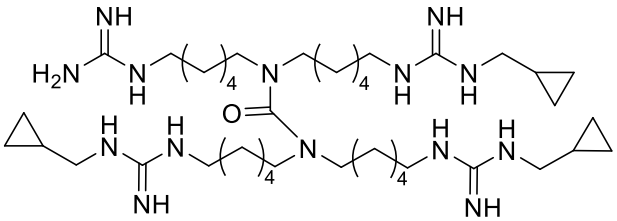
^{1}H NMR (CD_3OD) δ (ppm): 1.34 (m, 36h), 1.53 (m, 12H), 3.14 (q, 8H, *J*= 7.2 Hz), 3.26 (m, 8H). ^{13}C NMR (CD_3OD) δ (ppm): 26.7, 27.1, 28.0, 28.2, 28.8, 29.1, 29.2, 29.5, 49.7, 79.1, 82.9, 153.2, 156.0, 163.5, 165.8. LCMS *m/z* (ES+) = 369.5 $[M + 2H]^{2+}$, 246.6 $[M + 3H]^{3+}$.Yield: quantitative.

3,3-bis(8-carbamimidamidooctyl)-1,1-bis({8-[*N'*-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (8CC*/8HHs, 17)



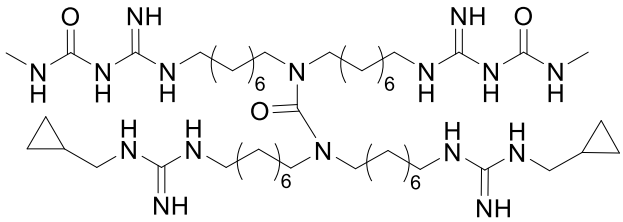
^{1}H NMR (CD_3OD) δ (ppm): 0.26 (m, 4H), 0.57 (m, 4H), 1.06 (m, 2H), 1.34 (m, 32H), 1.51 (m, 8H), 1.58 (m, 8H), 3.05 (d, 4H, *J*= 6.8 Hz), 3.15 (m, 16H). ^{13}C NMR (CD_3OD) δ (ppm): 3.4, 9.5, 26.2, 26.5, 27.5, 28.4, 28.5, 28.8, 28.9, 29.2, 41.0, 41.1, 45.8, 46.9, 47.1, 47.3, 47.5, 47.7, 47.9, 48.1, 155.6, 165.4. LCMS *m/z* (ES+) = 423.3 $[M + 2H]^{2+}$, 282.5 $[M + 3H]^{3+}$, 212.1 $[M + 4H]^{4+}$. Yied: quantitative.

1-(6-carbamimidamidohexyl)-1,3,3-tris({6-[*N'*-(cyclopropylmethyl)carbamimidamido]hexyl})urea trifluoroacetate salt (6CC*/6CHs, 18)



^{1}H NMR (CD_3OD) δ (ppm): 0.26 (q, 6H, *J*= 4.8 Hz), 0.57 (q, 6H, *J*= 8.0 Hz), 1.05 (m, 3H), 1.29 (m, 16H), 1.56 (m, 16H), 3.01 (d, 8H, *J*= 7.2 Hz), 3.06 (m, 16H). ^{13}C NMR (CD_3OD) δ (ppm): 3.64, 6.1, 9.5, 22.7, 26.1, 26.3, 27.5, 28.4, 29.3, 41.0, 42.2, 45.8, 155.6, 165.4. LCMS *m/z* (ES+) = 787.8 $[M + H]^{+}$, 394.6 $[M + 2H]^{2+}$, 263.3 $[M + 3H]^{3+}$. Yield: quantitative.

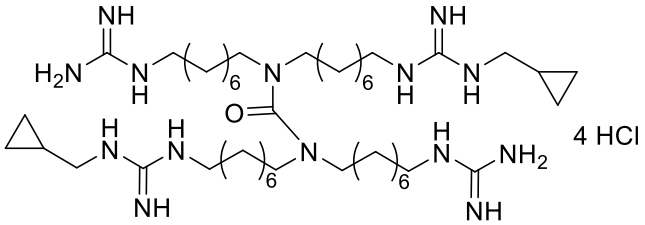
1,1-bis({8-[*N'*-(cyclopropylmethyl)carbamimidamido]octyl})-3,3-bis[8-({[(methylcarbamoyl)amino]methanimidoyl}amino)octyl]urea trifluoroacetate salt (8CC*/8UUs, 19)



^{1}H NMR (CD_3OD) δ (ppm): 0.26 (q, 4H, *J*= 4.8 Hz), 0.58 (q, 4H, *J*= 8.0 Hz), 1.06 (m, 2H), 1.34 (m, 32H), 1.52 (m, 8H), 1.60 (m, 8H), 2.76 (s, 6H), 3.05 (d, 4H, *J*= 7.2 Hz), 3.06 (t, 8H, *J*= 4.0 Hz), 3.18 (t, 4H, *J*= 6.8

Hz), 3.27 (t, 4H, *J*= 7.2 Hz). ^{13}C NMR (CD_3OD) δ (ppm): 3.5, 11.2, 26.7, 26.8, 28.9, 29.3, 30.4, 41.6, 42.2, 44.8, 50.5, 155.4, 156.6, 157.8, 164.5. LCMS *m/z* (ES+) 959.3 $[M + H]^+$, 480.1 $[M + 2H]^{2+}$, 320.1 $[M + 4H]^{4+}$. Yield: quantitative.

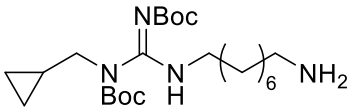
1,3-bis(8-carbamimidamidooctyl)-1,3-bis({8-[*N'*-(cyclopropylmethyl)carbamimidamido]octyl})urea hydrochloride salt (8CH8CH HCl, 19)**



^{1}H NMR (CD_3OD) δ (ppm): 0.29 (q, 4H, *J*= 4.8 Hz), 0.59 (q, 4H, *J*= 5.6 Hz), 1.07 (m, 2H), 1.37 (m, 32H), 1.48-1.55 (m, 8H), 1.59-1.60 (m, 8H), 3.08 (d, 4H, *J*= 6.8 Hz), 3.13-3.22 (m, 16H).^{13}C NMR (CD_3OD) δ (ppm): 3.4, 9.5, 26.2, 26.5, 27.5, 28.4, 28.4, 28.8, 28.9, 29.2, 41.0, 41.1, 45.8, 46.9, 47.1, 47.3, 47.5, 47.7, 47.9, 48.1, 155.6, 165.4. LCMS *m/z* (ES+) = 845.8 $[M + H]^+$, 423.1 $[M + 2H]^{2+}$, 282.5 $[M + 3H]^{3+}$, 212.1 $[M + 4H]^{4+}$. Yield: quantitative.

Preparation of *Arms-removed* derivatives 23-25

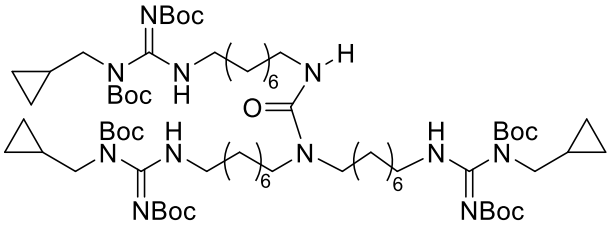
***tert*-butyl *N*-[[(8-aminooctyl)amino]({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (93)**



To a solution of 1,8-diaminooctane (586.0 mg, 4.07 mmol) in CH_3CN/MeOH 9/1 (10.0 mL), DIPEA (0.3 mL, 2.03 mmol) was added and the mixture was stirred at r.t. Then, a solution of *N,N*-Di-Boc-*N*-cyclopropylmethyl-pyrazole-1-carboxamidine (370.0 mg, 1.02 mmol) in CH_3CN/MeOH 9/1 (10.0 mL) was added dropwise through a syringe pump. at 45 °C for 16 h. Then, the solvent was evaporated and the crude product was purified by flash column chromatography (silica gel) (CH_3CN/MeOH/TEA 8/2/1), affording the product as a colourless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.20-0.22 (m, 2H), 0.41-0.43 (m, 2H), 1.02 (m, 1H), 1.28 (m, 10H), 1.44 (s, 9H), 1.46 (s, 9H), 1.57 (m, 2H), 2.08 (br, 2H), 2.65 (t, 2H, *J*= 8.0 Hz), 3.27 (t, 2H, *J*= 7.2 Hz), 3.51 (m, 2H), 8.23 (br, 1H), 11.50 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 10.5, 26.6, 26.8, 28.1, 29.1, 33.2, 41.9, 43.8, 52.0, 79.1, 81.7, 152.9, 158.0, 160.4. LCMS *m/z* (ES+) = 441.0 $[M + H]^+$. Yield: 70%.

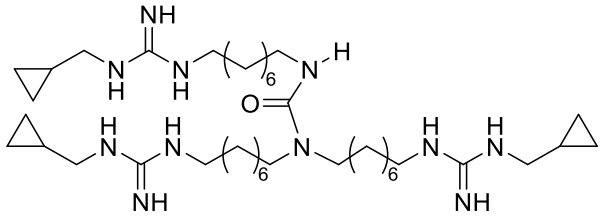
***tert*-butyl *N*-[({8-[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)[(8-{[{[(*tert*-**

butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)carbamoyl]amino]octyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (8CC*/8C(NH), 94)



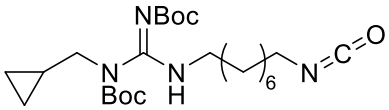
Same procedure for compounds **83-92**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (q, 6H, *J*= 7.2 Hz), 0.44 (q, 6H, *J*= 8.0 Hz), 1.04 (m, 3H), 1.31 (m, 24H), 1.43 (s, 27H), 1.48 (s, 27H), 1.60 (m, 14H), 3.14 (t, 4H, *J*= 7.6 Hz), 3.19 (q, 2H, *J*= 6.8 Hz), 3.30 (t, 6H, *J*= 4.8 Hz), 3.54 (6H), 4.24 (br, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 17.8, 26.8, 28.1, 28.6, 40.8, 43.8, 47.3, 52.1, 76.6, 76.9, 77.3, 77.3, 78.8, 151.3, 183.4. LCMS *m/z* (ES+) = 665.7 $[M + 2H]^{2+}$, 440.0 $[M + 3H]^{3+}$. Colourless oil. Yield: 50%.

1,3,3-tris({8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (8CC*/8C(NH)s, 23)



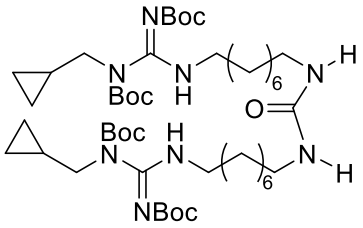
Same procedure for compounds **21-22**. ^{1}H NMR (CD_3OD) δ (ppm): 0.27 (q, 6H, *J*= 5.6 Hz), 0.58 (q, 6H, *J*= 5.2 Hz), 1.06 (m, 3H), 1.38-1.40 (m, 24H), 1.51 (m, 6H), 1.58 (m, 6H), 3.05 (d, 4H, *J*= 6.8 Hz), 3.14 (t, 6H, *J*= 8.0 Hz), 3.16-3.20 (m, 10H).^{13}C NMR (CD_3OD) δ (ppm): 3.5, 10.4, 16.9, 26.7, 28.2, 28.7, 40.0, 43.8, 47.7, 52.3, 76.5, 76.7, 77.7, 77.9, 78.7, 151.9, 183.2. LCMS *m/z* (ES+) = 365.0 $[M + 2H]^{2+}$, 244.0 $[M + 3H]^{3+}$. Colourless oil. Yield: quantitative.

***N*-(cyclopropylmethyl)-*N*-(8-isocyanatooctyl)guanidine (95)**



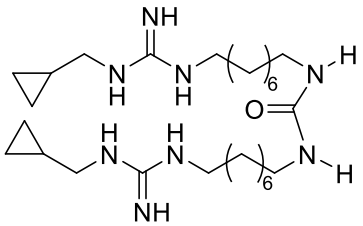
To a solution of **93** (40.0 mg, 0.09 mmol) in dry DCM (1.3 mL), DIPEA (16 µL, 0.09 mmol) was added under nitrogen atmosphere. Then, a solution of triphosgene (13.5 mg, 0.05 mmol) in dry DCM (1.3 mL) was added dropwise at 0 °C. The mixture was stirred 1 h at 0 °C and then it was allowed to reach r.t. for 0.5 h. Then $NaHCO_3$ s.s. was added to the reaction mixture and it was stirred for 10 min. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried on Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 9/1), affording the compound as oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (m, 2H), 0.44 (m, 2H), 1.04 (m, 1H), 1.30 (m, 8H), 1.46 (s, 9H), 1.48 (s, 9H), 1.59 (m, 4H), 3.15-3.30 (4H, m), 3.53 (m, 1H), 3.65 (m, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 7.1, 10.0, 26.7, 27.7, 28.0, 29.0, 29.2, 29.3, 41.0, 43.5, 114.7, 155.8. LCMS *m/z* (ES+) = 467.0 $[M + H]^{+}$. Colourless oil. Yield: 60%.

tert-butyl _N_-[[(8-{[(8-{[{[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl]amino}octyl)carbamoyl]amino}octyl)amino]({[(_tert_-butoxy)carbonyl]imino})methyl]-_N_-(cyclopropylmethyl)carbamate (8C(NH)*/8C(NH), 96)



To **95** (51.0 mg, 0.12 mmol) in a tube, a solution of **95** (45.0 mg, 0.10 mmol) in dry DCM (1.5 mL) and dry DIPEA (20 µL, 0.12 mmol) were added under nitrogen atmosphere. Then, the tube was sealed and the reaction mixture was stirred at 40 °C for 16 h. After cooling, DCM and $NaHCO_3$ s.s. were added to the mixture and it was stirred for 10 min. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried on Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 98/2), affording the compounds as colourless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (m, 4H), 0.44 (m, 4H), 1.04 (m, 2H), 1.30 (m, 16H), 1.46 (s, 18H), 1.48 (s, 18H), 1.60 (m, 8H), 3.13 (q, 4H, J= 8.0 Hz), 3.29 (m, 4H), 3.54 (m, 4H), 4.44 (br, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 26.6, 28.2, 28.9, 30.3, 40.4, 43.8, 48.9, 52.1, 151.4, 155.9. LCMS *m/z* (ES+) = 907.0 $[M + H]^+$, 929.0 $[M + Na]^+$, 454.5 $[M + 2H]^{2+}$. Colourless oil. Yield: 20%.

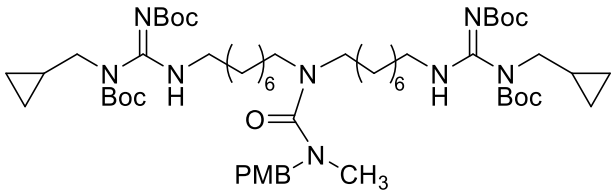
1,3-bis({8-[_N_-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (8C (NH)*/8C(NH)s, 24)



Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.27 (q, 4H, J= 5.2 Hz), 0.58 (q, 4H, J= 5.2 Hz), 1.06 (m, 2H), 1.35 (m, 16H), 1.46 (m, 4H), 1.59 (m, 4H), 3.05 (d, 4H, J= 7.2 Hz), 3.08 (t, 4H, J= 8.0 Hz), 3.18 (t, 4H, J= 6.8 Hz).^{13}C NMR (CD_3OD) δ (ppm): 3.5, 10.5, 26.6, 27.4, 28.0, 29.0, 29.2, 29.3, 41.0, 43.5, 114.0, 155.5. LCMS *m/z* (ES+) = 507.0 $[M + H]^+$, 254.0 $[M + 2H]^{2+}$, 169.0 $[M + 3H]^{3+}$. Colourless oil. Yield: quantitative.

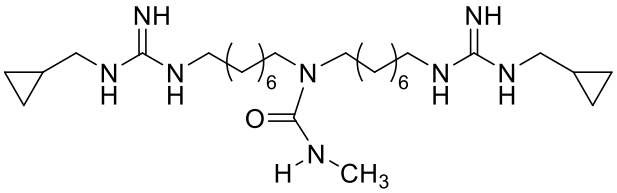
tert-butyl _N_-[({8-[(8-{[{[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl]amino}octyl)({[(4-

methoxyphenyl)methyl](methyl)carbamoyl})amino]octyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate ((NHMe)*/ 8CC, 97)



To a solution of **71** (26.0 mg, 0.12 mmol) in dry DCM (0.5 mL) in a tube, a solution of **93** (70.0 mg, 0.12 mmol) in dry DCM (1.0 mL) and dry DIPEA (20 µL, 0.12 mmol) were added under nitrogen atmosphere. Then, the tube was sealed and the reaction mixture was stirred at 40 °C for 16 h. After cooling, DCM and $NaHCO_3$ s.s. were added to the mixture and it was stirred for 10 min. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried on Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 98/2), affording the compounds as colourless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.22 (m, 4H), 0.43 (m, 4H), 1.03 (m, 2H), 1.28 (m, 20H), 1.45 (s, 18H), 1.47 (s, 18H), 1.59 (m, 4H), 2.67 (s, 3H), 3.10 (t, 4H, *J*= 8.0 Hz), 3.28 (t, 4H, *J*= 6.8 Hz), 3.53 (m, 4H), 3.77 (s, 3H), 4.26 (s, 2H), 6.83 (d, 2H, *J*= 8.0 Hz), 7.14 (d, 2H, *J*= 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 26.9, 28.0, 28.1, 29.2, 36.3, 43.8, 48.2, 53.7, 55.2, 76.6, 77.0, 77.3, 113.8, 128.8, 130.0, 165.3. LCMS *m/z* (ES+) = 1041.0 $[M + H]^+$, 1063.0 $[M + Na]^+$, 521.0 $[M + 2H]^{2+}$, 543.0 $[M + H + Na]^{2+}$. Colourless oil. Yield: 60%.

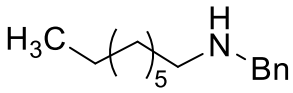
1,1-bis({8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl})-3-methylurea trifluoroacetate salt ((NHMe)*/ 8CCs, 25)



Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.27 (m 4H), 0.59 (m, 4H), 1.06 (m, 2H), 1.36 (m, 16H), 1.52 (m, 4H), 1.59 (m, 4H), 2.7 (s, 3H), 3.05 (d, 4H, *J*= 8.0 Hz), 3.18 (t, 8H, *J*= 8.0 Hz). ^{13}C NMR (CD_3OD) δ (ppm): 3.6, 10.2, 26.3, 26.8, 26.9, 27.4, 27.5, 27.7, 29.0, 29.1, 41.3, 43.6, 44.9, 155.8, 159.0. LCMS *m/z* (ES+) = 521.0 $[M + H]^+$, 261.0 $[M + 2H]^{2+}$. Colourless oil. Yield: quantitative.

Preparation of *Turned off*-guanidino derivatives 26-32.

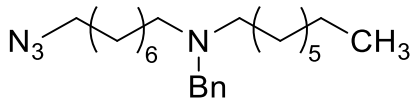
benzyl(octyl)amine (98)



To a solution of benzylamine (786.0 mL, 7.22 mmol) and potassium carbonate (1.5 g, 10.82 mmol) in *N*-butanol (15 mL) at 115 °C, a solution of 1-bromooctane (1.0 mL, 5.77 mmol) in *N*-butanol (11.0 mL) was added dropwise. The reaction mixture was stirred at 115°C for 24 h. After cooling, the reaction mixture was filtered and the white solid was washed with AcOEt. The organic phase was washed with

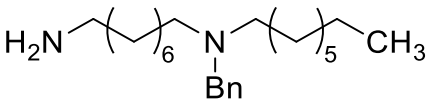
H_2O, dried over Na_2SO_4 and then evaporated under reduced pressure. The crude product was purified trough flash chromatography (silica gel) (AcOEt/MeOH 98/2), affording the compound as colourless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.87 (t, 3H, *J*= 6.8 Hz), 1.28 (m, 10H), 1.54 (m, 2H), 2.63 (t, 2H, *J*= 7.2 Hz), 2.96 (br, 1H), 3.80 (s, 2H), 7.24 (d, 1H, *J*= 6.4 Hz), 7.29-7.33 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.1, 22.7, 27.3, 29.3, 29.5, 29.7, 31.8, 49.2, 53.7, 76.9, 77.2, 77.5, 127.1, 128.4, 128.4, 139.5. LCMS *m/z* (ES+) = 332.0 $[M + H]^+$, 324.0 $[M + Na]^+$. Yield: 60%.

(8-azidooctyl)(benzyl)octylamine (99)



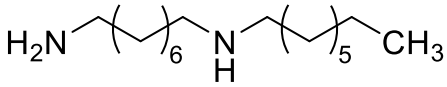
To a solution of **98** (340.0 mg, 1.55 mmol), potassium carbonate (321.0 mg, 2.33 mmol) and potassium iodide (90.0 mg, 0.54 mmol) in *N*-butanol (3.0 mL) at 115 °C, a solution of **46a** (365.0 mg, 1.55 mmol) in *N*-butanol (6.0 mL) was added. The reaction mixture was stirred at 115°C for 16 h. After cooling, the reaction mixture was filtered and the white solid was washed with DCM. The organic phase was washed with H_2O, dried over Na_2SO_4 and then evaporated under reduced pressure. The crude product was purified trough flash chromatography (silica gel) (DCM/MeOH 9/1), affording the compound as colourless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.84 (t, 3H, *J*= 7.2 Hz), 1.21 (m, 18H), 1.25 (m, 2H), 1.41 (m, 2H), 1.52 (qi, 2H, *J*= 6.8 Hz), 2.35 (t, 4H, *J*= 6.4 Hz), 3.16 (t, 4H, *J*= 6.8 Hz), 3.50 (s, 2H), 7.15 (d, 1H, *J*= 7.2 Hz), 7.21-7.28 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.2, 22.7, 26.7, 26.7, 27.0, 27.0, 27.3, 27.5, 28.4, 28.9, 28.9, 29.0, 29.2, 29.4, 29.4, 29.6, 29.8, 30.4, 31.9, 33.5, 51.4, 51.5, 53.8, 53.9, 58.7, 126.7, 128.1, 128.8, 128.9, 140.1. LCMS *m/z* (ES+) = 373.0 $[M + H]^+$, 395.0 $[M + Na]^+$. Yield: 80%.

(8-aminooctyl)(benzyl)octylamine (100)



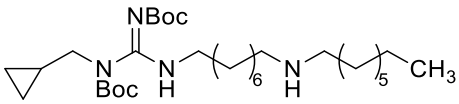
Same procedure for compounds **49a-c.** ^{1}H NMR (CD_3OD) δ (ppm): 0.89 (t, 3H, *J*= 6.8 Hz), 1.28 (m, 18H), 1.46 (m, 6H), 2.39 (t, 4H, *J*= 7.2 Hz), 2.60 (t, 2H, *J*= 7.6 Hz), 3.53 (s, 2H), 3.52 (s, 2H), 7.24-7.29 (m, 5H). ^{13}C NMR (CD_3OD) δ (ppm): 13.1, 13.3, 22.4, 26.3, 26.6, 27.2, 29.1, 29.2, 31.6, 32.3, 41.1, 49.4, 53.4, 58.3, 126.4, 126.7, 127.6, 127.9, 128.3, 128.7, 129.0, 139.1. LCMS *m/z* (ES+) = 347.0 $[M + H]^+$. Colourless oil. Yield: 80%.

(8-aminooctyl)(octyl)amine (101)



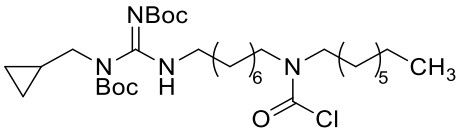
Same procedure for compounds **40a-c**. ^{1}H NMR (CD_3OD) δ (ppm): 0.91 (m, 3H), 1.35 (s, 18H), 1.52 (m, 6H), 2.56 (t, 4H, *J*= 7.2 Hz), 2.63 (t, 2 H, *J*= 6.8 Hz). ^{13}C NMR (CD_3OD) δ (ppm): 11.6, 20.8, 25.1, 25.5, 25.6, 27.5, 27.5, 27.6, 27.7, 30.1, 30.9, 39.6, 47.9. LCMS *m/z* (ES+) = 257.0 $[M + H]^+$. Yield: quantitative.

***tert*-butyl *N*-({[(*tert*-butoxy)carbonyl]imino}({[8-(octylamino)octyl]amino})methyl)-*N*-(cyclopropylmethyl)carbamate (8C8-, 102)**



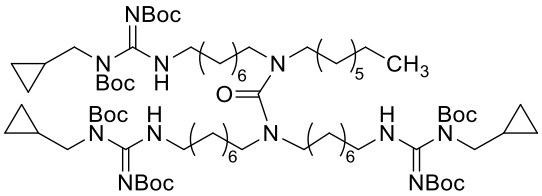
A solution of the guanylating agent **54** (368.0 mg, 1.01 mmol) in THF (2.0 mL) was added to the diamine **101** (215.6 mg, 0.84 mmol). DIPEA (146 µL, 0.84 mmol) was added and the reaction mixture was stirred at r.t. for 16 h. Then the mixture was diluted with DCM and washed with $NaHCO_3$ s.s. and brine. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 95/5), affording **102** as a yellowish oil. ^{1}H NMR ($CDCl_3$) δ (ppm): δ 0.23 (m, 2H), 0.44 (d, 2H, *J*= 7.6 Hz), 0.86 (t, 3H, *J*= 8.0 Hz), 1.04 (m, 1H), 1.25-1.31 (m, 18H), 1.45 (s, 9H), 1.48 (s, 9H), 1.60 (m, 6H), 2.68 (t, 4H, *J*= 7.6 Hz), 3.29 (m, 2H), 3.53 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.6, 14.1, 22.6, 26.9, 27.1, 27.2, 28.2, 28.8, 29.2, 29.2, 29.4, 31.8, 43.9, 49.3, 49.4, 79.1. LCMS *m/z* (ES+) = 553.0 $[M + H]^+$. Yield: 50%.

***tert*-butyl *N*-({[(*tert*-butoxy)carbonyl]imino}({8-[(chlorocarbonyl)(octyl)amino]octyl}amino)methyl)-*N*-(cyclopropylmethyl)carbamate (8C8-*, 103)**



Same procedure for compound **95**. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 98/2), affording **103** as a colourless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): δ 0.23 (m, 2H), 0.47 (m, 2H), 0.86 (t, 3H, *J*= 6.4 Hz), 1.04 (m, 1H), 1.28 (m, 20H), 1.46 (s, 9H), 1.48 (s, 9H), 1.61 (m, 6H), 3.31 and 3.36 (two overlapped t, 6H, *J*= 8.0 and 8.0 Hz), 3.54 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.1, 22.6, 26.8, 27.5, 28.2, 28.4, 29.1, 31.7, 53.4, 159.1. LCMS *m/z* (ES+) = 615.0 $[M + H]^+$, 637.0 $[Na + H]^+$. Yield: 91%.

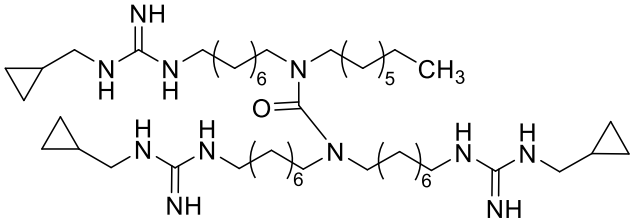
***tert*-butyl *N*-[({8-[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)(octyl)carbamoyl]amino]octyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (8C8-*/8CC, 104)**



To **52** (33.0 mg, 0.04 mmol) and sodium iodide (cat.) in a tube, a solution of **103** (52.5 mg, 0.06 mmol) in dry DCM (1.5 mL) and dry DIPEA (9 µL, 0.05 mmol) were added under nitrogen atmosphere. Then, the tube was sealed and the reaction mixture was stirred at 40 °C for 48 h. After cooling, DCM and $NaHCO_3$ s.s. were added to the mixture and it was stirred for 10 min. The aqueous phase was extracted with DCM and the combined organic layers were washed with brine, dried on Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified through flash chromatography (silica gel) (DCM/MeOH 98/2), affording the **104** as colourless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (q, 6H, *J*= 5.2 Hz), 0.45 (m, 6H), 0.87 (t, 3H, *J*= 6.8 Hz), 1.04 (m, 3H), 1.31 (m, 40H), 1.46 (s, 27H), 1.49 (s, 27H), 1.61 (m, 8H), 3.07 (t, 8H, *J*= 7.2 Hz), 3.30 (t, 6H, *J*= 6.8 Hz), 3.55 (m, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5,

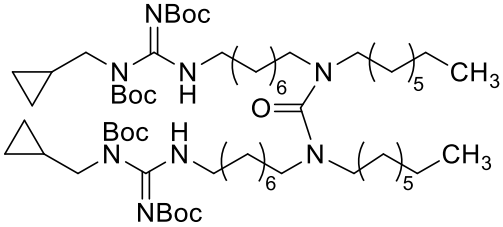
10.6, 14.1, 22.6, 26.8, 26.9, 27.1, 27.5, 28.2, 28.8, 29.2, 29.4, 31.7, 31.8, 43.9, 49.4, 53.4, 79.1, 159.1. LCMS *m/z* (ES+) = 1442.7 $[M + H]^+$, 732.8 $[M + H + Na]^{2+}$, 721.7 $[M + 2H]^{2+}$, 481.5 $[M + 3H]^{3+}$. Colourless oil. Yield: 50%.

1,1,3-tris({8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl})-3-octylurea trifluoroacetate salt (8C8-*/8CCs, 26)



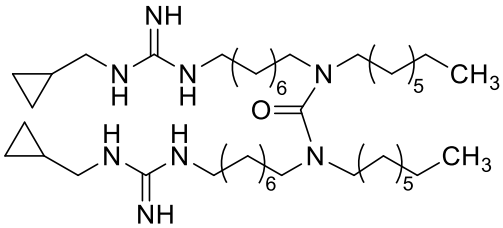
Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.27 (q, 6H, *J*= 5.2 Hz), 0.58 (q, 6H, *J*= 5.2 Hz), 0.90 (3H, t, *J*= 6.8 Hz), 1.06 (m, 3H), 1.29-1.40 (m, 34H), 1.52-1.59 (m, 14H), 3.05 (d, 6H, *J*= 7.2 Hz), 3.12-3.20 (m, 14H). ^{13}C NMR (CD_3OD) δ (ppm): 2.4, 9.5, 12.9, 22.2, 26.2, 26.6, 27.5, 28.2, 29.4, 31.5, 41.1, 45.8, 155.9. LCMS *m/z* (ES+) = 421.7 $[M + 2H]^{2+}$, 281.0 $[M + 3H]^{3+}$. Colourless oil. Yield: quantitative.

1,1,3-tris({8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl})-3-octylurea (8C8-*/8C8-, 105)



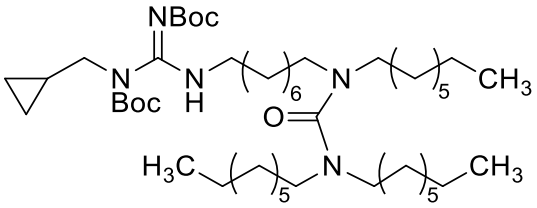
Same procedure for compound **104**. Coupling between **102** and **103**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (q, 4H, *J*= 5.2 Hz), 0.45 (q, 4H, *J*= 4.8 Hz), 0.87 (t, 6H, *J*= 7.2 Hz), 1.04 (m, 2H), 1.26 (m, 44H), 1.46 (s, 18H), 1.49 (s, 18H), 1.61 (qi, 4H, *J*= 7.2 Hz), 3.07 (t, 8H, *J*= 7.2 Hz), 3.30 (t, 4H, *J*= 6.8 Hz), 3.55 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.6, 10.6, 14.1, 22.7, 22.7, 27.0, 27.1, 28.0, 28.3, 29.3, 29.5, 29.7, 31.8, 43.9, 48.2, 79.1, 156.2, 165.4. LCMS *m/z* (ES+) = 1131.2 $[M + H]^+$, 1153.2 $[M + Na]^+$, 566.2 $[M + 2H]^{2+}$. Colourless oil. Yield: 30%.

1,3-bis({8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl})-1,3-dioctylurea trifluoroacetate salt (8C8-*/8C8-s, 27)



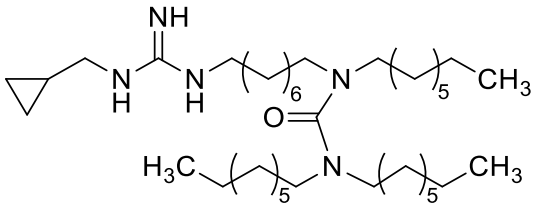
Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.30 (q, 4H, *J*= 5.2 Hz), 0.58 (q, 4H, *J*= 5.2 Hz), 0.90 (6H, t, *J*= 6.8 Hz), 1.06 (m, 2H), 1.29-1.39 (m, 34H), 1.49-1.55 (m, 8H), 1.58 (m, 4H), 3.05 (d, 4H, *J*= 7.2 Hz), 3.12-3.20 (m, 12 H). ^{13}C NMR (CD_3OD) δ (ppm): 2.4, 9.5, 12.9, 22.2, 26.6, 27.5, 28.5, 28.9, 31.5, 41.1, 45.8, 154.9. LCMS *m/z* (ES+) = 731.2 $[M + H]^+$, 366.1 $[M + 2H]^{2+}$. Colourless oil. Yield: quantitative.

tert-butyl _N_-({[(_tert_-butoxy)carbonyl]imino}({8-[(dioctylcarbamoyl)(octyl)amino]octyl}amino)methyl)-_N_-(cyclopropylmethyl)carbamate (8C8-*/8-8-, 106)



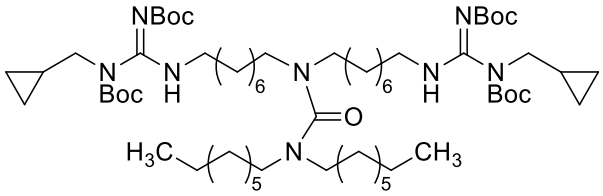
Same procedure for compound **104**. Coupling between bisoctylamine and **103**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.22 (q, 2H, *J*= 4.8 Hz), 0.45 (q, 2H, *J*= 7.6Hz), 0.87 (t, 9H, *J*= 6.8 Hz), 1.04 (m, 1H), 1.28 (m, 46H), 1.46 (s, 9H), 1.49 (s, 9H), 1.60 (qi, 2H, *J*= 7.2 Hz), 3.07 (t, 8H, *J*= 7.2 Hz), 3.30 (t, 2H, *J*= 7.2 Hz), 3.55 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.6, 14.1, 22.6, 27.0, 27.1, 28.0, 28.2, 29.3, 29.3, 29.4, 29.5, 29.7, 31.8, 43.9, 48.2, 48.3, 48.5, 81.9, 165.5. LCMS *m/z* (ES+) = 820.2 $[M + H]^+$, 842.2 $[M + Na]^+$. Colourless oil. Yield: 45%.

1-{8-[_N_-(cyclopropylmethyl)carbamimidamido]octyl}-1,3,3-trioctylurea trifluroacetate salt (8C8-*/8-8-s, 28)



Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.28 (q, 2H, *J*= 5.2 Hz), 0.60 (q, 2H, *J*= 5.6 Hz), 0.90 (9H, t, *J*= 6.8 Hz), 1.06 (m, 1H), 1.29-1.35 (m, 38H), 1.51 (qi, 8H, *J*= 6.8 Hz), 1.59 (qi, 2H, *J*= 7.2 Hz), 3.05 (d, 2H, *J*= 7.2 Hz), 3.14 (t, 8H, *J*= 7.2 Hz), 3.18 (t, 2H, *J*= 6.8 Hz) ^{13}C NMR (CD_3OD) δ (ppm): 2.4, 9.5, 12.9, 22.2, 26.2, 26.6, 27.5, 28.5, 28.8, 29.0, 29.0, 31.5, 41.1, 45.8, 165.9. LCMS *m/z* (ES+) = 620.2 $[M + H]^+$, 642.2 $[M + Na]^+$, 658.2 $[M + K]^+$. Colourless oil. Yield: quantitative.

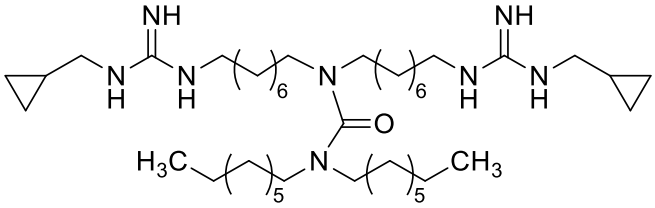
tert-butyl _N_-({[8-({8-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}(dioctylcarbamoyl)amino)octyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate _tert_-butyl _N_-({[8-({8-[({[(_tert_-butoxy)carbonyl](cyclopropylmethyl)amino}({[(_tert_-butoxy)carbonyl]imino})methyl)amino]octyl}(dioctylcarbamoyl)amino)octyl]amino}({[(_tert_-butoxy)carbonyl]imino})methyl)-_N_-(cyclopropylmethyl)carbamate (8CC*/8-8-, 107)



Same procedure for compound **104**. Coupling between bisoctylamine and **79**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.24 (q, 4H, *J*= 5.2 Hz), 0.45 (q, 4H, *J*= 4.8 Hz), 0.87 (t, 6H, *J*= 7.2 Hz), 1.04 (m, 2H), 1.29 (m, 44H), 1.46

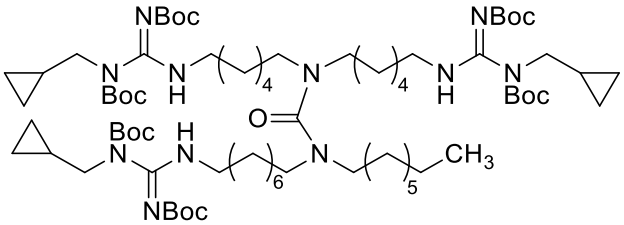
(s, 18H), 1.49 (s, 18H), 1.61 (qi, 4H, *J*= 7.2 Hz), 3.07 (m, 8H), 3.30 (t, 4H, *J*= 6.8 Hz), 3.55 (m, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm): 33.5, 10.6, 14.1, 22.6, 22.6, 27.0, 27.1, 28.0, 28.1, 28.2, 29.3, 29.4, 29.7, 31.8, 43.9, 48.3, 48.4, 53.4, 165.0. LCMS *m/z* (ES+) = 1131.2 $[M + H]^+$, 1153.2 $[M + Na]^+$, 1169.2 $[M + K]^+$. Colourless oil. Yield: 45%.

1,1-bis({8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl})-3,3-dioctylurea trifluoroacetate salt (8CC*/8-8-s, 29)



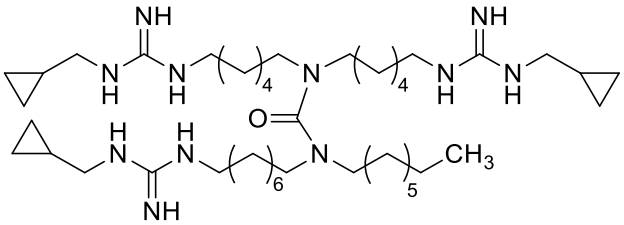
Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.28 (q, 4H, *J*= 5.2 Hz), 0.58 (q, 4H, *J*= 5.2 Hz), 0.90 (6H, t, *J*= 6.8 Hz), 1.06 (m, 2H), 1.29-1.40 (m, 34H), 1.49-1.55 (m, 8H), 1.58 (m, 4H), 3.05 (d, 4H, *J*= 7.2 Hz), 3.12-3.19 (m, 12 H). ^{13}C NMR (CD_3OD) δ (ppm): 2.4, 9.5, 12.9, 22.2, 26.2, 26.5, 27.5, 28.9, 31.5, 41.1, 45.8, 164.9. LCMS *m/z* (ES+) = 731.2 $[M + H]^+$, 366.1 $[M + 2H]^{2+}$. Colourless oil. Yield: quantitative.

***tert*-butyl *N*-[({6-[(6-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}hexyl)[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)(octyl)carbamoyl]amino]hexyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (6CC*/8C8-, 108)**



Same procedure for compound **104**. Coupling between **102** and **80**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (m, 6H), 0.45 (m, 6H), 0.86 (t, 3H, *J*= 6.8 Hz), 1.05 (m, 3H), 1.25 (m, 32H), 1.45 (s, 27H), 1.47 (s, 27H), 1.61 (m, 8H), 3.07 (m, 8H), 3.30 (m, 6H), 3.51 (m, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.6, 14.1, 22.6, 26.8, 27.1, 27.5, 28.2, 28.8, 29.2, 29.4, 31.7, 31.8, 43.9, 49.4, 53.4, 79.1, 159.1. LCMS *m/z* (ES+) = 1386.9 $[M + H]^+$, 1408.9 $[M + Na]^+$, 1425.9 $[M + K]^+$. Colourless oil. Yield: 77%.

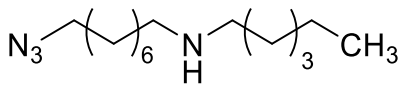
3,3-bis({6-[*N*-(cyclopropylmethyl)carbamimidamido]hexyl})-1-{8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl}-1-octylurea trifluoroacetae salt (6CC*/8C8-, 30)



Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.27 (q, 6H, *J*= 5.2 Hz), 0.58 (q, 6H, *J*= 5.2 Hz), 0.90 (3H, t, *J*= 6.8 Hz), 1.06 (m, 3H), 1.29-1.39 (m, 26H), 1.52-1.60 (m, 14H), 3.05 (d, 6H, *J*= 7.2

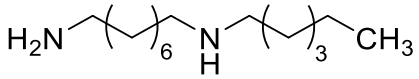
Hz), 3.12-3.20 (m, 14H). ^{13}C NMR (CD_3OD) δ (ppm): 2.6, 9.5, 12.9, 22.2, 26.1, 26.6, 28.2, 29.4, 31.5, 41.3, 45.8, 155.5. LCMS *m/z* (ES+) = 394.0 $[M + 2H]^{2+}$, 263.0 $[M + 3H]^{3+}$. Colourless oil. Yield: quantitative.

(8-azidooctyl)(hexyl)amine (109)



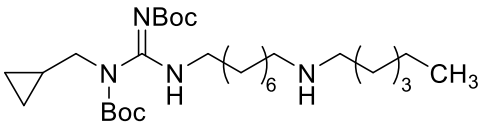
Same procedure for compound **47**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.84 (t, 3H, *J*= 7.2 Hz), 1.26 (m, 14H), 1.25 (m, 2H), 1.46 (m, 4H), 1.54 (m, 2H), 2.55 (t, 4H, *J*= 6.4 Hz), 3.20 (t, 2H, *J*= 6.8 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 115.0, 23.3, 27.6, 27.8, 28.1, 30.3, 30.9, 32.5, 34.5, 42.8, 50.4, 50.7, 51.1. LCMS *m/z* (ES+) = 253.0 $[M + H]^+$. Colourless oil. Yield: 30%.

(8-aminooctyl)(hexyl)amine (110)



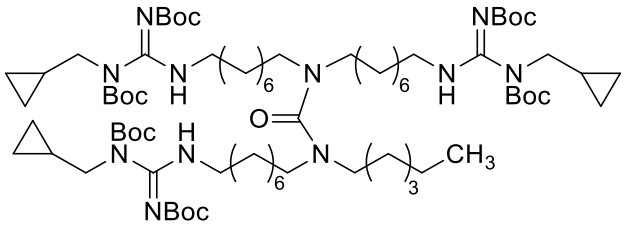
Same procedure for compound **99**. ^{1}H NMR (DMSO D_6) δ (ppm): 1.65 (t, 3H, *J*= 7.2 Hz), 2.04 (s, 14H), 2.15 (q, 6H, *J*= 6.0 Hz), 3.15 (br, 2H), 3.24 (t, 4H, *J*= 7.2 Hz), 3.32 (t, 2 H, *J*= 6.8 Hz). ^{13}C NMR (DMSO D_6) δ (ppm): 14.7, 23.0, 27.8, 28.1, 30.2, 30.9, 32.5, 42.7, 50.4, 51.0, 51.1. LCMS *m/z* (ES+) = 229.1 $[M + H]^+$. Colourless oil. Yield: quantitative.

***tert*-butyl *N*-({[(*tert*-butoxy)carbonyl]imino}({[8-(hexylamino)octyl]amino})methyl)-*N*-(cyclopropylmethyl)carbamate (8C6-,111)**



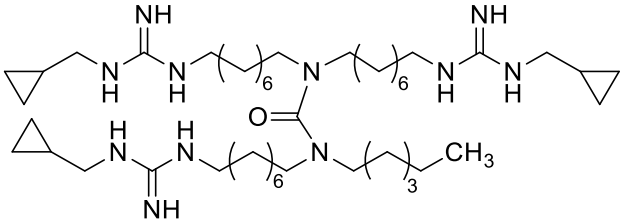
Same procedure for compound **102**.^{1}H NMR ($CDCl_3$) δ (ppm): δ 0.21 (m, 2H), 0.42 (m, 2H), 0.85 (t, 3H, *J*= 6.8 Hz), 1.02 (m, 1H), 1.28 (m, 18H), 1.44 (s, 9H), 1.46 (s, 9H), 1.59 (m, 2H), 2.58 (t, 4H, *J*= 7.6 Hz), 3.28 (m, 2H), 3.52 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.4, 10.5, 13.9, 22.5, 26.8, 26.9, 27.2, 28.1, 29.1, 29.1, 29.2, 29.7, 31.7, 43.8, 49.8, 49.9, 52.1, 53.3, 78.8, 79.0, 81.8. LCMS *m/z* (ES+) = 525.1 $[M + H]^+$, 547.1 $[M + Na]^+$. Colourless oil. Yield: 55%.

***tert*-butyl *N*-[({8-[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)(hexyl)carbamoyl]amino]octyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (8CC*/8C6-, 112)**



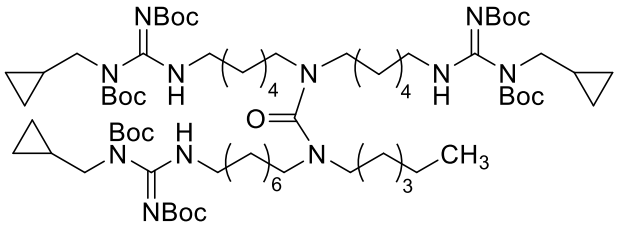
Same procedure for compound **104**. Coupling between **111** and **79**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (m, 6H), 0.44 (m, 6H), 0.87 (t, 3H, *J*= 6.8 Hz), 1.04 (m, 3H), 1.30 (m, 36H), 1.46 (s, 27H), 1.48 (s, 27H), 1.60 (m, 8H), 3.07 (m, 8H), 3.30 (m, 6H), 3.55 (m, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 13.9, 22.5, 22.5, 26.7, 26.9, 27.0, 27.9, 28.1, 29.2, 29.6, 31.6, 43.8, 48.3, 77.3, 151.3, 165.3. LCMS *m/z* (ES+) = 1414.9 $[M + H]^+$, 1437.9 $[M + Na]^+$, 1453.8 $[M + K]^+$. Colourless oil. Yield: 68%.

1,1,3-tris({8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl})-3-hexylurea trifluoroacetate salt (8CC*/8C6-s, 31)



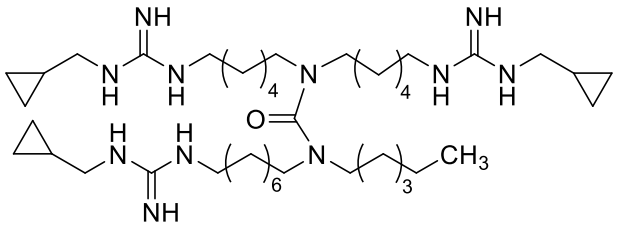
Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.27 (m, 6H), 0.58 (m, 6H), 0.90 (3H, t, *J*= 6.8 Hz), 1.06 (m, 3H), 1.29-1.39 (m, 30H), 1.51 (m, 8H), 1.58 (m, 6H), 3.05 (d, 6H, *J*= 7.2 Hz), 3.12-3.19 (m, 14H). ^{13}C NMR (CD_3OD) δ (ppm): 2.8, 9.5, 12.3, 22.2, 26.1, 26.6, 27.5, 28.2, 29.4, 31.5, 41.3, 45.8, 155.0. LCMS *m/z* (ES+) = 814.0 $[M + H]^+$, 407.5 $[M + 2H]^{2+}$, 272.2 $[M + 3H]^{3+}$. Colourless oil. Yield: quantitative.

***tert*-butyl *N*-[({6-[(6-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}hexyl)[(8-{[{[(*tert*-butoxy)carbonyl](cyclopropylmethyl)amino}({[(*tert*-butoxy)carbonyl]imino})methyl]amino}octyl)(hexyl)carbamoyl]amino]hexyl}amino)({[(*tert*-butoxy)carbonyl]imino})methyl]-*N*-(cyclopropylmethyl)carbamate (6CC*/8C6-, 113)**



Same procedure for compound **104**. Coupling between **111** and **80**. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.23 (m, 6H), 0.44 (m, 6H), 0.87 (t, 3H, *J*= 6.8 Hz), 1.04 (m, 3H), 1.30 (m, 28H), 1.46 (s, 27H), 1.48 (s, 27H), 1.60 (m, 8H), 3.07 (m, 8H), 3.30 (m, 6H), 3.55 (m, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 3.5, 10.5, 13.9, 22.5, 22.5, 26.7, 26.9, 27.0, 27.8, 27.9, 28.1, 29.6, 31.6, 43.9, 48.3, 151.3, 164.3. LCMS *m/z* (ES+) = 1358.8 $[M + H]^+$, 1381.8 $[M + Na]^+$, 680.8 $[M + 2H]^{2+}$, 453.8 $[M + 3H]^{3+}$. Colourless oil. Yield: 89%.

1,1-bis({6-[*N*-(cyclopropylmethyl)carbamimidamido]hexyl})-3-{8-[*N*-(cyclopropylmethyl)carbamimidamido]octyl}-3-hexylurea trifluoroacetate salt (6CC*/8C6-s, 32)



Same procedure for compounds **21-22**.^{1}H NMR (CD_3OD) δ (ppm): 0.27 (m, 6H), 0.58 (m, 6H), 0.90 (3H, t, *J*= 6.8 Hz), 1.06 (m, 3H), 1.29-1.39 (m, 22H), 1.52-1.60 (m, 14H), 3.05 (d, 6H, *J*= 7.2 Hz), 3.12-3.19 (m, 14H). ^{13}C NMR (CD_3OD) δ (ppm): 2.7, 9.5, 12.2, 22.3, 26.1, 27.5, 28.02, 29.4, 31.5, 41.3, 45.7, 156.2. LCMS *m/z* (ES+) = 759.0 $[M + H]^+$, 380.0 $[M + 2H]^{2+}$, 253.7 $[M + 3H]^{3+}$. Colourless oil. Yield: quantitative.

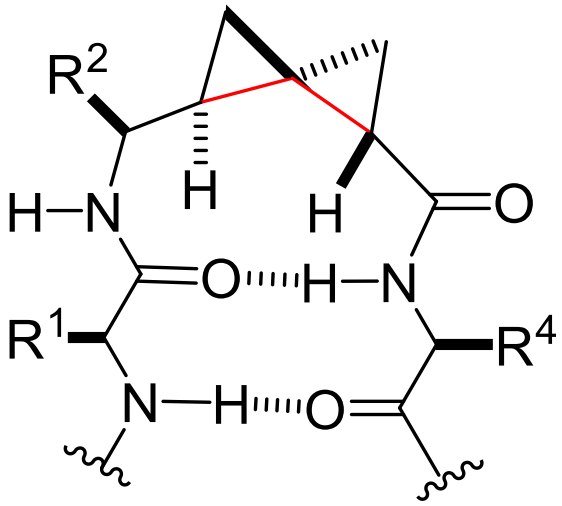
Additional issues

Biological experiments were performed by the research groups of Prof. Jean-Denis Docquier (Department of Medical Biotechnology, University of Siena, Siena, Italy) and of Prof. Paolo Visca (Department of Sciences, University of Roma Tre, Rome, Italy).

The analyses on the original mixture were performed by Prof. Elena Dreassi (Department of Biotechnology, Chemistry, and Pharmacy, University of Siena, Siena, Italy) and Dr. Claudio Zamperini (Lead Discovery Siena s.r.l., Via Vittorio Alfieri 31, Castelnuovo Berardenga, Italy)

Preliminary ADME characterization was performed by the analytical team of Prof. Maurizio Botta and Dr. Claudio Zamperini.

Peptide surrogates



Introduction and Discussion6-

In the last decades, medicinal and bioorganic chemistry put efforts in a new drug development approach based on the preparation of peptidomimetics. This term refers to compounds that are designed to mimic a peptide structure and/or functions.
In particular, several research groups[219–224] focused their attention on the design of peptide surrogates able to fold miming protein secondary structures. The interest in these compounds is enormous and it is driven forward two different directions. The first one is their possible application in medicinal chemistry as safe and effective alternatives of natural peptides which are considered not *druggable*, while the second route is developed in the biochemistry field with the purpose to gain enhanced information and understanding of the protein folding and the consequent protein-protein interactions. The studies of peptidomimetics as chemotherapeutics is based on the principle that they are endowed with protein-like properties, resembling proteins without being actually proteins. Indeed, natural or physiological peptides cannot be employed in clinics since their low absorption, poor oral bioavailability and metabolic instability hamper and do not allow their administration in humans. In brief, proteins do not obey Lipinski's rule of five that is a useful parameter to predict the suitability of a molecule as a drug. In particular, the large molecular weight and vast hydrogen bond patterns impair the crossing of cytoplasmatic membranes, making them unable to reach the therapeutic targets. Moreover, peptide bonds are very labile since they are easily cleaved by plasmatic enzymes, such as peptidases and proteases. Hence, the metabolism and, in particular, the catabolism of peptides are very fast and they are rapidly cleared from the body by renal filtration.
In the last two decades, antimicrobial peptides (AMPs), attracted researchers as possible candidates for innovative antibiotic therapy. Although they often display broad-spectrum antibacterial activity, including MDR pathogens, any synthetic AMPs have received the FDA approval. Rational designs involved some modifications on the AMPs structure by means of the incorporation of non-standard amino acids or sequences. Thus, by maintaining the pharmacophores of AMPs, the secondary features of the molecules such as backbone, side chain functions, hydrophobicity, and polarity are subjected to optimization studies resulting in the development of peptidomimetic antibiotics.[224] Currently, many peptidomimetics have been developed as antibacterial agents.[219–224] For example, Murepavadin, developed by Polyphor Ltd, has been recently approved by FDA and can be considered the first peptidomimetic antibiotic which targets an outer membrane protein with an innovative non-lytic mode of action. Its structure looks like the cationic antimicrobial peptide protegrin I (PG-1) being a cyclic beta hairpin peptidomimetic.[225]
The other reason that justifies the rise in interest in peptidomimetics, especially in the mimics of protein secondary structures, is the significant flexibility of small size peptides that could be useful tools for the study of the whole protein which they belong to. Indeed, in order to study large proteins and enzymes gaining access to relevant information for both physiological studies and therapeutic purpose, biochemists usually isolate small protein sub-unit. Unfortunately, as already mentioned, small peptides are conformationally flexible in solution because their amino acids interact and fold up according to the polarity of their side chains, not retaining the secondary structure of the native protein and not allowing to be considered as a model of the whole protein. In fact, the high degree of the local order of proteins is due to the pattern of hydrogen bonds between the amino acids and these characteristic interactions define the secondary structures of proteins, such as β-sheets, β-turns, and α-helices, responsible for their three-dimensional structure and the consequent folding and bioactivity.

Hence the need to design compounds or small peptide sequences able to mimic the protein native folding.

For years, the research group of Professor J. S. Clark has been involved in the synthesis of small constrained mimetics able to adopt a *β*-turn motif (data not published).

During my STSM founded by COST Action CM1407, I worked on the synthesis of new conformationally constrained peptide surrogates with a spiro-fused dicyclopropane (spiro[2.2]pentane) isostere scaffold as reported in **Figure 1**, with the aim to incorporate it into small peptides and to understand if it could induce the formation of a *β*-turn structure in the hybrid peptide system.

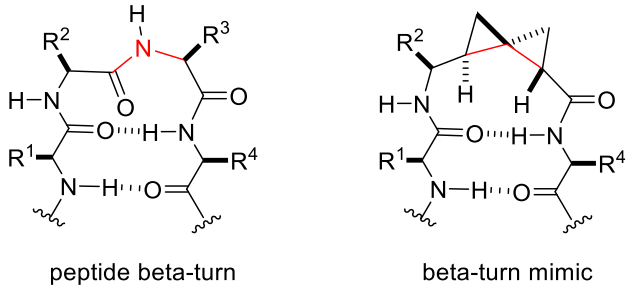
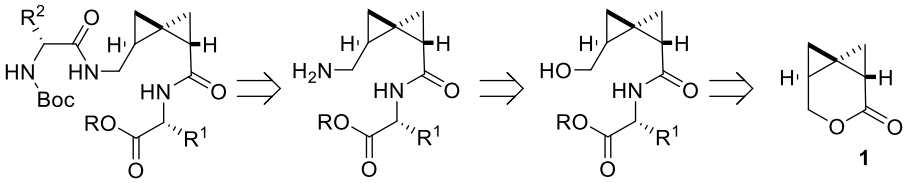


Figure 1. Comparison between the natural and the mimic protein secondary structures.

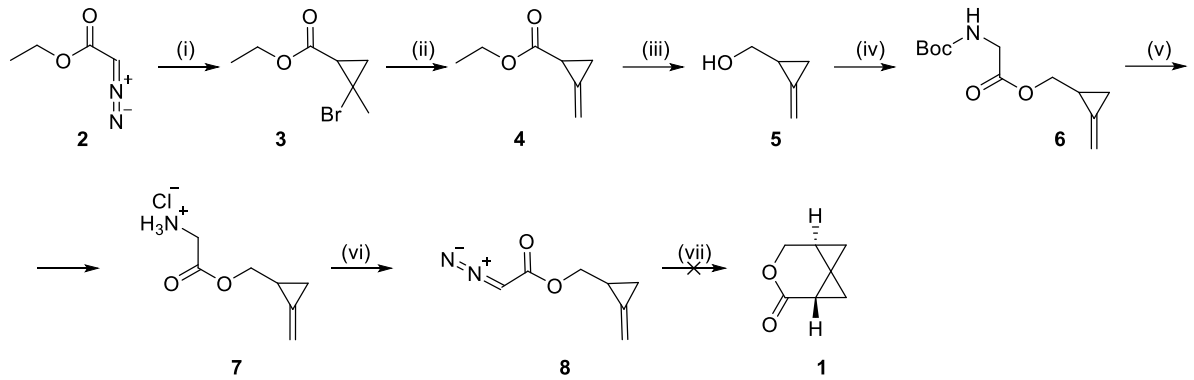
The retrosynthetic approach to obtain the desired peptidomimetic (**Scheme 1**) starts from the tricyclic lactone **1**.



Scheme 1. Retrosynthetic approach to preparing the spiro-fused dicyclopropane peptidomimetic.

The preparation of compound **1** is very challenging; in fact, only two research groups[226,227] have synthesized it but with low yields and very long synthetic pathways. While the first approach was not easily reproducible in a research laboratory because of the use of ozone, the second one is more accessible even if the volatile nature of most of the intermediates may cause concern about their manipulability.

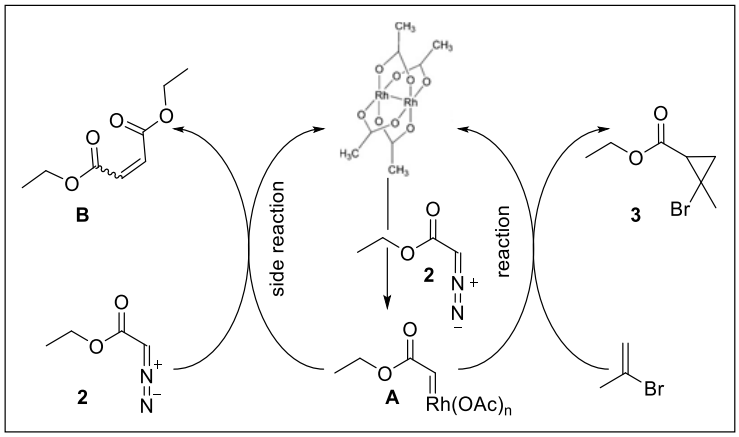
Initially, the synthetic strategy for preparing the tricyclic lactone **1** was as reported in the following scheme (**Scheme 2**).



Scheme 2. Synthetic approach for tricyclic lactone **1** *Reagents and conditions:* (i) 2-Bromopropene, $Rh_2(OAc)_4$ (0.02mol%), r.t., 20 h; (ii) NaH, dry Et_2O, 40 °C, 16 h; (iii) reductive conditions (iv) DCC, DMAP, dry DCM, 0 °C-r.t., 3 h; (v) HCl $_{conc.}$, Et_2O, 0 °C-r.t., 16 h; (vi) $NaNO_2$, $H_2SO_{4\ conc.}$, NaOAc 0.03 M pentane/H2O, -15 – 10 °C, 0.5 h; (vii) MLn (0.2 mol%), Ln, dry DCM, 40 °C, 20 h.

Procedures for the cyclopropanation step (i)[228–232] in which solvent-free conditions was performed by using a diazo-derived metal carbenoid[229–232] furnished the product **3** in low yield. Better results were obtained by increasing the excess of 2-bromopropene and by using dry DCM as a solvent (81.3%). The product was obtained as a mixture of diastereomers (detected by NMR analysis).

Cyclopropanation reactions rise enormous interest because of the versatility of the substrates that can be used and consequently the products obtained. The most known strategy involves the formal addition of a carbene group belonging to a diazo compound to an unsaturated double bond.[233,234] The presence of transition metal catalysts, such as $Rh_2(OAc)_4$ is essential to allow the chemoselectivity of the reaction. In fact, during this transformation, different products and isomers can be formed. The reaction occurs through the formation of a transient metallocarbene intermediate (**A**) furnished by the interaction between the catalyst and the diazo ethylacetate.[235,236] Usually, this intermediate **A** can react with another molecule of diazo compound leading to the undesired coupling products, such as diethyl maleate and diethyl fumarate **B**. To reduce this side reaction, the diazo compound **2** was added slowly to the solution of catalyst and 2-bromopropene, resulting in an improved chemoselectivity toward cyclopropanes (**Scheme 3**).



Scheme 3. Mechanism of cyclopropanation and the side product formation.

The *β*-elimination can occur through different mechanisms: $\mathbf{E_1}$, $\mathbf{E_2}$ or $\mathbf{E_{1cB}}$. The E_1 mechanism involves the unimolecular ionization of the starting material, generating a stable tertiary carbocation intermediate **A**. The elimination is then completed by removal of a *β*-proton. In contrast, the E_2 route occurs through a bimolecular transition state in which the removal of a *β*-proton, initiated by the base, and the departure of the leaving group bromine is concerted when anti-periplanar geometry between

the eliminated proton and leaving group is possible. In the end, E_{1cB} is similar to E_1, involving a two step-mechanism but the order is reversed: the *β*-deprotonation generates the carbanion intermediate **B** and precedes the departure of the bromine. (**Figure 2A**).

Considering the reaction conditions used for the alkene **4** preparation, all the routes are possible but the E_1 mechanism is believed to be the main elimination pathway due to the stable nature of the carbocation **A**.

Besides the desired alkene **4**, more possible byproducts could be formed (**Figure 2B**) according to Zaitsev's rule. The major *β*-elimination product is always the most substituted alkene, being the most thermodynamically stable compound. In this case, the most stable product is the *α*, *β*-unsaturated derivative, followed by the disubstituted alkene, while product **3** is expected to be the minor product. However, the use of sodium hydride as the base increases the formation of **4** since it is insoluble in most organic solvents, such as THF, the reaction solvent. In fact, the deprotonation of **3** can happen only the least hindered *β*-hydrogen being the nearest to the base surface. In contrast, to obtain the Zaitsev's products small and more soluble bases such as NaOH, and NaOEt should be used. However, in order to speed up the deprotonation step, a catalytic amount of anhydrous ethanol was added to the mixture, improving the solubility of sodium hydride. Furthermore, both Zaitsev's product and substituted alkene are not the favored reaction products since the olefin bond should be into the already strained cyclopropane. However, taking care of the anhydrous conditions of the reaction set up and limiting the possibility of sodium hydroxide generation due to residual water, any traces of these side products were detected in the reaction crude.

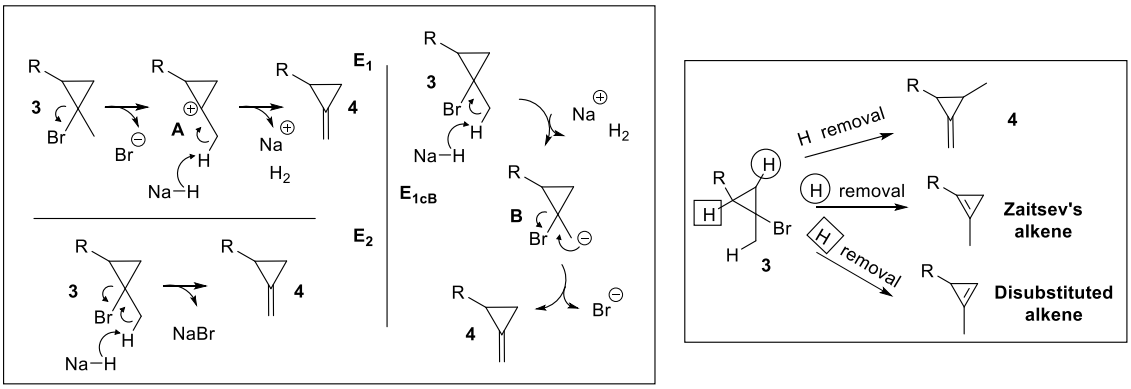
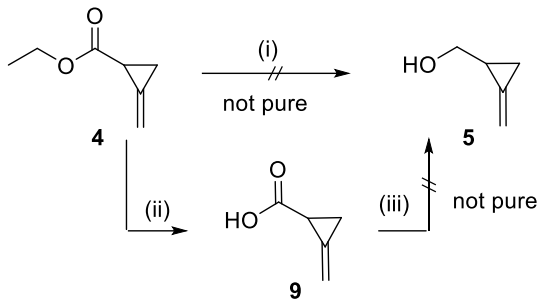


Figure 2. Left, mechanism of elimination, E_1 (with carbanion intermediate A), E_2(concerted), and E_{1cB} (with carbocation intermediate B); right, other alkene derivatives from β-elimination of **3**.

Hence, the dehydrohalogenation step (ii) was performed according to the literature,[228] but a mixture of product (**4**) and byproducts was obtained in most cases. Attempts to reduce the formation of byproducts were made by changing the reaction conditions (solvents, such as Et_2O, THF or DMSO; molarity; equivalents, temperature)[228,230,231,237] and the reactions were carried out by using flame-dried glassware and inert Ar atmosphere in order to lower the risk of generating other alkenes according to Zaitsev's rule. A low-pressure (20 mbar) bulb-to-bulb distillation at 50 °C was performed, giving only 36.4% of pure **4**. Increasing the distillation temperature (60-100 °C) resulted in the formation of a mixture of **4** (major product) and impurities. Both chromatographic purification and Vigreux column distillation did not allow to isolate the alkene **4** because of its volatile nature. Thus, the impure material was used for the reduction step by using LAH or DIBAL as reducing agents and the crude evaporation was conducted under several conditions, such as high temperature-distillation or concentration in vacuum at r.t. through Toxic Buchi. However, any efforts to isolate **5** as pure compound failed.

To remove the impurities and improve the reduction yield, the ester function of **4** was hydrolyzed[237] and the resulting carboxylic acid **9** was reduced with LAH or borane. However, both the attempts to perform the reduction reaction produced an impure product (**Scheme 4**).



Scheme 4. Synthesis of alcohol derivative **2** via ester hydrolysis and reduction. *Reagents and conditions*: (i) LAH, dry Et_2O, -78-40 °C, 16 h or DIBAL(H), dry THF, -78 °C-r.t., 3 h; (ii) KOH, MeOH/H_2O 4/1, 0 °C-r.t., 15 h; (iii) LAH, dry Et_2O, 0 °C-r.t., 16 h or BH_3-DMS, dry THF, 0 °C-r.t., 1 h.

Thus, the impure material **5** was reacted with Boc-glycine via Steglich esterification (iv) and the Boc protecting group was cleaved, furnishing the hydrochloride salt **7** (v).[227] The diazotization of the amine **7** (vi) was very challenging due to the high propensity of **8** to undergo decomposition. Following the published protocol,[227] a mixture of byproducts was obtained and column chromatography did not allow **8** to be isolated with high purity. Better results were obtained by using pentane and NaOAc as buffer to avoid ester hydrolysis.

The diazo ester formation mechanism begins with an *in situ* generation of nitrous acid via protonation of nitrate by a catalytic amount of sulfuric acid.. Its overprotonation produces an intermediate that is fast attacked by the primary amine **7**, forming the tautomers **A** and **B**. The following elimination of water by **C** leads to the diazo function, **D**. In the end, the elimination of the acidic *α*-H allowed the obtainment of the product **8** (**Figure 3**).

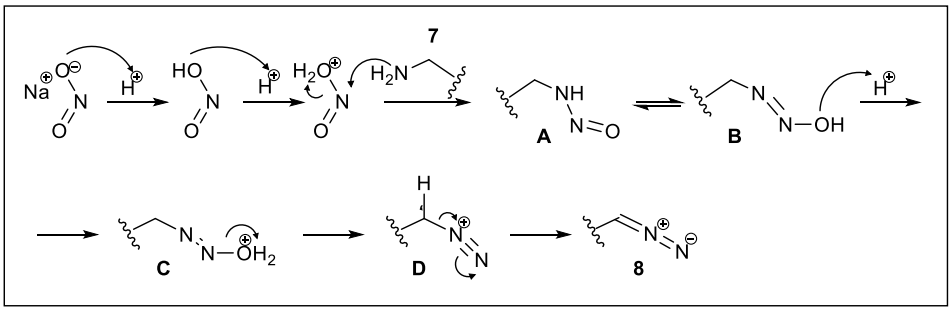


Figure 3. Mechanism of diazo function formation on compound **8**.

However, even though impurities were detected by ^{1}H NMR, the spectra showed the characteristic signal at 4.70 ppm corresponding to CHN_2. Thus, the cleanest compound batches were used for the intramolecular cyclopropanation (vii) catalyzed by $Rh_2(Oct)_4$, $Cu(MeCN)_4PF_6$, $Rh_2(pfb)_4$ or $Cu(hfacac)_2$[238,239] but the spiropentane **1** was not obtained. The intramolecular cyclisation should happen through a similar mechanism as the cyclopropanation step: firstly, the metal carbenoid **A** is generated in presence of the catalyst, then the formation of two possible products can occur, the desired product **1** and another compound by C-H insertion. (**Figure 4**).

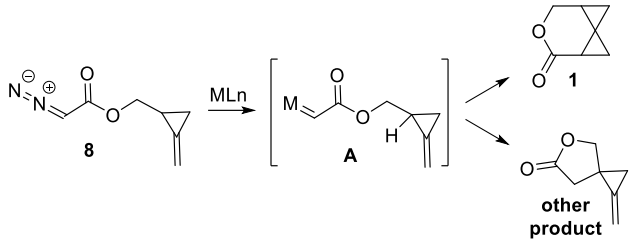
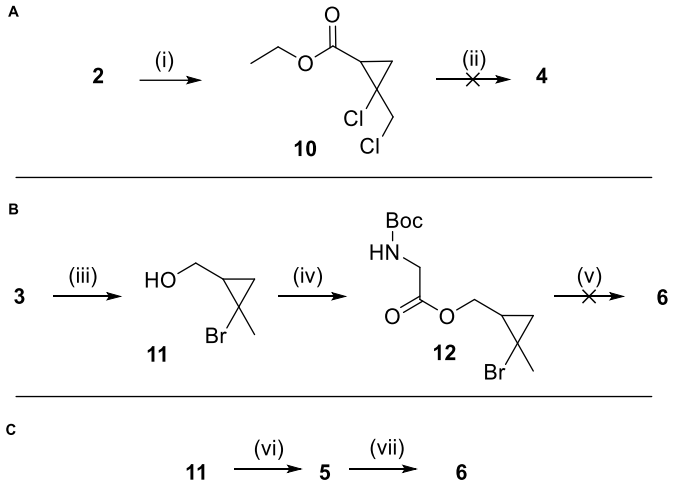


Figure .4. Intramolecular cyclopropanation.

Taking into account the difficulties encountered when attempting to purify the previously described intermediates due to their volatile nature, the synthesis of **4** was designed to proceed through a non-volatile dichloro-derivative (**Scheme 5A**, **10**).[237,240] Unfortunately, the dihalogeno reductive elimination[241] did not occur in this case. Thus, another approach was planned (**Scheme 5B**): the ester **3** was reduced with DIBAL, to furnish a non-volatile alcohol **11**.[229,230] The alcohol was esterified with the Boc-glycine to give the adduct **12**. Unfortunately, the *β*-elimination reaction conducted on **12** did not furnish the desired product **6**; ester hydrolysis occurred instead, giving back the alcohol **10**. In the end, the alcohol derivative **11** was subject to *β*-elimination via NAH in DMSO which furnished the adduct **6** as a pure yellowish oil (**Scheme 5C**).



Scheme 5. Different synthetic approaches. *Reagents and conditions:* (i) 1,2-Dichloropropene, $Rh_2(OAc)_4$ (0.02 mol%), r.t., 15 h; (ii) Zn dust, AcOH, Et_2O, 0 °C or 40 °C, 15 h. (iii) DIBAL(H), dry DCM, -78 °C-r.t., 3 h; (iv) DCC, DMAP, dry DCM, 0 °C-r.t., 15 h; (v) NaH, dry Et_2O, 40 °C, 15 h; (iv) NaH, dry DMSO, -78 °C-r.t., 2 h.

During the preparation of the diazo derivative **8**, the volatile nature of the early intermediates impaired the reaction yields and the purification of the compounds. In fact, while the yield of the cyclopropanation of 2-bromopropene with the commercial ethyl diazoacetate (**2**) was improved by the dilution of the starting materials with dry DCM, the *β*-elimination reaction conducted on the derivative **3** gave a mixture of inseparable compounds despite many attempts to avoid byproduct formation that were made by performing small changes in the reaction conditions. Moreover, the impurities produced in this reaction were not removed in subsequent steps, making the design of a new procedure necessary. Two easy synthetic strategies (**Scheme 5A** and **5B**) were planned with the aim of producing non-volatile and synthetically useful intermediates. Unfortunately, these routes did not furnish the desired compounds. However, alcohol **2**, a key intermediate, was produced as a pure compound by reversing the order of the ester reduction and the *β*-elimination steps (**Scheme 5C**). The diazotization of the free amine of **7** was improved by the addition of a buffer to control the acidic conditions of the reaction, thereby avoiding ester hydrolysis. However, this step needs to be studied in greater depth in order to produce the pure diazo derivative **8** in higher yield. In the end, the intramolecular

cyclopropanation of the diazo derivative **8** mediated by any of the four catalysts failed and spiropentane **1** was not obtained.

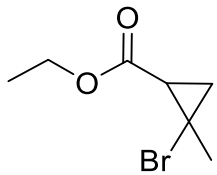
Material and Methods

General chemistry

All commercially available chemicals and solvents were used as purchased. Anhydrous reactions were performed under an inert atmosphere of argon in flame-dried glassware. Tetrahydrofuran, dichloromethane and diethyl ether were dried using a Pure-Solv™ solvent purification system. Absolute ethanol and dry reagents were purchased from commercial suppliers and used without further purification. Reactions were monitored by TLC on Merck silica gel 60 plates, then TLCs were visualized under UV light and stained with acidic ethanolic anisaldehyde solution. Chromatographic purifications were performed on columns packed with silica gel (Fluorochem LC60A, 35–70 micron) under forced flow. ^{1}H NMR and ^{13}C NMR were recorded at 400 and 100 MHz respectively on a Bruker Avance III 400 MHz spectrometer at ambient temperature. Spectra are reported in parts per million (δ scale) and internally referenced to the $CDCl_3$ or D_2O signal, respectively at δ 7.26 ppm and 4.79 ppm. Chemical shifts for carbon are reported in parts per million (δ scale) and referenced to the carbon resonances of the solvent ($CDCl_3$ at δ 77.00). Data are shown as following: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet and/or multiplet resonances, br = broad), coupling constant (*J*) in Hertz (Hz) and integration.

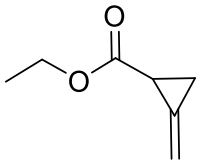
Characterization of synthesized compounds 3-12.

Ethyl 2-bromo-2-methylcyclopropane-1-carboxylate (3)



To a solution of $Rh_2(OAc)_4$ (0.2 mol%) and 2-Bromopropene (10.0 ml, 11.25 mol) in dry DCM (93.5 mL), ethyl diazoacetate **2** (6.0 mL, 5.16 mmol) was added dropwise through a syringe pump with a flux of 0.25 mL/h under argon. The reaction mixture was stirred for additional 3h. Then, the excess of 2-bromopropene and DCM were distilled off at 60°C. The pure compound **3** was obtained as a colorless oil after distillation bulb-to-bulb (Kugelrohr) at 100 °C under vacuum. ^{1}H NMR ($CDCl_3$)δ (ppm): 4.15 (dq, *J* = 7.2, 1.4 Hz, 2H isomer), 4.10 (q, *J* = 7.1 Hz, 2H trans isomer), 2.24 (dd, *J* = 9.3, 6.8 Hz, 2H trans isomer), 1.81 (s, 3H, trans isomer), 1.77 (s, *J* = 3.4 Hz, 1H cis isomer), 1.75–1.64 (m, 2H cis isomer), 1.53 (dd, *J* = 9.3, 6.3 Hz, 1H trans isomer), 1.37 (t, *J* = 6.6 Hz, 1H trans isomer), 1.23 (t, *J* = 7.1 Hz, 1H cis isomer), 1.22 (t, *J* = 7.1 Hz, 1H trans isomer), 1.16 (dd, *J* = 6.9, 4.6 Hz, 1H cis). ^{13}C NMR ($CDCl_3$) δ (ppm): 170.21, 169.30, 61.17, 61.04, 33.32, 32.97, 30.76, 29.61, 28.73, 24.30, 23.87, 22.91, 14.35, 14.27. Yield: 81.3%. Ratio trans/cis: 3/2.

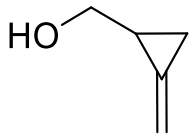
Ethyl 2-methylenecyclopropane-1-carboxylate (4)



To a suspension of NaH (60% in mineral oil;1.255 g, 31.38 mmol, not previously washed) in dry Et_2O (22.0 mL), a solution of compound **3** (3.620g, 17.43 mmol) in dry Et_2O (2.0 mL) was added dropwise via

cannula under argon in an ice bath. Then, the suspension was kindly stirred at 40 °C and a catalytic amount of abs ethanol was added. The brown mixture was stirred at reflux for 16 h. After cooling, the mixture was filtered through a cake of celite and the solid was washed with Et_2O. The yellowish filtrate was concentrated at 45 °C at atmospheric pressure. Then, the bulb-to-bulb distillation under vacuum allowed the isolation of the product **4** as a yellowish oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.55-5.36 (m, 2H), 4.08 (q, *J* = 7.1 Hz, 2H), 2.21-2.17 (m, 2H), 1.77-1.72 (m, 1H), 1.59-1.53 (m, 1H), 1.20 (t, *J* = 7.1 Hz, 3H).^{13}C NMR ($CDCl_3$) δ (ppm): 172.17, 130.31, 104.60, 60.74, 18.12, 14.22, 11.50. Yield: 36.4%.

(2-methylenecyclopropyl)methanol (5)



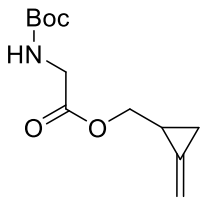
Via reduction of ester **4**:

To a solution of **4** (1.040 g, 8.25 mmol) in dry DCM () DIBAL(H) (1M in DCM; 20.6 mL, 30.6 mmol) was added dropwise under argon at -78 C. After 1 h the reaction mixture was left slowly warm up to r.t. and stir for 3 more hours. Then, NH_4Clss was added dropwise at -78 C and the mixture was left to stir at r.t. for 1 h. Afterward, the aqueous layer was extracted several times with DCM, washed with brine and dried over magnesium sulfate. To minimize the loss of product the obtained organic layer was concentrated using Toxic Buchi at 0 °C reducing the pressure.

Via dehydrohaloelimination of **11**:

To a solution of **11** (1 g, 6.06 mmol) in dry DMSO (10.1 mL), NaH (60% in mineral oil;340 mg, 8.49 mmol, not previously washed) was added portion-wise under argon in an ice bath. Then, the suspension was kindly stirred at 90 °C and a catalytic amount of abs ethanol was added. The dark mixture was stirred for 1 h. After cooling, the mixture was treated with HCl 1 M and extracted with Et_2O. The combined organic layers were washed several times with a solution of LiCl 5% to remove DMSO. Then, they were dried over magnesium sulfate and filtered. Then, the bulb-to-bulb distillation under vacuum allowed the isolation of the product **4** as a yellowish oil. ^{1}H NMR spectrum was identical to those reported by Yu and Lin.[242] Yield: 64%.

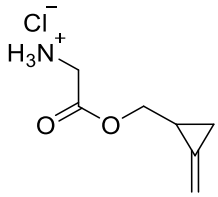
(2-methylenecyclopropyl)methyl (*N*-tert-butoxycarbonyl)glycinate (6)



To the alcohol derivative **5** (206 mg, 2.45 mol) in dry DCM (37 mL), DMAP (30.0 mg, 0.25 mmol) and DCC (557 mg, 2.70 mmol) were added under argon. Then, Boc-glycine (450 mg, 2.57 mmol) was added dropwise at 0 °C as a solution in dry DCM (0.8 mL). The reaction mixture was stirred at r.t. for 3 h. Afterward, the white solid (DCU) was filtered off over cotton and washed with DCM. The yellowish filtrate was concentrated under vacuum, re-dissolved and filtered again to remove the solid residue. The obtained solution was treated with $NaHCO_3$ ss and extracted with DCM. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and evaporated. The product was purified by using silica gel flash chromatography (PE/ Et_2O 8/2) to obtain the adduct as a colorless oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.52-5.51 (m, 1H), 5.48 (d, *J* = 2.1 Hz, 1H), 5.02 (br s, 1H), 4.18 (dd, *J* = 11.4, 6.5 Hz, 1H), 4.01 (dd, *J* = 11.4, 8.3 Hz, 1H), 3.97 (d, *J* = 5.6 Hz, 2H), 1.88-1.80 (m, 1H), 1.41 (tt, *J* = 8.6,

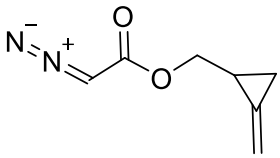
2.1 Hz, 1H), 1.09- 1.4(m, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): δ 170.59, 153.73, 131.75, 105.13, 67.93, 42.47, 28.33, 14.18, 8.89. Yield: 68%.

(2-Methylenecyclopropyl)methyl glycinate hydrochloride (7)



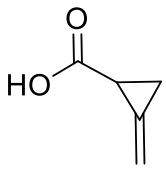
To a solution of compound **6** (200 mg, 0.83 mmol) in Et_2O (5.2 mL), HCl (37%, 3.1 mL) was added at 0 C. The mixture was stirred vigorously for 16h. Then, the mixture was evaporated to give a hygroscopic whitish solid. Yield: 80%. ^{1}H NMR (D_2O) δ (ppm): 5.47-5.46 (m, 1H), 5.42 (d, *J* = 2.1 Hz, 1H), 4.19 (dd, *J* = 11.3, 6.8 Hz, 1H), 4.06 (dd, *J* = 11.3, 8.1 Hz, 1H), 3.87 (s, 2H), 1.87-1.79 (m, 1H), 1.37 (tt, *J* = 9.0, 2.3 Hz, 1H), 1.05-1.01 (m, 1H). ^{13}C NMR (D_2O) δ (ppm): 173.60, 132.31, 104.77, 69.48, 40.10, 13.38, 8.12. Yield: 90%.

(2-methylenecyclopropyl)methyl 2-diazoacetate (8)



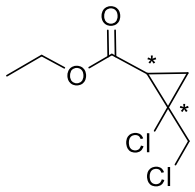
To a solution of amine **7** (160 mg, 0.90 mmol), a solution of NaOAc 0.03 M (300 µL) was added. This mixture was stirred and cooled to -15 °C and pentane (400 µL) was added. A solution of nitric acid was prepared from $NaNO_2$ (99.1 mg, 1.44 mmol) in H_2O (30 µL) and H_2SO_{4conc} (30 µL) at 10 °C. This solution was added to the previously described mixture of **7** at -15 °C. After 10 minutes stirring, additional pentane and water were added and the organic phase was extracted, treated with $NaHCO_3$ to adjust the pH at 7.0 and extracted again. The combined organic layers were dried over magnesium sulfate, filtered and evaporated at 0 °C under vacuum. ^{1}H NMR spectrum showed the characteristic broad signal at 4.70 ppm of the CHN_2, thus, the cleanest compound batches were used for the following step. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.42 (m, 1H), 5.38 (d, *J* = 2.0 Hz, 1H), 4.70 (s, 1H), 4.09 (dd, *J* = 11.4, 6.4 Hz, 1H), 3.93 (dd, *J* = 11.4, 8.2 Hz, 1H), 1.79-1.73 (m, 1H), 1.31 (tt, *J* = 9.0 Hz, 1H), 0.99-0.96 (m, 1H).

2-methylenecyclopropane-1-carboxylic acid (9)



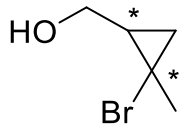
To the ester derivative **8** (480 mg, 3.8 mmol) in MeOH/H_2O 4/1 (5.0 mL), KOH (920 mg, 1.75 mmol) was added at 0 °C. The mixture became more lipid and was stirred at r.t. for 16 h. Then, the volatile byproducts were removed by evaporation at reduced pressure; the white residue was dissolved in water and extracted with Et_2O. Then, the aqueous layers were combined and HCl 2N was added dropwise at 0°C to adjust the pH at 2.0. Then it was retro-extracted with Et_2O, were washed with brine, dried over sodium sulfate, filtered and evaporated. The product was furnished as a yellowish oil.^{1}H NMR ($CDCl_3$) δ (ppm): 5.57-5.56 (m, 2H), 2.29-2.25 (m, 1H), 1.91-1.86 (m, 1H), 1.75-1.69 (m, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 178.03, 129.91, 105.12, 17.74, 12.28. Yield: 75%.

Ethyl 2-chloro-2-(chloromethyl)cyclopropane-1-carboxylate (10)



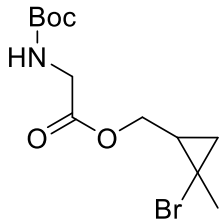
A solution of ethyl diazoacetate (0.5 mL, 4.3 mmol) in dry DCM (100 µL) was added dropwise to a stirred mixture of 2,3-dichloro-1-propene (0.65 mL, 8.0 mmol) and $Rh_2(OAc)_4$ (0.25 mol%) in dry DCM (400 µL) with the aid of a syringe pump at a rate of 0.25 mL/h at r.t. under argon. Then, the excess of 2,3-dichloro-1-propene and DCM were distilled off at 60°C. Water and $KMnO_4$ were added to the residue into an ice bath. The mixture was stirred for 2 hours and excess of $KMnO_4$ was quenched by addition of $Na_2S_2O_3$. The mixture was extracted with Et_2O, the combined organic layers were washed with $NaHCO_3$ ss and brine. Then it was dried over magnesium sulfate, filtered and evaporated to give **10** as a yellow oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 4.26 (two overlapped q, *J* = 7.2 Hz, 4H), 4.11 (dd, *J* = 12.3, 0.7, 1H isomer 1), 4.01 (d, *J* = 12.3 Hz, 1H isomer 1), 3.85-3.78 (m, 1H isomer 2), 2.39 (t, *J* = 8.0 Hz, 1H isomer 1), 2.21 (dd, *J* = 9.2, 7.2 Hz, 1H isomer 2), 1.88 (t, *J* = 4.0 Hz, 1H isomer 2), 1.75 (d, *J* = 8.0 Hz, 2H isomer 2), 1.54 (dd, *J* = 9.2, 6.7 Hz, 2H isomer 1), 1.33 (two overlapped t, *J* = 7.2 Hz, 6H).^{13}C NMR ($CDCl_3$) δ (ppm):169.83, 68.23, 61.65, 61.54, 51.53, 48.64, 46.66, 29.92, 27.63, 24.46, 20.31, 14.13. Yield: 65%.

(2-bromo-2-methylcyclopropyl)methanol (11)



To a solution of **3** (500 g, 2.4 mmol) in dry DCM (1.8 mL) DIBAL(H) (1M in DCM; 6.0 mL, 6.0 mmol) was added dropwise under argon at -78 C. After 1 h the reaction mixture was left slowly warm up to r.t. and stir for 3 more hours. Then, water was added dropwise at -78 C and the mixture was left to stir at r.t. for 1 h. Afterward, the aqueous layer was extracted several times with Et_2O, washed with NH_4Clss and HCl 1 N, dried over magnesium sulfate and filtrated. After evaporation, compound **11** was obtained as yiellowish oil. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.52-5.50 (m, 1H), 5.47-5.45 (m, 1H), 3.65 (dd, *J* = 11.4, 6.5 Hz, 1H), 3.54 (dd, *J* = 11.4, 8.2 Hz, 1H), 1.87-1.77 (m, 1H), 1.35 (tt, *J* = 9.0 Hz, 1H), 1.02-0.97 (m, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 132.89, 104.27, 65.47, 18.06, 8.11. Yield: 76%.

(2-bromo-2-methylcyclopropyl)methyl (*N-tert*-butoxycarbonyl)glycinate (12)



Same procedure for compound **6** furnished compound **12** as colorless oil. ^{1}H NMR ($CDCl_3$ δ (ppm): 4.99 (s, 1H), 4.28 (dd, *J* = 11.9, 6.8 Hz, 1H), 3.98 (dd, *J* = 11.9, 6.8 Hz, 1H), 3.94 (d, *J* = 5.4 Hz, 2H), 1.86 (ddt, *J* = 10.0, 8.8, 6.6 Hz, 1H), 1.78 (s, 3H), 1.46 (s, 9H), 1.38 (dd, *J* = 10.0, 6.6 Hz, 1H), 0.71 (t, *J* = 6.6 Hz, 1H). Yield: 63%.

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Appendix 1

Scientific Production

- Zamperini, C.; Maccari, G.; Deodato, D.; Pasero, C.; D'Agostino, I.; Orofino, F.; De Luca, F.; Dreassi, E.; et al. Identification, Synthesis and Biological Activity of Alkyl- Guanidine Oligomers as Potent Antibacterial Agents. *Sci. Rep.* **2017**, *7* (July), 1–11. doi: 10.1038/s41598-017-08749-6.
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Congress participation

- Poster Presentation at **VII EWDSy** – 7th European Workshop in Drug Synthesis, Siena (Italia), (May 2018): *From a serendipitous discovery to new alkyl-guanidine oligomers as perspective antibacterial agents*, Carolina Pasero, Ilaria D'Agostino, Claudio Zamperini, Davide Deodato and Maurizio Botta.
- Organizing committee member of **VII EWDSy** – 7th European Workshop in Drug Synthesis, Siena (Italy), (May 2018).
- Poster Presentation at **EFMC-YMCS 2017** – 4th EFCM Young Medicinal Chemist Symposium, Vienna (Austria), (September 2017): *From a serendipitous discovery to new alkyl-guanidine oligomers as perspective antibacterial agents*, Carolina Pasero, Ilaria D'Agostino, Claudio Zamperini, Davide Deodato and Maurizio Botta.
- Poster Presentation at **EFMC-ASMC 2017** – 7th edition of EFMC International Symposium on Advances in Synthetic and Medicinal Chemistry, Wien (Austria), (August 2017): *From a serendipitous discovery to new alkyl-guanidine oligomers as perspective antibacterial agents*, Carolina Pasero, Ilaria D'Agostino, Claudio Zamperini, Davide Deodato, and Maurizio Botta.
- Participation to **XI EWDD** – 11th European Workshop in Drug Design, Siena (Italy), (May 2017):
- Poster Presentation at **MYCS** – Merck Young Chemists Symposium, Rimini (Italy), (October 2016): *Synthesis of linear guazatine derivatives as antibacterial agents*, Ilaria D'Agostino, Davide Deodato and Maurizio Botta.
- Poster Presentation at **VI EWDSy** – 6th European Workshop in Drug Synthesis, Siena (Italy), (May 2016): *Fluorescent probe: target fishing of novel antifungal macrocyclic compounds*, Ilaria D'Agostino, Giuseppina I. Truglio, Alexandru Casian and Maurizio Botta.
- Organizing committee member of **VI EWDSy** – 6th European Workshop in Drug Synthesis, Siena (Italy), (May 2016).

Period abroad

- Short-Term Scientific Mission in COST Action **CM1407**, laboratory of Prof. J. Stephen Clark, School of Chemistry, University of Glasgow (UK).

Appendix 2

Part of this PhD thesis has been published as Research Articles:

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SCIENTIFIC REPORTS

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Identification, synthesis and biological activity of alkyl-guanidine oligomers as potent antibacterial agents

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Alkyl-guanidine Compounds as Potent Broad-Spectrum Antibacterial Agents: Chemical Library Extension and Biological Characterization

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