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# **Gemini surfactants derived from serine: synthesis, characterization, and application**

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# Gemini surfactants derived from serine: synthesis, characterization, and application

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## Abstract

In recent decades, the emergence of resistant bacterial strains has increased, becoming something of great concern for society. As existing antibiotics are becoming less effective, it is urgent to develop new compounds with antimicrobial potential.

Among the existing bacteria, *Staphylococcus aureus* is one that has been showing high resistance to antibiotics, making it a target of study. *S. aureus* causes several infections mainly in catheters and transplants due to its ability to form biofilms.

So, due to their history of infections and worsening resistance to conventional antibiotics, we sought to find a viable alternative.

The amino acid-derived gemini surfactants represent a new generation of surfactants, with improved physicochemical (interfacial and self-aggregating) and biological properties when compared to conventional surfactants. The introduction of biomolecules in its structure, such as amino acids, increased its biocompatibility and reduced its toxicity.

The study of properties, namely the *critical aggregation concentration*, size, surface charge and the concentration of surfactants in the form of aggregates are important indicators for the study of biological activity. Thus, the physical properties and biological activity of the two surfactants derived from the amide series (amide 5C and amide 12C) were studied.

In addition to these compounds (amide 5C and amide 12C) varying in terms of spacer size, they also form different aggregation structures, with amide 5C forming micelles and amide 12C forming vesicles. As for the study carried out to evaluate these compounds for their activity, the antimicrobial activity of amide 5C and amide 12C was carried out in three different strains of *Staphylococcus aureus* (*S. aureus* DSM 2569, MRSA ATCC 33592, *S. aureus* ATCC 6538 - biofilm formation) in the planktonic state and in a biofilm structure. The two surfactants were shown to be bactericidal against the three strains of *S. aureus*, since after 24 hours the bacteria was dead. Only amide 5C, in the MRSA ATCC 33592 strain, was shown to have better activity, as only 5.08 µg/mL is needed to obtain the minimum bactericidal concentration. In the other strains for the two compounds, a concentration of 10.16 µg/mL was required. Both surfactants caused a decrease in biofilm viability, although amide 5C was more effective than amide 12C on planktonic bacteria, the difference between the two compounds was not significant. Thus, both compounds demonstrated activity of *S. aureus* in its planktonic state and in the biofilm structure.

After these promising results, studies on the effect of these compounds on hemolytic and cytotoxic activities were carried out. Both compounds have shown promising properties for use as antimicrobial agents.

**Keywords:** gemini surfactants, serine, synthesis, characterization, antimicrobial agents, anti-biofilm agents, *Staphylococcus aureus*

## Resumo

Nas últimas décadas o surgimento de estirpes bacterianas resistentes acentuou-se tornando-se algo bastante preocupante para a sociedade. Uma vez que, os antibióticos existentes estão a tornar-se menos eficazes, torna-se urgente desenvolver novos compostos com potencial antimicrobiano.

Entre as bactérias existentes, a *Staphylococcus aureus* é uma das que tem vindo a demonstrar elevada resistência a antibióticos, tornando-se um alvo de estudo. A *S. aureus* provoca várias infeções principalmente em cateteres e transplantes devido a sua capacidade em formar biofilmes.

Assim, devido ao seu historial de infeções e do agravamento da resistência aos antibióticos convencionais, procurou-se encontrar uma alternativa viável.

Os tensioativos gemini derivados de aminoácidos representam uma nova geração de tensioativos, com melhoradas propriedades físico-químicas (interfaciais e de auto-agregação) e biológicas quando comparados aos tensioativos convencionais. A introdução de biomoléculas na sua estrutura, tais como aminoácidos, veio aumentar a sua biocompatibilidade e diminuir a sua toxicidade.

No presente projeto sintetizou-se três novas séries de tensioativos derivados de serina: a série das amidas, dos ésteres e das aminas. Em todos os compostos, a cadeia alifática foi introduzida no grupo hidroxila da serina e o espaçador foi introduzido entre os grupos de ácido carboxílico (série amida e éster) ou os grupos amino (série amina). A síntese da série das amidas foi realizada com sucesso, obtendo-se compostos com espaçadores de cinco e doze carbonos. Para as séries éster e amina, os tensioativos gemini catiónicos de amónio foram obtidos, entretanto, a transformação desses sais nos correspondentes sais quaternários de amónio ainda não teve sucesso.

O estudo das propriedades, nomeadamente a concentração de agregação crítica, tamanho, carga superficial e a concentração dos tensioativos em forma de agregados são indicadores importantes para o estudo da atividade biológica. Assim, foram estudados quanto as propriedades físicas e quanto atividade biológica os dois tensioativos derivados da série das amidas (amida 5C e amida 12C).

Para além destes compostos (amida 5C e amida 12C) variarem em termos do tamanho do espaçador, também formam estruturas de agregação diferentes, sendo que a amida 5C forma micelas e a amida 12C forma vesículas. Quanto ao estudo realizado para avaliar estes compostos quanto a sua atividade, foi feita a avaliação da atividade antimicrobiana da amida 5C e da amida 12C em três diferentes estirpes de *Staphylococcus*

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Os dois surfactantes mostraram-se bactericidas contra as três estirpes de *S. aureus*, uma vez que, após 24 horas a bactéria estava morta. Apenas a amida 5C, na estirpe MRSA ATCC 33592, mostrou ter melhor atividade, pois apenas 5,08 ug/mL são necessários para obter a concentração bactericida mínima. Nas outras estirpes para os dois compostos, foi necessária uma concentração de 10,16 µg/mL.

Ambos os surfactantes causaram uma diminuição na viabilidade do biofilme, embora a amida 5C tenha sido mais eficaz do que a amida 12C em bactérias planctônicas, a diferença entre os dois compostos não foi significativa. Assim, ambos os compostos demonstraram atividade da *S. aureus* no seu estado planctônico e na estrutura do biofilme.

Após estes resultados promissores, estudos sobre o efeito desses compostos nas atividades hemolítica e citotóxica foram realizados.

Ambos os compostos têm demonstrado propriedades promissoras para serem utilizados como agentes antimicrobianos.

**Palavras-chave:** tensioativos gemini, serina, síntese, caracterização, agentes antimicrobianos, agentes anti-biofilmes, *Staphylococcus aureus*

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## List of symbols and abbreviations

|                  |                                                                                                |
|------------------|------------------------------------------------------------------------------------------------|
| $^1\text{H-NMR}$ | Proton nuclear magnetic resonance                                                              |
| AASs             | Amino acid-based surfactants                                                                   |
| AMPs             | Antimicrobial Peptides                                                                         |
| API              | Atmospheric pressure ionization                                                                |
| aq               | Aqueous                                                                                        |
| Boc              | <i>tert</i> -butyloxycarbonyl group                                                            |
| CA-MRSA          | Community-associated Methicillin-resistance <i>Staphylococcus aureus</i>                       |
| <i>cac</i>       | <i>Critical aggregation concentration</i>                                                      |
| CCD              | Charge-Coupled Device                                                                          |
| CFU              | Colony-forming units                                                                           |
| CLSM             | Confocal laser scanning microscopy                                                             |
| COMU             | (1-Cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate |
| cr               | hydrated crystals                                                                              |
| CV               | Crystal violet                                                                                 |
| DCE              | Dichloroethane                                                                                 |
| DCM              | Dichloromethane                                                                                |
| DIEA             | <i>N,N</i> -Diisopropylethylamine                                                              |
| DMEM             | Dulbecco's Modified Eagle Medium                                                               |
| DMF              | <i>N,N</i> -Dimethylformamide                                                                  |
| DNA              | Deoxyribonucleic acid                                                                          |
| DOPE             | Dioleoylphosphatidylethanolamine                                                               |
| DTAB             | Dodecyltrimethylammonium bromide                                                               |
| ELS              | Electrophoretic Light Scattering                                                               |
| eq               | equivalents                                                                                    |
| ESI              | Electrospray ionization                                                                        |
| ESI-MS           | Electrospray Ionisation Mass Spectrometry                                                      |

|                  |                                                                          |
|------------------|--------------------------------------------------------------------------|
| EU/EEA           | European Union/European Economic Area                                    |
| FBS              | Fetal bovine serum                                                       |
| Fmoc             | 9-Fluorenylmethyloxycarbonyl group                                       |
| GrlA             | DNA topoisomerase (ATP-hydrolyzing)                                      |
| GyrA             | DNA gyrase subunit A                                                     |
| h                | hour                                                                     |
| HA-MRSA          | Hospital-acquired Methicillin-resistance <i>Staphylococcus aureus</i>    |
| HEPES            | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid                       |
| HLB              | Hydrophilic-lipophilic balance                                           |
| hRBC             | Human red blood cells                                                    |
| Hz               | Hertz                                                                    |
| IC <sub>50</sub> | Median inhibitory concentration                                          |
| <i>J</i>         | Coupling constants                                                       |
| L                | Isotropic solution phase                                                 |
| L $\alpha$       | lamellar phase                                                           |
| LA-MRSA          | Livestock-associated Methicillin-resistance <i>Staphylococcus aureus</i> |
| M                | Molarity                                                                 |
| MBC              | Minimum Bactericidal Concentration                                       |
| MBEC             | Minimum Biofilm Eradication Concentration                                |
| MBIC             | Minimum Biofilm Inhibitory Concentration                                 |
| MeOH             | Methanol                                                                 |
| MHB              | Müller-Hinton Broth medium                                               |
| MHC-II           | Major histocompatibility complex – class II                              |
| MHz              | Mega-hertz                                                               |
| MIC              | Minimum Inhibitory Concentration                                         |
| MRCRAMMs         | Microbial Surface Components Recognizing Adhesive Matrix Molecules       |
| MRSA             | Methicillin-resistance <i>Staphylococcus aureus</i>                      |
| MSSA             | Methicillin-sensitive <i>Staphylococcus aureus</i>                       |
| mV               | millivolt                                                                |
| nm               | nanometer                                                                |

|                  |                                                                               |
|------------------|-------------------------------------------------------------------------------|
| NMO              | <i>N</i> -Methylmorpholine <i>N</i> -oxide                                    |
| NMR              | Nuclear magnetic resonance                                                    |
| NTA              | Nanoparticle tracking analysis                                                |
| PALS             | Light Scattering Phase Analysis                                               |
| PBS              | Phosphate-buffered saline                                                     |
| PI               | Propidium iodide                                                              |
| ppm              | Parts per million                                                             |
| PyBOP            | Benzotriazole-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate         |
| Quats            | Quaternary ammonium salts                                                     |
| R <sub>f</sub>   | Chromatographic retention factor                                              |
| RNA              | Ribonucleic acid                                                              |
| rt               | Room temperature                                                              |
| <i>S. aureus</i> | <i>Staphylococcus aureus</i>                                                  |
| SD               | Standard deviation                                                            |
| SDS              | Sodium dodecylsulfate                                                         |
| STI-like         | Sexually transmitted infections                                               |
| T3P              | 2,4,6-Tripropyl-1,3,5,2,4,6-trioxatriphosphorinane-2,4,6-trioxide solution    |
| TBTU             | O-(Benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium tetrafluoroborate |
| TFA              | Trifluoroacetic acid                                                          |
| TLC              | Thin-layer chromatography                                                     |
| TMSCl            | Trimethylsilyl chloride                                                       |
| TMU              | Tetramethylurea                                                               |
| TPAP             | Tetrapropylammonium perruthenate                                              |
| UK               | United Kingdom                                                                |
| USA              | United States of America                                                      |
| v/v              | volume-volume                                                                 |
| VISA             | Vancomycin Intermediate <i>Staphylococcus aureus</i>                          |
| VRSA             | Vancomycin Resistant <i>Staphylococcus aureus</i>                             |
| w/v              | Weight-volume                                                                 |
| ZP               | Zeta-potential                                                                |

|           |                 |
|-----------|-----------------|
| $\beta$   | Beta            |
| $\delta$  | Chemical shifts |
| $\gamma$  | Surface tension |
| $\lambda$ | Wave-length     |
| $\eta$    | Yield           |

## 1. Objectives and organization of the thesis

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## 1.1. Motivation and objectives of the project

In recent decades, the resistance of bacteria to antibiotics with the emergence of resistant strains has increasingly become a problem for society. As existing antibiotics are becoming ineffective, there is an urgent need to develop new compounds with bactericidal potential.<sup>1</sup>

Among several strategies, there are some examples in the literature of gemini surfactants derived from amino acids that have antimicrobial activity on gram-positive (*Micrococcus luteus*, *Bacillus cereus*, *Staphylococcus aureus*) and gram-negative (*Klebsiella pneumoniae*, *Escherichia coli*) bacteria.<sup>2</sup> However, and despite their reported efficacy, the progress has been very slow and yet no clinical treatment is available.<sup>2</sup>

Particularly, *Staphylococcus aureus* is the leading cause of hospital-acquired infections, therefore, it is recognized as an opportunistic and adaptable pathogen with the ability to infect, invade, persist and replicate in any human tissue, including skin, bone, visceral organs or vasculature.<sup>3</sup> Only in 2017, 119 000 people had *Staphylococcus aureus* bloodstream infections in the United States. Of that number, almost 20,000 people died.<sup>4</sup>

Thus, antimicrobial resistance is a serious problem. Effective bacterial control through extensive monitoring and careful use of antibiotics is required. In addition, it is being important to discover new compounds with antimicrobial capacity particularly against resistant strains to common antibiotics.<sup>1</sup>

This project has as its general objective the design, synthesis and application of three new families of serine-based surfactants as antimicrobial agents. In all the compounds the aliphatic chain will be introduced at the hydroxyl group of serine and the spacer will be introduced either between the carboxylic acid groups (amide and ester series) or the amino groups (amine series). The different series obtained will allow the establishment of relationships between the compounds structure, physico properties and biological activity.

The proposed work plan involved the following tasks:

1. Design and synthesis of three series of gemini surfactants using spacers with different chain length.

2. Evaluation of the physical properties of the synthesized gemini quats surfactants, namely (i) determination of interfacial parameters by tensiometry, (ii) determination of phase mapping by optical microscopy and (iii) determination of size and charge of aggregates by nanoparticle tracking analysis technique and electrophoretic light scattering, respectively.

3. Evaluation of the antimicrobial activity of all compounds in three different strains of *Staphylococcus aureus* (*S. aureus* DSM 2569, MRSA ATCC 33592, *S. aureus* ATCC 6538) in the planktonic state and a biofilm structure.

4. Evaluation of the toxicity of all compounds in a reference human fibroblast cell line (L929) and evaluation of their haemolytic activity.

## 1.2. Structure

This thesis is outlined in 6 chapters.

Chapter 1 includes the motivation for the work, general objectives and a description of the organization and structure of the thesis.

A general discussion of the properties of surfactants, in particular of the amino acid-derived gemini surfactants, is found in Chapter 2. Chapter 3 describes the principals' methodologies used during the course of laboratory work.

In chapter 4, the synthesis of gemini compounds as well as the evaluation of the physicochemical properties will be presented and discussed. In chapter 5, the characterization of gemini surfactants and the evaluation of the antimicrobial activity and toxicity of the compounds are described.

Chapter 6 corresponds to the final considerations and future perspectives, that is, it consists of a brief conclusion about all the studies carried out in this dissertation and some proposals for future work to continue the work carried out.

The current project was carried out at FFUP in the MB2 “Molecular Biophysics and Biotechnology” part of Nanoplatfoms for Life group of LAQV REQUIMTE, at FFUP, research group, under the supervision of Professor Maria de la Salette Reis and co-supervision of Professor Cláudia Nunes and Doctor Catarina Seabra. The project was performed in collaboration with Professor Luísa Vale and Doctor Sandra Silva members of the research group ORCHIDS “Organic Chemistry-Inspired Design and Synthesis of Bioactive Molecules” part of the Molecular Synthesis group of LAQV REQUIMTE, at FCUP.

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## 2. Introduction

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## 2.1. Surfactants overview

Surfactants are a class of organic molecules with great importance in society, as they are present in the constitution of several products daily used, such as detergents, industrial oils, cosmetics, pharmaceutical formulations, food additives and agrochemicals, among others. Moreover, surfactants are also present in the human body. Phospholipids e.g. are the main components of the cell membranes and sustain life by providing a scaffold for cells and are responsible for the regulation of the permeation of compounds and regulate some chemical reactions.<sup>1, 2</sup>

The first surfactants were soaps, that were obtained from saponification. They were produced from natural raw materials, commonly animal fats and ashes, in order to obtain carboxylates of fatty acids. It was only later that synthetic surfactants began to appear as substitutes for soaps – known as detergents.<sup>2, 3</sup>

These compounds are amphiphilic molecules because they have in their structure two regions of different polarity (**Figure 2.1.**): a polar headgroup (hydrophilic) and a nonpolar (lipophilic) region, consisting generally of a long alkyl chain.<sup>4</sup>

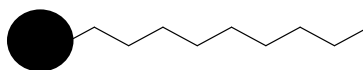


**Figure 2.1.** Illustration of molecular surfactant structure. Adapted from <sup>4</sup>. (Created using ChemDraw® 20.0).

Due to their amphiphilic nature, surfactants have two fundamental properties: they have the ability (1) to adsorb at interfaces and (2) to self-assemble in solution, forming different types of colloidal assemblies.

Considering the ability to adsorb at interfaces in a water/air interface, there is a limit to the reduction of the interfacial tension that is normally reached when the solution is saturated with monomers. At this point occurs, the surfactant concentration reaches a critical value called *critical aggregation concentration (cac)* and the surfactant starts to aggregate in solution. This aggregation process is called surfactant self-assembly. When in solution at different concentrations, amphiphiles can form different aggregates (**Figure 2.2.**) An ideal phase sequence would be the micellar solution – cubic phase – hexagonal phase – cubic phase – lamellar phase – reverse phases (hexagonal, cubic and micellar). However, this sequence is not observed to the full extent for all surfactant systems. For example, single-chained surfactants which are characterized by having one polar group attached to one long alkyl chain tend to form, as first aggregates micelles, while double-

chained monomeric surfactants, consisting of two alkyl chains linked to the polar head group, are known to form bilayer structures (liposomes).<sup>2</sup>

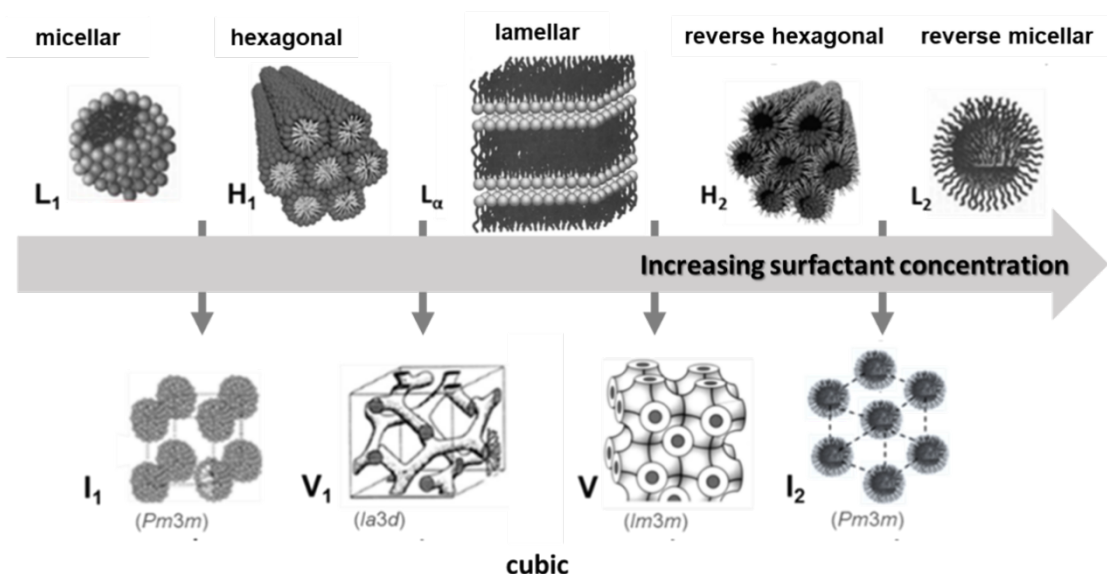


**Figure 2.2.** The Fontel scheme: the ideal sequence of self-assembly of surfactants.

Legend: L<sub>1</sub> – micellar phase, H<sub>1</sub> – hexagonal phase, L<sub>α</sub> - lamellar phase, H<sub>2</sub> - reverse hexagonal phase, L<sub>2</sub> – reverse micellar phase, I<sub>1</sub> and I<sub>2</sub>- cubic arrangement of globular micelles, V<sub>1</sub> and V- cubic arrangement of bicontinuous structure. Adapted from <sup>4, 5</sup>

Surfactants can be also classified according to the nature of their polar group as anionic, cationic, non-ionic or zwitterionic. Each of these classes contains typical functional groups in the polar region of the amphiphilic molecule and interfacial properties of different nature.<sup>4</sup> In **Figure 2.3.** some representative examples of each class of surfactants are presented.

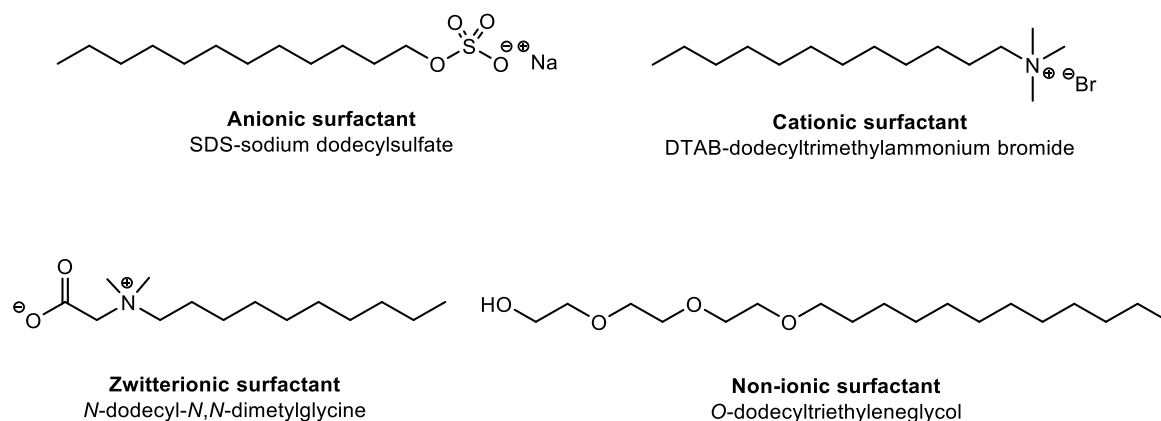


**Figure 2.3.** Examples of surfactants of the different classes according to the nature of their polar group as anionic, cationic, non-ionic or zwitterionic. Adapted from <sup>6</sup>. (Created using ChemDraw® 20.0).

Anionic surfactants possess a negatively charged polar headgroup. The most common anionic surfactants are carboxylates, sulfates, sulfonates and phosphates. The counterions may be sodium, potassium, ammonium, calcium and several protonated alkyl amines.<sup>4</sup> Most of these surfactants find use in detergent and cosmetic formulations. From all the classes of surfactants, these are the most used and are less expensive due to their easy preparation.<sup>7</sup> For example, alkyl/alkenyl carboxylates (also known as soaps) can be readily prepared by saponification of triglycerides obtained from natural oils (usually tallow, coconut and palm) or fats. However, one of the major problems of carboxylates is their low hard water stability. Numerous attempts have been made to develop more soluble and less corrosive substitutes, which resulted in the emergence of alkyl benzene sulfonates, alkyl sulfates and phosphates.

Concerning cationic surfactants, the majority are long-chain alkyl amines (which become positively charged in acid media) and quaternary ammonium salts.<sup>4</sup> Due to their positively charged head group, cationic surfactants strongly adsorb at negatively charged surfaces, such as tissues, hair and bacterial cell membranes. In this way, they can be used as dispersants, hair conditioners, antibacterial agents and more recently their potential as gene transfection agents has also been described.<sup>1, 2, 7</sup>

Non-ionic surfactants are uncharged amphiphilic molecules that may present either a polyether or a polyhydroxyl unit as a polar headgroup. The polyethers are constituted by several oxyethylene groups obtained by polymerization of ethylene oxide.<sup>4, 7</sup> These amphiphiles represent the second most used class of surfactants, finding applications in the food and pharmaceutical industries and in personal health care products. These compounds are not sensitive to hard water (contrary to anionic surfactants) and present high detergency and foaming capacity.<sup>4</sup>

Zwitterionic surfactants have both a positive and a negative charge in the polar headgroup and thus are electrically neutral. The cationic charge is due to a quaternary ammonium group, while the negative charge is most frequently associated with a carboxylate or phosphate group. These surfactants are generally pH-sensitive, especially those which are amphoteric in nature. From all classes of surfactants, zwitterionic surfactants are less studied and less used due to their higher cost of preparation. Zwitterionic amphiphiles are not harmful to the skin or irritating to the eyes and are also not sensitive to hard water. Moreover, due to their relevant dermatological properties, they are widely used in personal care products.<sup>4, 7</sup>

In the last decades, the increasing demand for surfactants (**Figure 2.4.**), with improved properties in relation to those already existing on the market, led to the emergence of new types of surfactants, the bolaform, and gemini surfactants.<sup>6</sup>

The bolaforms are made up of two polar groups joined by an aliphatic chain.<sup>6</sup> Gemini surfactants are slightly more complex molecules, as they have two polar head groups linked to two non-polar alkyl chains, and are covalently linked, usually at the level of the polar groups, by a spacer of variable size and flexibility.<sup>8</sup> These latter ones will be described in more detail as they are the subject of the present dissertation.

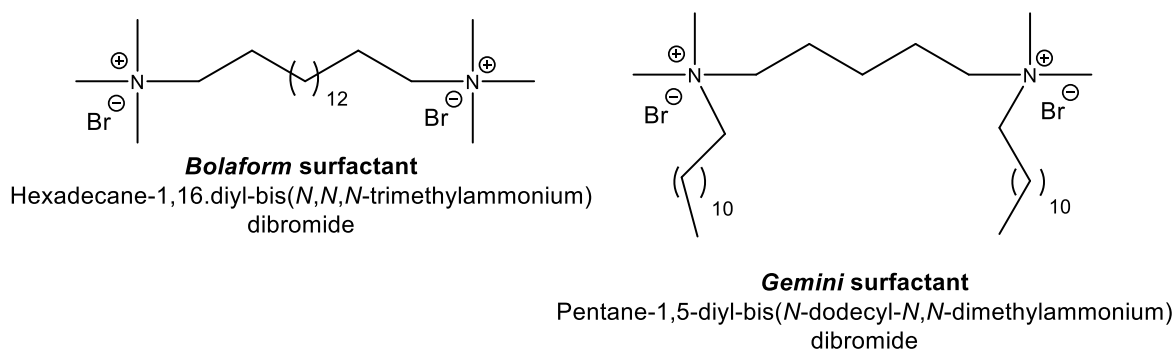


**Figure 2.4.** Examples of new types of surfactants, the bolaform, and gemini surfactants, are described in the literature. Adapted from <sup>8</sup>. (Created using ChemDraw® 20.0).

## 2.2. Gemini surfactants

Gemini (or dimeric) surfactants have been first reported in 1971 by Bunton *et al.* and named "Gemini" in 1991 by Menger and Littau.<sup>9, 10</sup>

Gemini surfactants are most interesting because of their enhanced interfacial/aggregation behaviour in comparison with monomeric analogues. These compounds exhibit a number of unique properties that result from their structure: their *critical aggregation concentration (cac)* is usually 1 to 2 orders of magnitude lower than that of the corresponding monomers; they are more efficient at lowering surface or interfacial tension compared to conventional surfactants; they present better solubilizing power, better viscoelasticity and better gelification ability.<sup>2, 8</sup>

As previously mentioned, gemini surfactants possess two hydrophilic tails and two hydrophobic headgroups connected by a rigid or flexible spacer near or at the headgroups (**Figure 2.5.**). When both hydrophobic parts and hydrophilic groups are identical, the gemini surfactant forms a symmetrical structure.<sup>8</sup>

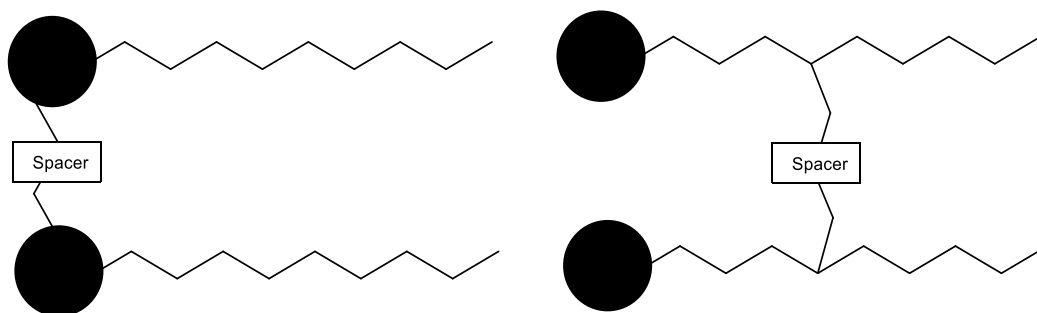


**Figure 2.5.** Structure of gemini surfactants with spacer at the headgroups (left) and near the headgroups (in the hydrophobic part) (right). Adapted from <sup>8</sup>. (Created using ChemDraw® 20.0).

There are also heterogeminis which consist of two equal or different polar headgroups attached to two different or equal hydrophobic groups (**Figure 2.6.**).<sup>4, 8</sup>

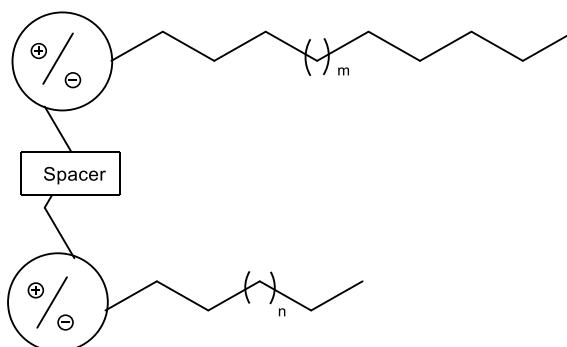
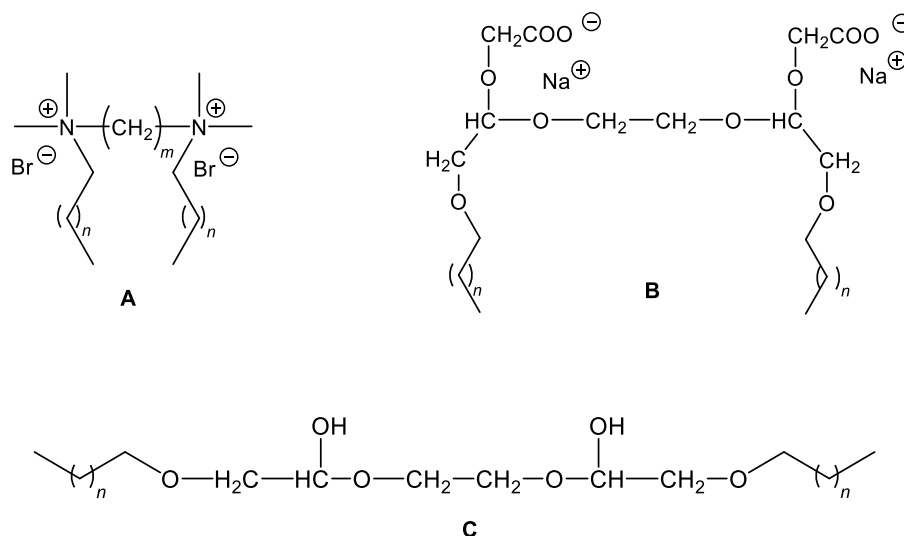


**Figure 2.6.** Structure of heterogemini surfactants. Adapted from <sup>8</sup>. (Created using ChemDraw® 20.0).

Like monomeric surfactants, gemini surfactants can be classified as cationic (quaternary ammonium ions), anionic (carboxylates, sulphates, phosphates), non-ionic (polyethers) or zwitterionic. **Figure 2.7.** are depicted some representative examples of gemini surfactants described in the literature.



$n$  = number of methylene groups the chain  
 $m$  = number of methylene groups in the spacer

**Figure 2.7.** Examples of gemini surfactants are described in the literature. Cationic gemini (A), Anionic gemini, (B) Non-ionic gemini containing oxyethylene spacer (C). Adapted from <sup>5</sup>. (Created using ChemDraw® 20.0).

Amongst all the gemini surfactants, quaternary ammonium salts, also known as *bis-quats*, are the most widely studied (**Figure 2.7A**).<sup>11</sup> They are known to present antimicrobial activity showing a broad spectrum of action; they are described to be able to inhibit the growth of bacteria, yeasts, fungi and virus.<sup>11, 12</sup> The mechanism via which these cationic gemini amphiphiles disrupt the bacterial membrane has been described as a combination of electrostatic and hydrophobic interactions. Initially, the surfactants adsorb onto the negatively charged bacteria membrane and then, their alkyl chains create pores and channels leading to severe damage or death of bacteria.<sup>13-15</sup>

Also, these surfactants have been evaluated as drug/gene delivery systems. Their use as DNA transfection agents has become widespread ever since it was shown that cationic lipids, known as cytofectins, were capable of delivering functional genes. It has been proved that bis-quats bind and compact DNA efficiently; however, their applicability is somewhat limited by their toxicity profiles.<sup>13, 16</sup> To overcome this issue, great effort has been devoted to the development of increasingly biocompatible amphiphiles and several gemini surfactants derived from sugars, amino acids and peptides have been designed and synthesized, with many of them showing attractive biological features.<sup>11, 17, 18</sup>

### 2.3. Surfactants derived from amino acids

Surfactants containing natural structural motifs, such as amino acids, sugars and fatty acids are promising compounds, due to their improved biocompatibility, low toxicity, greater biodegradability in the environment, good interfacial performance and interesting self-aggregation properties. In fact, the different industrial applications of surfactants increasingly demand the use of natural and renewable starting materials.<sup>11, 17, 18</sup>

Amino acids are used as preservatives for medical and cosmetic applications.<sup>11</sup> Later, they were shown to be active against various diseases caused by bacteria, viruses and tumors.<sup>19, 20</sup> With this discovery, amino acid-based surfactants (AAS) have become an important class of natural surface-active molecules with great interest in organic and physical chemists, as well as to biologists, due to the diverse number of fundamental and industrial applications they possess.<sup>21</sup> AAS can be synthesized using renewable sources and their ease of degradability and harmless by-products make them safer for the environment. In addition, they present improved surface properties, rapid biodegradation, and excellent antimicrobial and antifungal activities compared to conventional surfactants.<sup>17, 21</sup>

The large variety of amino acid structures allied to the also large variety of fatty acid structures endows the obtention of a great diversity of AAS, with different, and easy to tailor, physicochemical and biological properties (**Figure 2.8.**)

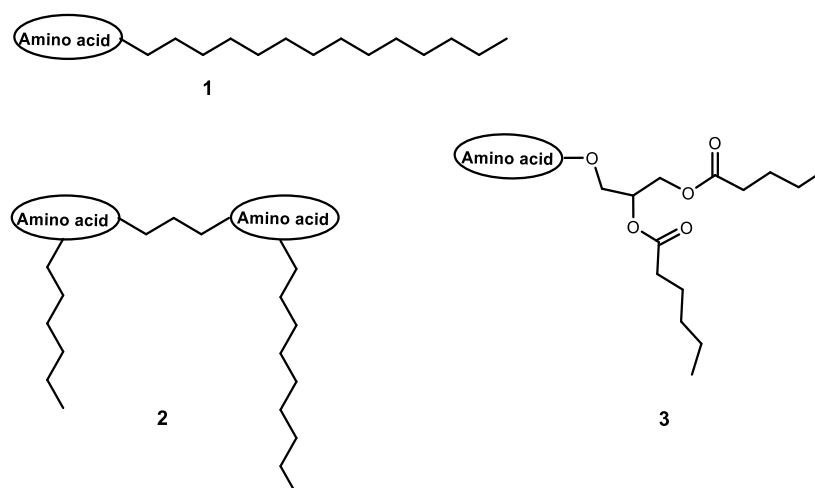


**Figure 2.8.** Schematic representation of some categories of AAS. 1. linear or single-chained, 2. dimeric or gemini and 3. glycerolipid-similar structures. Adapted from <sup>19</sup>. (Created using ChemDraw® 20.0).

The adsorption, aggregation and biological properties depend strongly on the portion of amino acid in the surfactant. Depending on the free functional groups of the amino

acid, cationic, anionic, nonionic and amphoteric surfactants can be obtained. It is the further modifications of these groups (introduction of alkyl chains of variable length, introduction of one or more alkyl chains, type of linkage to the alkyl-amino acid chain) that allow for a fine adjustment in the physicochemical properties to suit various applications, such as antimicrobial activity, drug delivery, transfection vectors.<sup>11</sup>

## 2.4. Biological activity of amino acid-based gemini surfactants

Gemini surfactants derived from amino acids are a family of surfactants with increasing use in cosmetic, pharmaceutical and biomedical applications. Generally, they are non-toxic, environmentally friendly and with excellent biodegradability.<sup>21</sup> Several studies have demonstrated that amino acid-based gemini surfactants present less toxicity and less irritating effects on the skin compared to either the monomeric analogue or conventional surfactants.<sup>17, 18, 20, 22</sup>

Most surfactant toxicity tests found in the literature aim to discover the intrinsic toxicity of surfactants without considering any specific application. Several examples of *in vitro* procedures to evaluate the cytotoxicity of surfactants are found in the literature; among the most mentioned are: hemolytic activity, lactate dehydrogenase release, Alamar blue assay, and cell viability by MTT.<sup>14, 21-29</sup> However, all tests carried out on *in vitro* systems fail to fully predict the effect of these compounds on the human body. It is, therefore, crucial to start *in vivo* testing in order to confirm and guarantee the security of these compounds.<sup>21</sup>

On the other side, the development of new antimicrobial agents has become urgent, due to the rapid growth of bacteria and fungi resistant to existing drugs. In this context, amino acid-based gemini surfactants stand as a promising alternative as antibacterial and antifungal agents. These compounds can present different modes of action and different targets according to their structure, thus becoming more valuable compared to conventional existing antibiotics.<sup>18</sup> Contrary to conventional antibiotics that usually target specific enzymes or DNA, these gemini surfactants possess two alkyl chains that may interact with cell membranes, leading to depolarization, lysis and cell death, probably resulting from advantageous incorporation in the phospholipid bilayer, which hinders bacterial resistance.<sup>18</sup> However, some studies suggest a more complex mechanism of action on membranes. Gemini surfactants are thought to be able to disturb the membrane structure, but not necessarily to break it. Some studies suggest that these compounds penetrate the plasma membrane and are able to enter the cell, interact with the mitochondrial membrane and inhibit respiratory enzymes. It was also proposed that surfactants may be incorporated in the plasma membrane forming pores for influx.<sup>13, 22</sup>

Several authors have demonstrated that lysine and arginine gemini surfactants exhibited antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria.<sup>17</sup> Gemini surfactants based on arginine had strong antibacterial properties mainly in gram-positive bacteria, as mentioned earlier, modulated by the length of the spacer (the longer the spacer, the less the activity)<sup>17</sup>. It was also possible to notice that when the acyl chain was kept constant, antimicrobial activity decreased with long spacer chains, that is, the longer the spacers, the greater the capacity to form viscous solutions involving large aggregates. These structures are unlikely to interact with the membrane, so gemini surfactants with long spacers are less hemolytic.<sup>18</sup> Tavano *et al.* reported a series of cationic surfactants based on arginine, that showed antimicrobial efficiency at concentrations below those in which hemolysis occurs.<sup>30</sup> As for surfactants derived from lysine, Colomer *et al.* studied the influence of the number and position of cationic charges, as well as the number of alkyl chains on antibacterial activity, and concluded that the most active surfactants against bacteria were those with cationic charge in the trimethylated amino group.<sup>21</sup>

Other authors have also reported the existence of a strong antimicrobial activity exhibited by glycine and alanine derivatives. In a study of alanine-derived surfactants, the authors, when evaluating the effects of the counterion, concluded that bromides were more effective when compared to chlorides. These compounds inhibited the growth and viability of bacteria (for example, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*) and yeast-like fungi (*Candida albicans* and *Rhodotorula mucilaginosa*), and surprisingly, they were highly active against *Pseudomonas aeruginosa* - species commonly resistant to various antibacterial compounds.<sup>13</sup>

There is a significant difference in the tolerance to surfactants between Gram-positive and Gram-negative bacteria. Gram-positive bacteria tend to be more sensitive, while Gram-negative bacteria are often resistant to these compounds. This fact is due to the many modifications of the external membrane of Gram-negative bacteria which contribute to the active efflux of surfactants, leading to resistance to drugs and disinfectants.<sup>13</sup> Furthermore, the antimicrobial activity generally increases in the presence of hydroxyl groups, cyclopropane residues or unsaturation in the acyl chain. Castillo *et al.* stated that the length of the acyl chains and the positive charge that determine the molecules' hydrophilic-lipophilic balance (HLB) affect their membrane-breaking properties.

In general, amino acid-based on gemini surfactants are promising compounds for biomedical applications and many were shown to present a broad spectrum of antimicrobial activity while being non-cytotoxic.<sup>14, 20-29</sup>

## 2.5. *Staphylococcus aureus*

Recently, with the increase in the number of organ and marrow transplants, as well as invasive diagnostic tests, sepsis events are on the rise. *S. aureus* ranks first among bacteria causing bloodstream infections and is the leading cause of nosocomial pneumonia. *S. aureus* is the leading cause of hospital-acquired infections, therefore, it is recognized as an opportunistic and adaptable pathogen with the ability to infect, invade, persist and replicate in any human tissue, including skin, bone, visceral organs or vasculature.<sup>31</sup>

The resistance of bacteria to antibiotics has been recognized since the development of the first antibiotics, however, in the last few decades, the emergence of resistant strains has become more and more of a problem for society. Since existing antibiotics are becoming ineffective, there is an urgent need to develop new compounds with bactericidal potential.<sup>32</sup>

### ***Staphylococcus aureus* in Europe**

Surveillance data from European countries show that the percentage of Methicillin-resistant *Staphylococcus aureus* (MRSA) weighted by the EU/EEA population is decreasing, however, there is a general upward trend in the incidence of MRSA (**Figure 2.9.**). It is believed that various infection control practices and the use of antimicrobials contribute to the observed differences.<sup>33</sup>

Despite this positive development, MRSA remains an important pathogen in Europe. *S. aureus* is one of the most common causes of bloodstream infections, exhibiting a high burden in terms of morbidity and mortality.<sup>33</sup>

In order to slow the spread of MRSA in Europe, comprehensive MRSA strategies targeting all health sectors remain essential. Monitoring for MRSA in animals and food is currently voluntary and only performed in a limited number of countries. Recently, LA-MRSA has gained increasing attention as it poses a zoonotic risk, especially for those working in close contact with livestock.<sup>33, 34</sup>

A 2019 study showed a prevalence of MRSA of around 34.8% in Portugal, thus revealing a trend towards a decrease in the prevalence of MRSA. However, the prevalence of MRSA in Portugal is worrying and studies indicate that it will increase if the proper infection control precautions and proper prescription of antibiotics are not taken. It is worth emphasizing the importance of these surveillance studies so that the burden of MRSA in Portugal is known and that the necessary precautions are taken.<sup>33</sup>

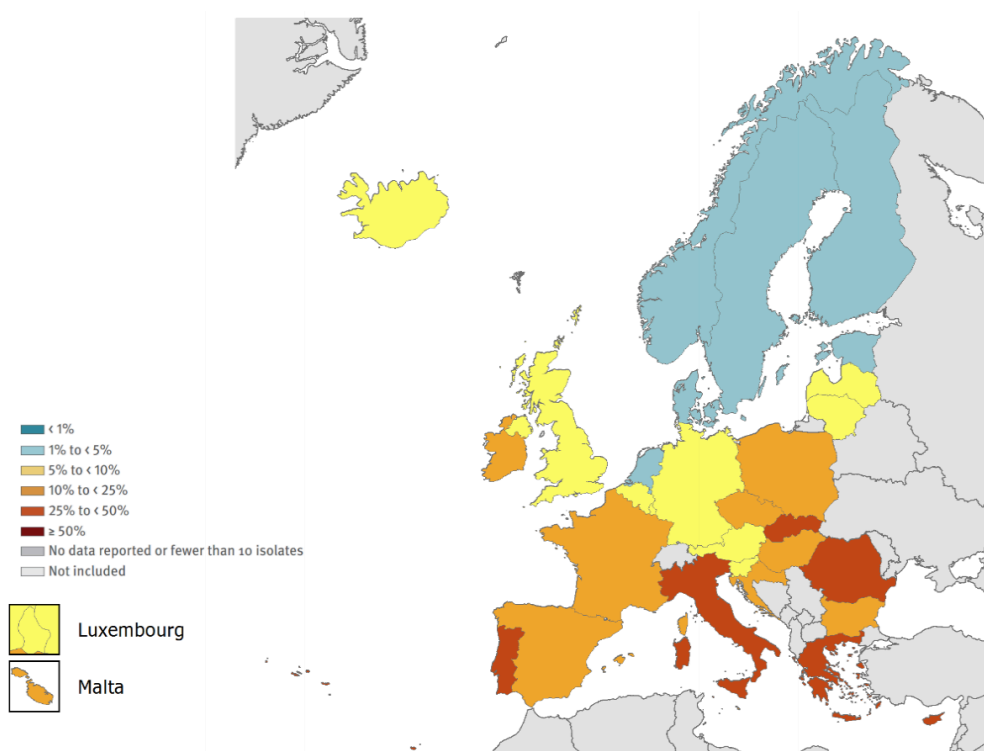


**Figure 2.9.** *Staphylococcus aureus*. Percentage of invasive isolates resistant to methicillin (MRSA), by country, EU/EEA, 2019. From <sup>33</sup>.

## Morphology and structure

The genus *Staphylococcus* belongs to the bacterial family *Staphylococcaceae*. Members of the *Staphylococcus* genus inhabit the skin, skin glands and mucous membranes of humans, animals and birds. *S. aureus* are Gram-positive and have a spherical coccoid shape with sizes ranging from 0.5 to 1.5  $\mu\text{m}$  in diameter and a smooth surface. It is a facultative aerobic and anaerobic organism that forms large yellow or white colonies on nutrient-rich agar media.<sup>35, 36</sup>

*S. aureus* strains have two states: planktonic and biofilm states with different specifications.<sup>36</sup>

## Colonization and virulence factors

Normally, the human host is infected by bacteria that colonize its skin or mucosal surface, such as *S. aureus*. Colonization by *S. aureus*, in most cases, precedes the development of the infection. Infections that occur without known colonization by these bacteria are less frequent and are usually induced by contamination of catheters or open surgical wounds because of poor compliance with infection control protocols.<sup>34</sup>

Some studies have identified three temporal patterns of *S. aureus* colonization, including Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA).<sup>34</sup> Persistent colonization was found in 15% of individuals, intermittent colonization was present in about 70% of participants and in 15% the bacteria was not found.<sup>34</sup>

*S. aureus* has numerous virulence factors (**Figure 2.10**) that vary from strain to strain, a variability that is reflected in the high diversity of infections that this bacterium can cause. Most genes expressing these virulence factors are present in mobile genetic elements, thus their combination differs substantially between strains and even closely related clones. Many virulence factors remain, in a way, to be investigated, as they are specific to humans, and it is not possible to study them in an animal model.<sup>34, 36</sup>

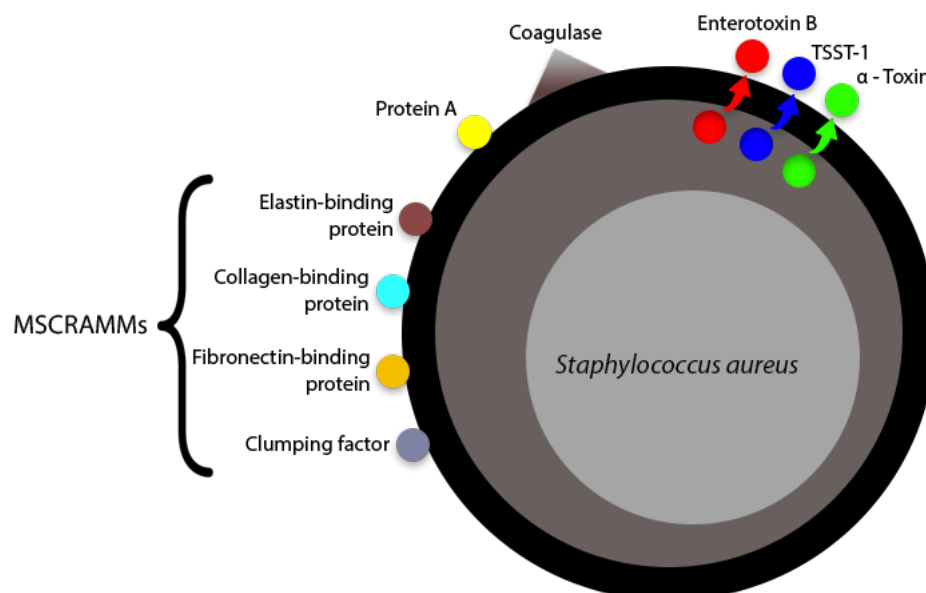


**Figure 2.10.** Virulence factors of *S. aureus*. Adapted from <sup>37</sup>.

These factors allow the organism to succeed as a pathogen, as it causes a wide range of infections in humans and animals. Virulence factors aid in host cell binding, breaking the host's immune shield, tissue invasion, inhibiting phagocytosis and toxin-mediated host tissue damage. This is the basis for persistent *staphylococcal* infections without a strong host immune response. *Staphylococcal* virulence factors are classified as shown in the **Table 2.1**.<sup>36, 38</sup>

**Table 2.1.** Virulence factors produced by *S. aureus*. Adapted from <sup>34, 39</sup>.

| Virulence factors                                                                | Activity in host tissue                                                                                                                               |
|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cell wall components                                                             |                                                                                                                                                       |
| Microbial surface components that recognize adhesive matrix molecules (MSCRAMMs) | Cell wall proteins that interact with host molecules such as collagen, fibronectin, fibrinogen, and elastin. These facilitate adhesion to host tissue |
| Polysaccharide capsule                                                           | Inhibits phagocytosis                                                                                                                                 |
| Protein A                                                                        | Binds to the fragment crystallizable region of the immunoglobulin                                                                                     |
| Fibronectin, fibrinogen, collagen binding protein                                | Promotes binding to mucosal cells and tissue matrices                                                                                                 |
| Extracellular toxins and enzymes                                                 |                                                                                                                                                       |
| Enzyme catalase                                                                  | Conversion of hydrogen peroxide to water and oxygen                                                                                                   |
| Coagulase enzyme and agglutination factor                                        | Catalyzes conversion of fibrinogen to fibrin                                                                                                          |
| Proteases                                                                        | Degrade proteins                                                                                                                                      |
| Exotoxins: comprises four toxins $\alpha$ , $\beta$ , $\gamma$ , $\delta$        | Cytotoxic to tissue cells and leukocytes                                                                                                              |
| Panton-Valentine Leukocidin                                                      | Destroy leukocytes                                                                                                                                    |
| Enterotoxins                                                                     | Superantigen                                                                                                                                          |

Studies found in the literature indicate that there is a divergence of opinions as to whether coagulase is a virulence factor or not.<sup>34, 36, 38, 40</sup> Coagulase is known to be a marker of *S. aureus*, and to play a significant role in biofilm formation during *S. aureus* infections. Coagulase binds to host prothrombin and forms active *staphylothrombin* complexes, which convert soluble monomeric fibrinogen to self-curing insoluble fibrin and activate a coagulation cascade. Over the years *S. aureus* has evolved to interact with various components of the coagulation cascade, allowing it to take advantage of a system that is normally part of the host's defence against bacterial pathogens. For example, fibrin clots form in cracks in the skin, in part to trap invading bacteria and prevent them from spreading throughout the body. However, in the presence of *S. aureus*, it secretes its coagulases, which allows it to use the host's resources to build a fibrin shield around abscess

communities, protecting the bacteria from neutrophil infiltration. In addition to catalyzing the deposition of a fibrin wall, *S. aureus* also has several fibrinogen-binding proteins anchored and secreted in the cell wall.<sup>38, 40</sup>

This phenomenon has been used in clinical microbiology laboratories as a coagulase test for the identification of *Staphylococcus* species. In recent years, some reports have suggested that *S. aureus* uses fibrin and fibrinogen recruited by coagulase to form the biofilm structure.<sup>38, 40</sup>

## Resistance Mechanisms

Just one year after the introduction of penicillin, in 1941 and its widespread use, strains of *S. aureus* resistant to this antibiotic emerged through the production of  $\beta$ -lactamases (penicillinases), enzymes that cleave the  $\beta$ -lactam ring of penicillin, inactivating the antibiotic. About 80% of *S. aureus* isolates acquired in the community and hospitals were resistant to penicillin in the late 1960s. Methicillin, a penicillinase-stable semi-synthetic  $\beta$ -lactam antibiotic, was introduced in 1959 to overcome the problems caused by the crescent prevalence of resistant strains. However, in 1961, MRSA was first described, it was resistant to all  $\beta$ -lactam antibiotics including penicillins, cephalosporins and carbapenems. The rise in MRSA infections in hospitals has resulted in high morbidity and mortality and an increase in the cost of health care.<sup>36</sup>

Currently, methicillin, due to its high toxicity, is no longer used in humans, however, the term "resistant to methicillin" is still in use today, having achieved notoriety among the media over the years and general population.<sup>34</sup>

A few years ago, strains with intermediate susceptibility or complete resistance to vancomycin (VISA and VRSA respectively) emerged. VISA strains were first reported in Japan and later identified in the United States of America (USA) and Europe. Vancomycin resistance is mediated by the *vanA* gene. Resistance to new antibiotics for the treatment of MRSA infections such as linezolid and daptomycin has already emerged although only confined to a minority of cases.<sup>36, 41</sup>

Thus and in addition to the scarcity of therapeutic options for the elimination of MRSA, these bacteria tend to acquire resistance to new antibiotics.

Initially, MRSA bacteria were considered a concern for hospitalized patients (HA-MRSA) and in these cases, they are particularly related to the use of invasive procedures or invasive devices such as catheters, certainly due to their ability to survive and form biofilms. However, in the last two decades, new strains of MRSA have emerged in the community (CA-MRSA) with the ability to infect healthy adults. These strains are associated

with aggressive virulence factors and strong resistance to antibiotics, becoming a public health concern.<sup>34, 36</sup>

MRSA bacteria are typically transmitted through skin contact, with public spaces such as schools or hospitals being places that can present significant risks of transmission. More recently, LA-MRSA strains have been identified that cause infection in individuals with contact with animals or, less commonly, environmental or food-borne contamination.<sup>34</sup>

## Current treatment options

Treatment for *S. aureus* infections has undergone numerous changes over the decades due to the enormous adaptive potential of the bacterium to various antibiotics and antimicrobial agents. The therapeutic approach depends on the type of infection, patient age, the clinical manifestation of the disease, comorbidity, antibacterial susceptibility of the infecting organism and hospitalization.<sup>36</sup> Several single-agent drugs and drug combinations have been used to treat *S. aureus* infections. It is currently recommended to use an antibiotic effective against this organism for the empirical treatment of severe *staphylococcal* infections or HA-MRSA strains where the prevalence of MRSA is greater than 20%. The choice, route of administration and duration of antibiotic therapy are determined by the location and severity of the infection. Treatment should then be adjusted based on subsequent results of cultures and susceptibility testing. Vancomycin, linezolid and daptomycin can be used for severe sexually transmitted infections (STI-like infections). Clindamycin, trimethoprim-sulfamethoxazole, and doxycycline are choices for the treatment of mild to moderate infections, depending on antibiotic susceptibility testing.<sup>34</sup>

For non-complex skin abscesses, the use of these latter antibiotics in conjunction with incision and drainage has been shown to improve clinical cure rates in the emergency department and other outpatient settings.<sup>34</sup> Oral therapy, as a rule, should be avoided in the initial treatment of serious infections. When the infection is related to a medical device such as catheters, these should be removed or replaced. Vancomycin remains the mainstay for the intravenous treatment of serious systemic MRSA infections, first because of its safety profile and, second, because of the lack of other fully approved alternatives. Of note, glycopeptides (vancomycin and teicoplanin) have slower bactericidal activity than  $\beta$ -lactam agents and tissue penetration is poor. Studies continue to be done to improve these two antibiotics.<sup>34</sup>

Alternative anti-MRSA antibiotics are being used, increasingly, but it is important to note that they can have adverse effects, particularly linezolid.<sup>34</sup>

Combination therapy to treat *S. aureus* bacteremia (including MRSA) has been used in an attempt to increase bacterial death, particularly for endocarditis therapy. However,

evidence that combination therapy improves outcomes is lacking. Combinations of vancomycin and rifampicin have also been studied, particularly in the context of biofilm infections. However, a clinical trial evaluated the addition of rifampicin and found no overall benefit. On the other hand, the combination of vancomycin and  $\beta$ -lactam antibiotics showed synergistic bacterial death in vitro, but sufficient clinical evidence in favour of this combination is lacking.<sup>34</sup>

Several new agents have been approved for the treatment of skin and soft tissue infections and, in some cases, for pneumonia (Ceftaroline, Ceftobiprole, Telavancin, Dalbavancin, Oritavancin, among others).<sup>34, 36</sup> However, the efficacy and safety of these antibiotics for invasive infections, for which there is a real need, have largely not been demonstrated. New treatment options have some advantages over older agents and are likely to play a role in the therapy of serious MRSA infections in the near future.<sup>34, 36</sup>

Ceftaroline and ceftobiprole are the first  $\beta$ -lactam antibiotics with anti-MRSA activity, they were recently approved for the treatment of skin and soft tissue infections and, in some cases, pneumonia. Its use in combination with daptomycin in the prevention of MRSA endocarditis in animals has been studied with only promising results. Its efficacy and safety in real cases of severe invasive infection have not been demonstrated. However, resistance to ceftaroline has been observed in MRSA strains.<sup>34</sup>

The development of a vaccine against *S. aureus* is currently a reality and may prevent future infections.<sup>34</sup>

## Biofilm

Biofilm development represents a mode of growth that allows bacteria to survive in hostile environments and colonize new niches by various dispersed mechanisms. Biofilm is a structured microbial community that is attached to a surface and surrounded by an extracellular matrix.<sup>39, 42</sup>

The matrix of self-produced biofilm or extracellular polymeric substance contains mainly polysaccharides, proteins and extracellular DNA, the main function of which is to protect cells from harmful environmental factors, including antibiotics and the host's immune system. Biofilm formation is estimated to be involved in 65–80% of bacterial infections in humans.<sup>42</sup> Some staphylococcal infections that involve biofilm formation occur in internal devices, including catheters, artificial joints, and pacemakers.<sup>38</sup>

The biofilm life cycle has distinct phases including the adhesion of planktonic cells to a surface, the growth of cells into a mature colony (biofilm) and the dispersal of cells belonging to the biofilm to the surrounding environment. All steps involve complex physical,

chemical and biological processes that govern bacterial adhesion, biofilm formation and persistent cohesion.<sup>39</sup>

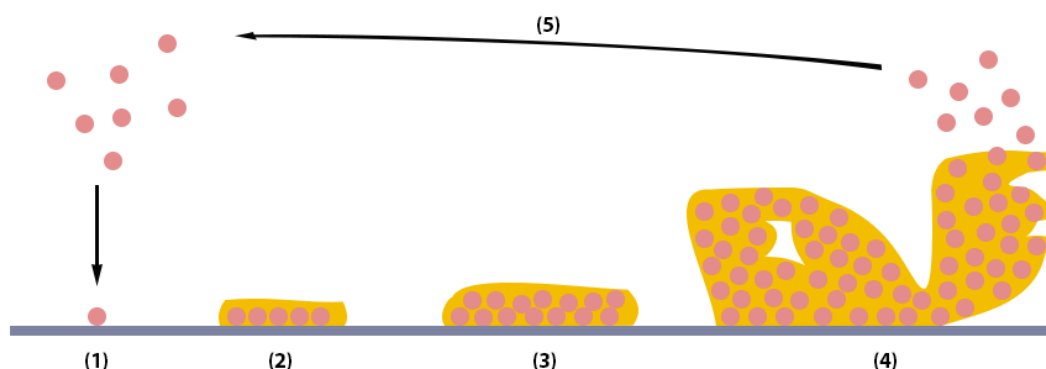


**Figure 2.11.** Schematic representation of a biofilm formation. (1) – Attachment to the surface, (2) – Formation of monolayer and production of the matrix, (3) – Microcolony formation, multilayer, (4) – Mature biofilm, with characteristic “mushroom” formed of polysaccharide, (5) – Detachment and reversion to planktonic growth, starting a new cycle. Adapted from <sup>43</sup>.

It is known that the life cycle of *Staphylococcal* biofilm occurs in several stages (**Figure 2.11**): (1), as a fixation of planktonic bacteria to a surface that is often reversible by van der Waals forces, steric interactions, electrostatic interaction (double layer) and irreversibly as a result of the hydrophobic and hydrophilic interaction between the bacteria and the surface, (2), the bacteria adhere and form a monolayer that binds irreversibly, producing an extracellular matrix, (3), formation of microcolonies of bacteria and, (4), the maturation of microcolonies under ideal conditions of growth. Some cells start to detach, and the biofilm will disperse (5).<sup>31</sup>

After the biofilm is formed, it provides nutrition and resistance to the microorganism community against the host's immune system and also against various antimicrobials. Protection over the human immune system is achieved thanks to the decreased bactericidal activity of neutrophils. Regarding the decrease in the effectiveness of antibiotics and antimicrobials, it is known that penetration into the biofilm by these agents is severely compromised, as well as the physiological changes that occur in certain bacteria, thus giving them resistance.<sup>42</sup>

One of the main challenges associated with the treatment of biofilm infections is its resistance to antimicrobials. General, cells within a biofilm are 10 to 1000 times more tolerant to antibiotics and antimicrobial peptides (AMPs) than planktonic cells. Although some antibiotics and AMPs show a partial decrease in their ability to penetrate the biofilm

matrix, the high tolerance of biofilms to therapeutic agents is mainly attributed to altered cellular metabolism within a biofilm. However, the exact mechanism of biofilm resistance to antibiotics is not yet known.<sup>39</sup>

The antibiotic rifampicin has a high rate of diffusion within biofilms, host cells and bone tissue, as well as bactericidal activity against *S. aureus* and *S. epidermidis* in the stationary growth phase. Although rifampicin is highly effective against *Staphylococcal* biofilms and is often proposed for the treatment of *Staphylococci*, there is also a high risk of emergence of resistance to rifampicin during treatment and, therefore, it is given only in combination with other antimicrobial agents.<sup>39</sup>

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### 3. Theoretical concepts of techniques used

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In this chapter, there is a brief explanation of the different techniques used in the characterization of gemini surfactants with regard to size, concentration and charge. A short description is also made of all the techniques used to assess the antimicrobial, anti-biofilm and cytotoxicity activities of gemini surfactants.

### 3.1. Characterization of compounds

#### 3.1.1. Tensiometry

The *critical aggregation concentration* (*cac*) is the minimum concentration at which a surfactant start to form aggregates.<sup>1</sup> It is an important parameter since it allows to predict their activity at surfaces, such as solubilization, its ability to disrupt and interact with biological membranes, among others. Specifically, is essential for understanding the concentration range in which these compounds can be tested in experiments, in order to successfully assess the influence of their organization in solution (monomer or aggregate) on its biological/biophysical properties.

The *cac* can be determined by measuring the surface tension ( $\gamma$ ) of a solution with increasing surfactant concentration. The addition of surfactant increases the number of monomers in solution and due to their tendency to accumulate at the surface, the surface tension decreases until the critical concentration is reached. After the *cac*, the surface tension stabilizes because, at this point, the concentration of free monomer is roughly constant and represents the *cac* value. Thus, this concentration can be determined as the "break points" in the  $\gamma$  vs.  $\ln(w/w^0)$  curves as illustrated in **Figure 3.1**.

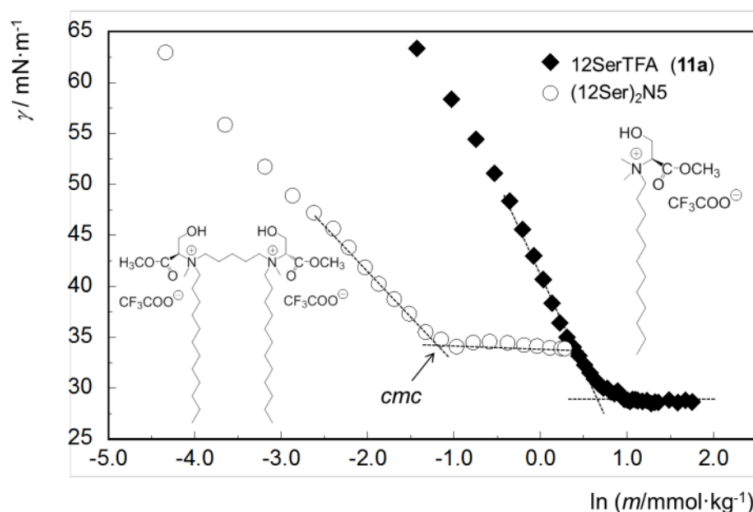


**Figure 3.1.** Example of surface tension versus  $\ln(m/mmol.kg^{-1})$  curves. From <sup>2</sup>.

In this work, the surface tension was measured using the Wilhelmy plate method (**Figure 3.2.**).

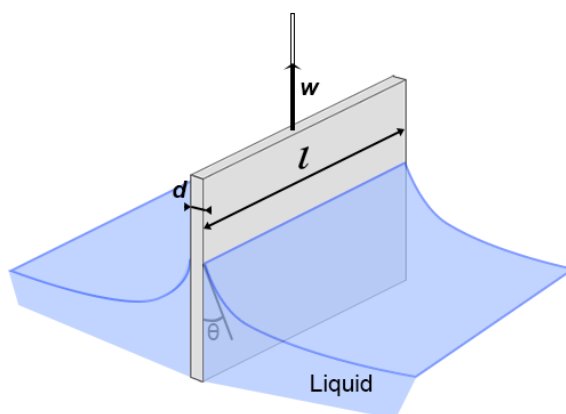


**Figure 3.2.** Schematic diagram of the Wilhelmy plate method. Adapted from <sup>3</sup>.

The platinum plate used is made of platinum-iridium alloy and present roughened surfaces (to minimize the contact angle,  $\theta$ ) and has the following geometric dimensions: length 10 mm, width 19.9 mm and thickness 0.2 mm. This method measures the force ( $F$ ) with which the platinum plate is pulled downward by an air/water interface, and it can be expressed according to the following equation (Eq.1).

$$\gamma = \frac{F_w}{2(l + d) \cos \theta} \quad (1)$$

where  $w$  is the meniscus' weight detected by balance, and  $l$  is the width and  $d$  the plate thickness, respectively (**Figure 3.2.**). Before each experiment the plate is thoroughly clean and flamed. The solutions used for the interfacial characterization were prepared on the same day of the experiment or the day before, in concentrations between 2-4 mM. The measurements were performed in a DCATT 11 surface tensiometer and the temperature was kept constant at the desired value ( $\pm 0.1^\circ\text{C}$ ) using a thermostatted Julabo water bath.

This method and equipment were used to measure the surface tension of the aqueous solution. Successive aliquots of the surfactants' stock solution, freshly prepared in buffer solution, were added to the thermostated vessel with 25 mL under constant stirring. The surface tension value in each measurement was allowed to vary only  $0.005 \text{ mN.m}^{-1}$ . Between the additions, the solution was stirred for 1.5 min and given a 5 min equilibrium time.

### 3.1.2. Polarized light microscopy

Polarized light microscopy is used to quantitatively or qualitatively characterize and identify optically anisotropic materials.

A sample is anisotropic due to molecular order, as found in crystals. That is, in materials with molecular order, the absorption, refraction and scattering of light normally become dependent on the material's orientation in relation to the polarization of light. Polarized light microscopy exploits this dependence and provides a sensitive tool to analyze the alignment of molecular bonds, so an anisotropic sample rotates the plane of polarization and appears illuminated on the dark background. This technique allows the analysis of fixed or live transparent samples, such as living cells and tissues, without the use of dyes.<sup>4, 5</sup>

Polarizing microscopes differ from a standard transillumination microscope in that they add a polarizer before the condenser; a compensator and analyzer slot behind the objective lens; voltage-free optics; a graduated and rotating internship; centring lens mounts; crosshairs in the eyepiece aligned parallel or at  $45^\circ$  to the polarizer axes; and a focusable Bertrand lens that can be inserted for conoscopic observation of interference patterns at the posterior aperture of the objective lens. The **Figure 3.3.** shows the diagram of the traditional polarized light microscope.<sup>4</sup>

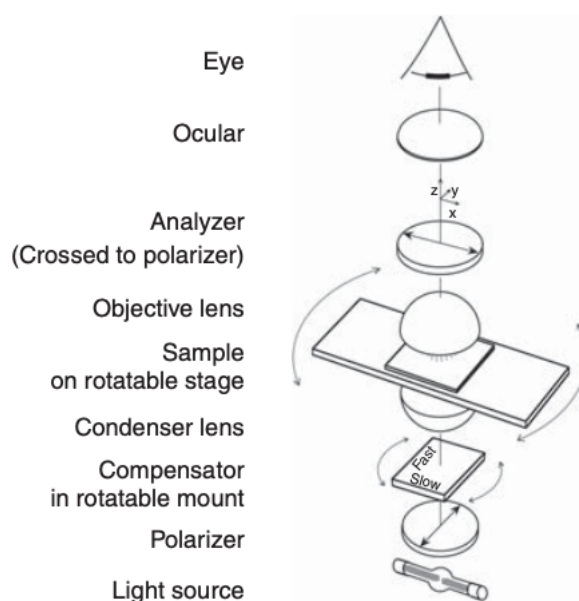


**Figure 3.3.** Schematic of the optical arrangement of a conventional polarizing microscope. Adapted from <sup>4</sup>.

In the characterization of surfactants, this technique allows obtaining information about the three-dimensional structure. Making it a relatively inexpensive and accessible tool.<sup>5</sup>

## 3.2. Physical characterization

### 3.2.1. Size and concentration by Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) is one of the few methods capable of visualizing the size and measuring the number of nanoparticles in a liquid suspension in the range of 10 to 1000 nm based on light scattering properties and Brownian motion analysis.<sup>6</sup>

This technique performs hydrodynamic diameter measurements on all particles individually and simultaneously (particle by particle) and is ideal for real-time analysis.<sup>7</sup> In addition, it is also allowed to acquire information about the concentration of nanoparticles, the relative intensity of scattered light and also visualize and analyze particles labelled with fluorescent probes. The technique is non-destructive and the sample can be retrieved if necessary.<sup>7</sup>

NTA determines the particles size by analyzing the path of the particles that pass through a chamber at a constant flow. Sample particles are visualized by illumination with a laser beam.<sup>6</sup> The light scattered by the particles is recorded with a light-sensitive CCD camera, which is arranged at a 90° angle to the plane of irradiation (**Figure 3.4.**). Using a special algorithm, particles are detected and their path recorded.<sup>8</sup>

The camera operating at approximately 30 frames per second, is used to capture a video with a field of view approximately 100  $\mu\text{m}$  x 80  $\mu\text{m}$ .<sup>7</sup>

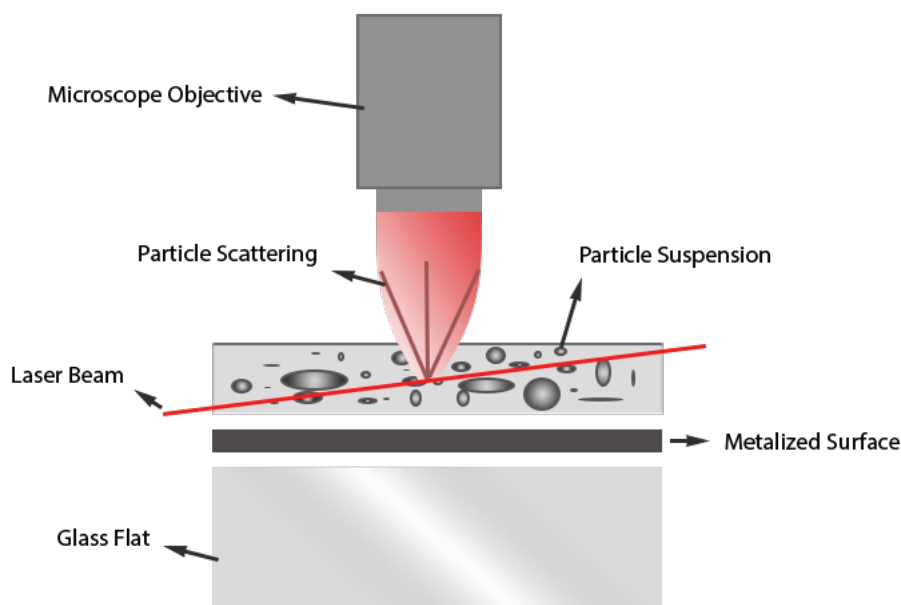


**Figure 3.4.** Schematic representation of the NanoSight® instrument configuration. Adapted from <sup>9</sup>.

The concentration is calculated by averaging and dividing by the volume used in which the number of particles is measured. A sufficient number of particles must be analyzed within the test time to ensure that the data produced is statistically robust.<sup>8</sup>

### 3.2.2. Surface charge by Electrophoretic Light Scattering

The zeta potential is a very important measure, as it indicates the stability of the particles in suspension. In a solvent, under the influence of an electric field applied to the sample, the particles move to the opposite electrode and acquire a surface charge (stern layer), essentially by ionizable surface groups or adsorption of charged species. These surface charges modify the distribution of the surrounding ions, creating a layer around the particle, called the sliding plane, as shown in **Figure 3.5**. The potential that exists in this "slipping plane" limit is called the zeta potential.<sup>10</sup>

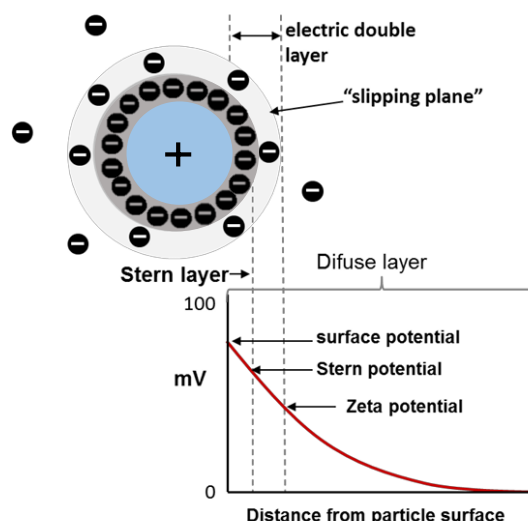


**Figure 3.5.** Schematic representation of zeta potential. Adapted from <sup>10, 11</sup>

The main factor that affects the zeta potential is pH, as it affects the surface charge and, therefore, the extent of repulsion between the particles.<sup>11</sup>

In general, particles in a suspension are considered colloidally stable if the value of the zeta potential is above +30mV or below -30mV, if the values are intermediate it indicates a colloidally unstable system.<sup>11</sup>

Light Scattering Phase Analysis (PALS), also known as Electrophoretic Light Scattering (ELS) is a well-established method, due to its sensitivity, precision and versatility, to measure the zeta potential.<sup>12</sup>

It is important to note that the measurements of the zeta potential must be optimized, that is, different measurements must be made with different sample concentrations in order to verify which one provides the best quality data. Dilutions must be made in the same buffer as the original material to preserve the surface charge, and attention must be paid to the conductivity of the sample, as it may affect the results.<sup>11</sup>

The electrophoretic mobility ( $\mu_e$ ) of the particles is calculated by Henry's equation (2):

$$\mu_e = \frac{2\varepsilon_r\varepsilon_0\zeta f(Ka)}{3\eta} \quad (2)$$

where  $\varepsilon_r$  is the relative permittivity/dielectric constant,  $\varepsilon_0$  is the permittivity of vacuum,  $\zeta$  is the zeta-potential value,  $f(Ka)$  is the Henry's or Helmholtz-Smoluchowski function, and  $\eta$  is the viscosity at the experimental temperature.

### 3.3. Biological characterization

#### 3.3.1. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The Minimum Inhibitory Concentration can be calculated by one of several methods, including agar dilution, broth microdilution, agar gradient dilution, or by one of several automated or semi-automatic methods.<sup>13</sup> The most used is the broth microdilution method, as it provides a quantitative and more accurate result. Minimum Inhibitory Concentration (MIC) assays determine the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism.<sup>13, 14</sup> A schematic representation is shown in the following **Figure 3.6.:**

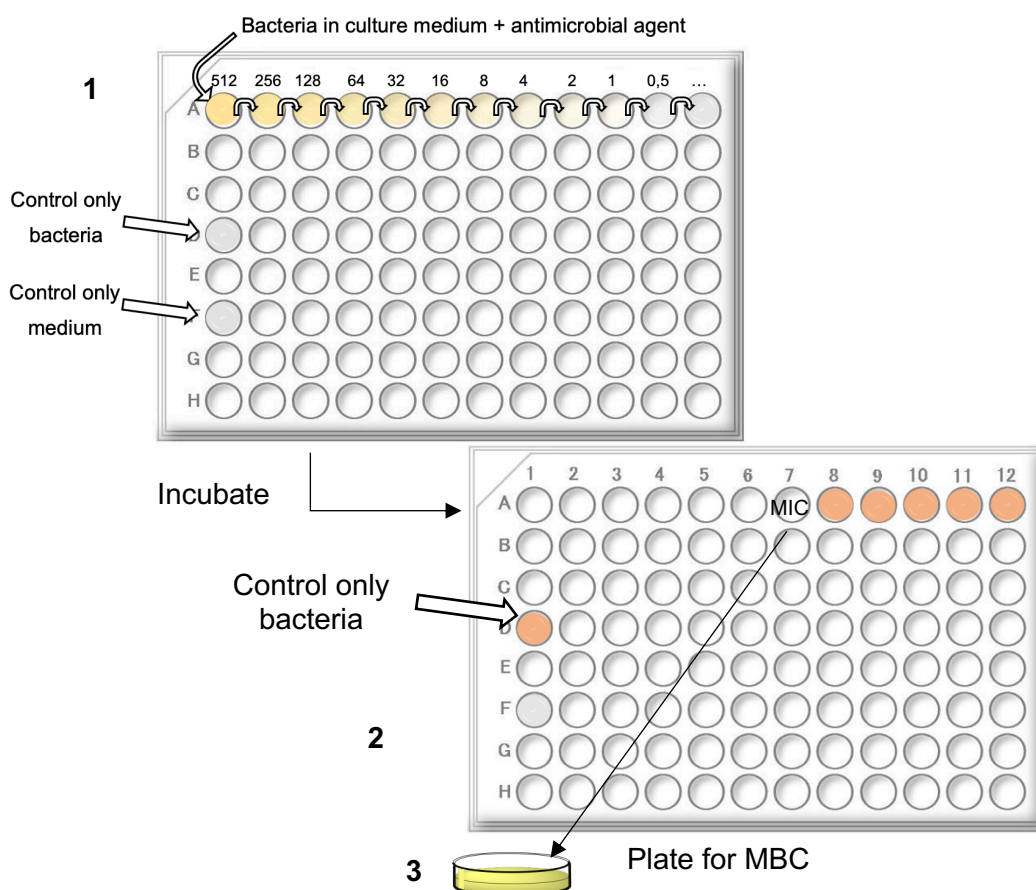


**Figure 3.6.** MIC and MBC Assays. (1) Dispense inoculum into microdilution plate, add compounds and make serial dilutions (512 µg/mL). Incubate the microdilution plate. (2) Analyze the microdilution plate to determine the MIC value. (3) Plate a portion of each well on an appropriate agar medium, incubate and check for colonies to determine MBC.

The MIC is considered the first well without visual pellet-formation on the well-bottom. The minimum bactericidal concentration (MBC) is determined by plating the content of the first three wells where visible growth was not observed.

### 3.3.2. Resazurin assay

The resazurin assay also known as the Alamar Blue Assay provides a simple, quick and sensitive measurement of cell and bacterial viability. Living cells are metabolically active and are able to reduce, via mitochondrial reductase, the non-fluorescent dye resazurin (blue) to the strongly fluorescent dye resorufin (pink). The time of the reduction, i.e. colour development after addition to treated cells depends on the metabolic rates of the bacteria and cell lines being tested.<sup>15, 16</sup>

It is a relatively inexpensive, reproducible method and is a sensitive method. The main disadvantage of this method is that the conversion to resorufin depends on the enzymatic conversion, that is, it makes this method sensitive when quantifying the fluorescence or absorbance.<sup>15, 16</sup>

### 3.3.3. Crystal violet assay – CV

The crystal violet (CV) assay is one of the most used and well-optimized methods in microbiology to quantify the total biomass of biofilms. The method consists of treating a sample of a bacterial culture grown with the crystal violet dye. The most used dye, the triarylmethane dye, is one of the primary components in bacterial staining. It is permeable to the cell membrane in Gram-positive and Gram-negative bacteria.<sup>17</sup>

Although the crystal violet assay consists of several steps, it is relatively easy to perform, reproducible, and allows you to analyze multiple samples simultaneously. In addition to being a relatively inexpensive method, it does not require the purchase of specialized equipment, and the dye is cheap with a shelf life of years.<sup>17</sup>

The main disadvantage of this assay is its non-specific nature, that is, this assay does not distinguish between matrix, living and dead cells. Another disadvantage of this assay is the many variables (incubation times, incubation temperatures, etc.) that can introduce variability into the assay results.<sup>17</sup>

### 3.4. Bioimaging technique - Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is an established light microscopy technique for generating high-resolution images of fluorescently labelled species with significant three-dimensional structure. Applications of confocal microscopy in the biomedical sciences include imaging the spatial distribution of macromolecules in fixed or living cells, reconstructing 3D structures, imaging multiple species tagged with one or more fluorescent probes and measuring physiological events in living cells. In addition to the various applications, this technique allows you to obtain numerous information from the sample under analysis, such as the number of cells, dimensions and 3D structure.<sup>18</sup>

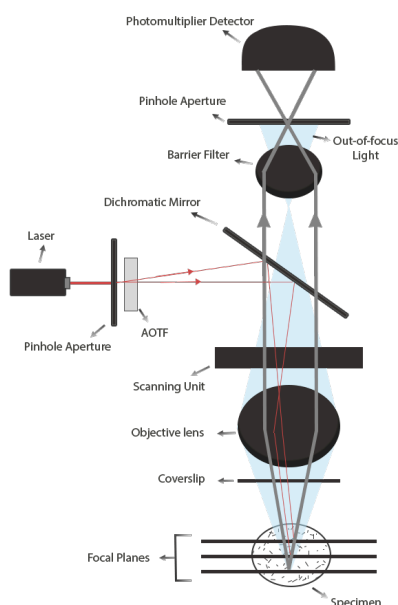


**Figure 3.7.** The main components of a modern laser scanning confocal microscope reflected light.

Adapted from <sup>19</sup>

As illustrated in **Figure 3.7.**, light from a laser passes through a pinhole, reflects off a dichromatic mirror, and passes to the scanning unit. A digitized beam enters the posterior focal plane of the objective lens, which focuses light on the sample. Any light that returns from the excitation of a fluorochrome at this point within the sample returns through the objective lens and scanning unit. As this light has a longer wavelength than the excitation light, it passes through the dichromatic mirror, is subsequently cleaned by a barrier filter, and is eventually focused on the second orifice. Any light that passes through the hole hits a low-noise photomultiplier detector, the signal from which it subsequently passes to the confocal microscope's computer imaging system. The use of different lasers ensures the ability to view multiple markers simultaneously.<sup>19</sup>

The advantage of the CLSM is its versatility and a large number of applications, combined with its relative ease of use for producing extremely high-quality images from specimens prepared for the optical microscope. Despite the advantages, this technique has some limitations, mainly the acquisition and maintenance costs.<sup>18, 20</sup>

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## 4. Synthesis and characterization of gemini surfactants derived from serine

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### Abstract

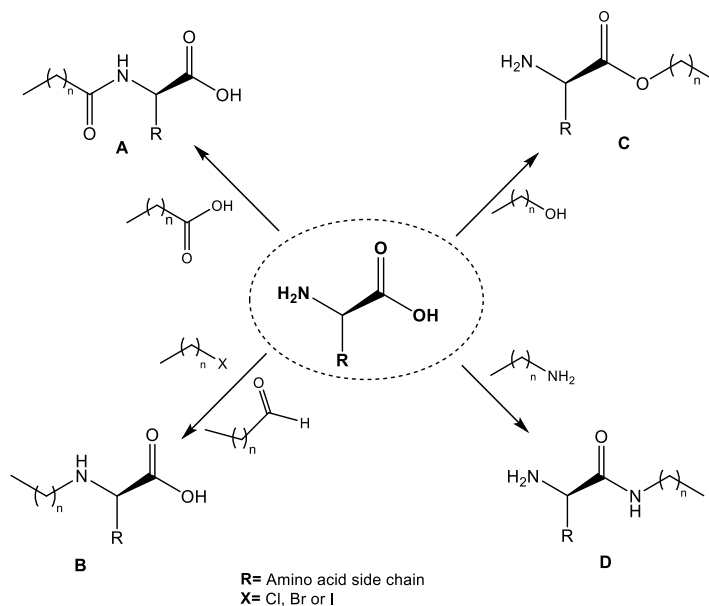
Amino acid-based surfactants are a promising alternative to conventional surfactants as they present better biocompatibility, biodegradability and cytotoxicity profiles. Specifically, gemini surfactants are of great interest due to their enhanced physicochemical properties which result from their novel structure. Therefore, gemini surfactants have a broad range of applications (cosmetic and food formulations, drug and gene delivery systems, etc.)

In this project, the aim was to synthesize three different series of serine-based gemini surfactants, differing in the position and length of the spacer, to be evaluated for their biological activity. The compounds were designated as of the amine, amide or ester series, according to the bond “spacer-residue”. The synthesis of the amide series was accomplished, and compounds with a five and a twelve carbon spacer were obtained. For the ester and amine series, the gemini cationic ammonium surfactants were obtained, however, the transformation of these salts into the corresponding quaternary ammonium salts has not yet been successful.

Overall, four compounds of the amide series and one of each, the ester and the amine series were obtained in moderate to good yields.

## 4.1. Introduction

Amino acids are the building blocks of proteins, so they stand as a good choice to be used as raw materials in the design of biocompatible surfactants.<sup>1</sup> The large variety of amino acids as well as fatty acids allows for a high diversity of tailored molecules and, consequently, different physicochemical properties. In **Scheme 4.1**, some of the monomeric structures that can be obtained using amino acid as a polar headgroup are presented.<sup>1-3</sup>



**Scheme 4.1.** Monomeric precursors can be obtained using amino acids as raw materials.

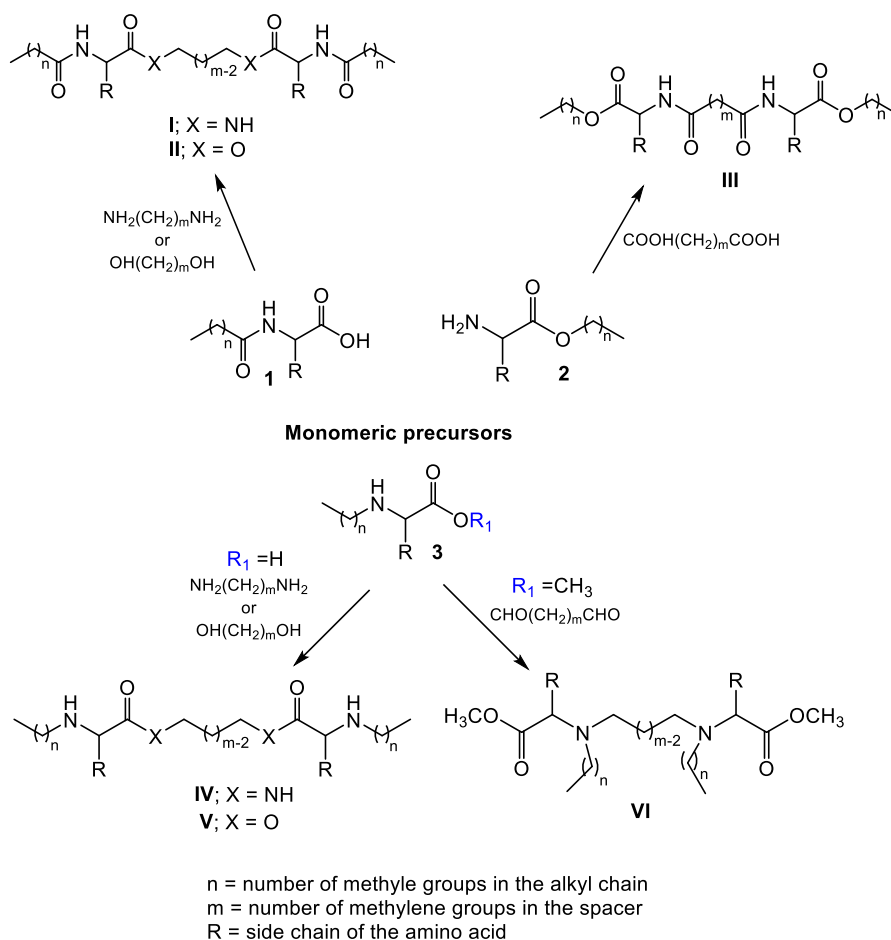
Adapted from <sup>2</sup>. (Created using ChemDraw® 20.0).

Derivatives **A** and **B** can be obtained by reaction of the  $\alpha$ -amino group of the amino acid with carboxylic acids and alkyl halides ( $S_N2$ ) or aldehydes (reductive amination), respectively. On the other hand, the carboxylic acid group of the amino acid can be transformed into the corresponding O-alkyl esters **C** or N-alkyl amides **D**, by reaction with fatty alcohols or fatty amines, respectively. The cationic surfactants can then be obtained by methylation of the amine functionality with an alkylating agent (**B**, **C** and **D**) while the anionic ones can be accessed by saponification (**A** and **B**). In addition, compounds of type **B** offer the possibility of preparing zwitterionic surfactants.<sup>3</sup>

The authors used glycine and alanine as polar headgroups to obtain N-acyl derivatives (**A**). Since then, a large number of monomeric surfactants have been synthesized and studied as e.g. N-alkanoyl derivatives (**A**) and N-alkylamides (**D**) based on arginine and lysine, O-alkyl esters (**C**) derived from arginine and glycerol, and N-alkyl amines (**B**) derived from serine, tyrosine and 4-hydroxyproline, amongst others.<sup>4-7</sup>

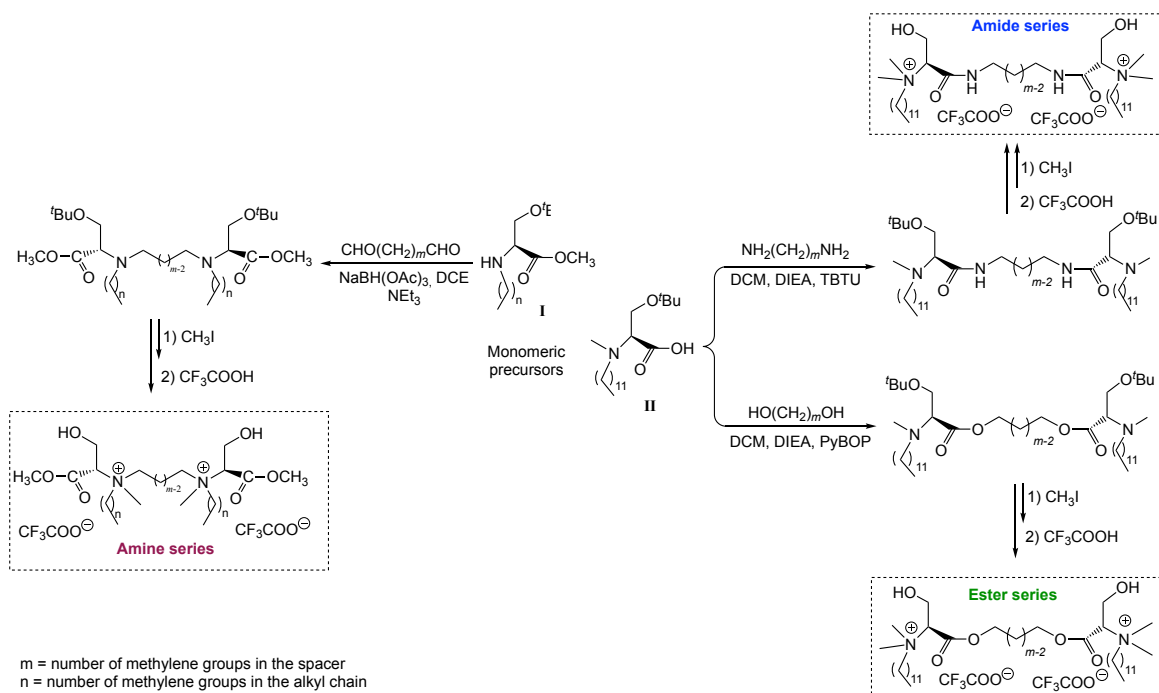
The variety of monomeric precursors, shown in **Scheme 4.1**, allows to access a high number of gemini/dimeric compounds (**Scheme 4.2.**).

Formally, a gemini surfactant is obtained by introducing a spacer between two monomeric precursors. The insertion of the spacer can be done using diamines, diols or carboxylic acids, depending on either on the precursor in question or on the place where the spacer is to be inserted (**Scheme 4.2**).<sup>8</sup> Gemini precursors can be transformed into anionic, cationic and zwitterionic surfactants. With regard to gemini precursors **I**, **II** and **III**, they can only acquire charge (positive or negative) if the side chain of the amino acid contains functional groups that can be protonated/alkylated (amino group) or neutralized (carboxylic acid group). On the other side, gemini precursors **IV**, **V**, **VI** can be transformed into the corresponding cationic surfactants by alkylation of the available amino groups with alkyl halides (e.g. iodomethane). Furthermore, dimeric precursors **VI** can be transformed into anionic amphiphiles, by alkaline hydrolysis, as well as be converted into zwitterionic surfactants, by alkylation of the amino group and subsequent saponification of the methyl ester.



**Scheme 4.2.**Obtention of gemini structures from monomeric precursors. Adapted from <sup>9</sup>. (Created using ChemDraw® 20.0).

An overview of methodologies of synthesis of some existing gemini surfactants is outlined in **Scheme 4.3**. We will focus our attention mainly on cationic gemini surfactants, especially on serine derivatives, since they are the subject of this work.



**Scheme 4.3.** Synthesis of serine-based cationic gemini surfactants.<sup>7, 10</sup> (Created using ChemDraw® 20.0).

The serine-based gemini surfactants described in the literature, by Silva et co-workers, are ammonium salts in which the alkyl chain is always linked to the nitrogen atom of the amino acid residue.<sup>7, 10, 11</sup> The synthetic methodology involved the introduction of the spacers into the corresponding monoalkylated/dialkylated monomeric precursors (**Scheme 4.3**). Three surfactant series have been obtained and were classified, according to the nature of the bond between the polar headgroup and the spacer, as amine, amide and ester series. In the case of the amine series, the spacer was introduced between the nitrogen atoms of the *N*-alkyl-*O*-*tert*-butylserine methyl ester (precursor **I**) via reductive amination of dialdehydes. On the other hand, the compounds of the amide/ester series were obtained by the introduction of the spacer between the carboxylic acid groups of *O*-*tert*-butyl-*N*-dodecyl-*N*-methyl serine (**II**). The reactions were performed according to conventional peptide condensation methods, using either TBTU or PyBOP as coupling agents. The final target compounds were obtained by methylation, with iodomethane, and subsequent removal of the *tert*-butyl group using trifluoroacetic acid.<sup>7, 10</sup>

For the amine derivatives, compounds with alkyl chains (12, 14, 16 and 18) and/or spacers (2, 5, 10 and 12) of different length were synthesized in order to evaluate the influence of these two parameters on the physicochemical properties of the surfactants. As expected, the *cac* values of the serine-based gemini surfactants decrease with increasing alkyl chain length due to the increased hydrophobicity of the surfactants. The influence of the spacer length, however, was not so remarkable. When compared with the conventional *bis-quats*, serine-based surfactants displayed enhanced performance, presenting (i) lower *cac* values (3-5 times) and (ii) higher interfacial performance.<sup>7, 10</sup>

Concerning the amide and ester derivatives, the same author's,<sup>7, 10</sup> synthesized 2 compounds of each series (12 carbon alkyl chains and 5 or 12 carbon spacer, each). The solution behaviour of these compounds was studied and compared with the results previously obtained for the amine series. All the compounds with a 5-carbon spacer formed micelles. However, a significant difference for the compounds with the 12-methylene spacer of the different series was observed: while the amine derivative formed micelles in solution, the amide and ester formed multilamellar vesicles.<sup>11</sup> The authors also evaluated the cytotoxic profile of the compounds, either by the colourimetric Alamar blue assay using HeLa cells or by the MTT assay using the HEK 293T cell line. All the serine-based surfactants were shown to be less cytotoxic than the conventional *bis-quats* (**Table 4.1**).<sup>12</sup> Yet in another study, the different families of serine-based dimeric surfactants proved to be efficient in DNA/RNA compaction and gene transfection in the formulations containing different helper lipids namely DOPE/cholesterol or monolein.<sup>8, 13</sup> Moreover, serine-based surfactants were shown to be excellent enhancers in permeation of anaesthetics, in dermal delivery approaches, presenting high levels of biocompatibility.<sup>14</sup>

**Table 4.1.** Critical aggregation concentration (*cac*) and  $IC_{50}$  values for different families of serine-based cationic gemini surfactants.

| Surfactants                  |                            | $Cac/$<br>$mmol \cdot kg^{-1}$ | Type of self-assembly  | $IC_{50}/ \mu M$<br>24 h | Cells used | Ref. |
|------------------------------|----------------------------|--------------------------------|------------------------|--------------------------|------------|------|
| <b>amine series</b>          | (12Ser) <sub>2</sub> N5    | 0.32                           | Micelles               | 253                      | HeLa       | 10   |
|                              | (14Ser) <sub>2</sub> N5    | 0.042                          | Micelles               | 40.2                     |            |      |
|                              | (16Ser) <sub>2</sub> N5    | 0.015                          | Micelles               | 7.65                     |            |      |
|                              | (18Ser) <sub>2</sub> N5    | --                             | Micelles               | 15.7                     |            |      |
|                              | (12Ser) <sub>2</sub> N12   | 0.077                          | Micelles               | >100                     | HEK293T    | 12   |
| <b>amide series</b>          | (12Ser) <sub>2</sub> CON5  | 0.40                           | Micelles               | 15.2                     | HeLa       | 10   |
|                              | (12Ser) <sub>2</sub> CON12 | 0.027                          | Multilamellar vesicles | 18                       | HEK 293T   | 12   |
| <b>ester series</b>          | (12Ser) <sub>2</sub> COO5  | 0.26                           | Micelles               | 19.5                     | HeLa       | 10   |
|                              | (12Ser) <sub>2</sub> COO12 | 0.014                          | Multilamellar vesicles | >100                     | HEK 293T   | 12   |
| <b>conventional bis-quat</b> | 12-5-12                    | 0.87                           | Micelles               | $6.83 \times 10^{-3}$    | HeLa       | 10   |
|                              | 14-5-14                    | 0.32                           | Micelles               | --                       |            |      |
|                              | 16-5-16                    | 0.038                          | Micelles               | --                       |            |      |
|                              | 12-12-12                   | 0.22                           | Micelles               | --                       |            |      |

Some reports of cationic gemini surfactants derived from lysine, arginine, alanine and cysteine, amongst others, can also be found in the literature.<sup>24, 27-31</sup>

## 4.2. Materials and methods

### 4.2.1. Materials and reagents

In all reactions the solvents used were of quality for analysis. Commercial reagents were used without prior purification. **Table 4.2.** shows all the reagents used in this work.

**Table 4.2.** Chemical information regarding the reagents used in the synthesis reactions.

| Reagents                                                                       | Molecular weight    | Density            | Brand             |
|--------------------------------------------------------------------------------|---------------------|--------------------|-------------------|
|                                                                                | $\text{g.mol}^{-1}$ | $\text{g.mL}^{-1}$ |                   |
| 1,5-Diaminopentane                                                             | 102,18              | 0,873              | Sigma-Aldrich     |
| 1,12-Dodecanediamine                                                           | 200,36              | -                  | Sigma-Aldrich     |
| 1,12-Dodecanediol                                                              | 202,37              | -                  | Sigma-Aldrich     |
| 2-(1 <i>H</i> -Benzotriazol-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate | 321,09              | -                  | Merck             |
| 2-Nitrobenzenesulfonyl chloride                                                | 221,62              | -                  | TCI               |
| 4-Methylmorpholine <i>N</i> -oxide                                             | 117,15              | -                  | Sigma-Aldrich     |
| Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate             | 520,39              | -                  | Merck             |
| Chlorotrimethylsilane                                                          | 108,64              | 0,86               | Sigma-Aldrich     |
| Di- <i>tert</i> -butyl dicarbonate                                             | 218,25              | 1,02               | Sigma-Aldrich     |
| Dodecanoyl chloride                                                            | 218,76              | 0,946              | Sigma-Aldrich     |
| Formaldehyde solution 37%                                                      | 30,03               | 1,09               | Fisher Scientific |

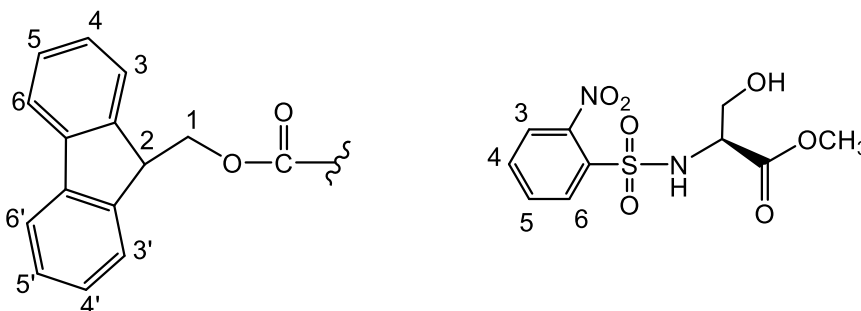
**Table 4.2.** Chemical information regarding the reagents used in the synthesis reactions (cont.).

| Reagents                          | Molecular weight    | Density            | Brand         |
|-----------------------------------|---------------------|--------------------|---------------|
|                                   | g.mol <sup>-1</sup> | g.mL <sup>-1</sup> |               |
| Hexadecanoyl chloride             | 274,87              | 0,91               | Sigma-Aldrich |
| Iodomethane                       | 141,94              | 2,28               | Sigma-Aldrich |
| <i>N,N</i> -Diisopropylethylamine | 129,24              | 0,742              | Sigma-Aldrich |
| <i>N,N</i> -Dimethylformamide     | 73,09               | 0,94               | Merck         |
| Piperidine                        | 85,15               | 0,86               | Sigma-Aldrich |
| Potassium carbonate               | 138,21              | -                  | Panreac       |
| Sodium carbonate                  | 105,99              | -                  | Sigma-Aldrich |
| Sodium carbonate anhydrous        | 105,99              | -                  | Panreac       |
| Sodium triacetoxyborohydride      | 211,94              | -                  | Sigma-Aldrich |
| Tetradecanoyl chloride            | 246,82              | 0,908              | Sigma-Aldrich |
| Tetrapropylammonium perruthenate  | 351,43              | -                  | Sigma-Aldrich |
| Thiophenol                        | 110,18              | 1,08               | Sigma-Aldrich |
| Triethylamine                     | 101,19              | 0,726              | Sigma-Aldrich |
| Trifluoroacetic acid              | 114,02              | 1,49               | Sigma-Aldrich |

The syntheses were performed according to procedures already described in literature.<sup>15-17</sup> All reactions and purifications were monitored by thin layer chromatography (TLC) on silica gel 60 F<sub>254</sub> pre-coated aluminum sheets of 0.25 mm thickness, from Merck. Phosphomolybdic acid was used as stain for TLC. For the purification of the compounds by adsorption column chromatography SDS silica gel was used (60 Å, 30-70 µm, surface area of 550 m<sup>2</sup>/g). Column chromatography was performed under pressure (flash chromatography), with collection of eluent fractions of 5 to 20 mL. The eluents use in each case are referenced in the experimental section.

The characterization of the compounds was performed by NMR, at CEMUP (UP) and MS, at DQB/FCUP. <sup>1</sup>H and <sup>13</sup>C NMR spectra were specifically obtained on a Bruker

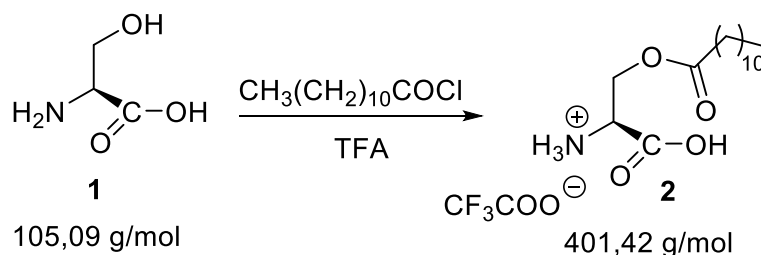
Avance III 400 spectrometer. Chemical shifts ( $\delta$ ) are presented in ppm and coupling constants (J) are quoted in Hz. The signal multiplicity is represented as follows: s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), dt (doublet of triplets), q (quartet); m (multiplet). For NMR proposes the fmoc subunit and sulfonamide subunit in the corresponding derivatives were numbered according to the following:



Mass spectra were recorded on a Finnigan Surveyor instrument, fitted with a Finnigan LCQ DECA XP MX mass detector (Finnigan Corp. San José, Calif. USA) and Atmospheric Pressure Ionization (API) with an Elettrospray ionization (ESI) interface. The samples were analysed by direct injection and the spectra were obtained in positive mode ( $m/z$  50-1500).

#### 4.2.2. Synthesis of the amide series

##### A) Synthesis of *O*-dodecanoylserine (**2**)



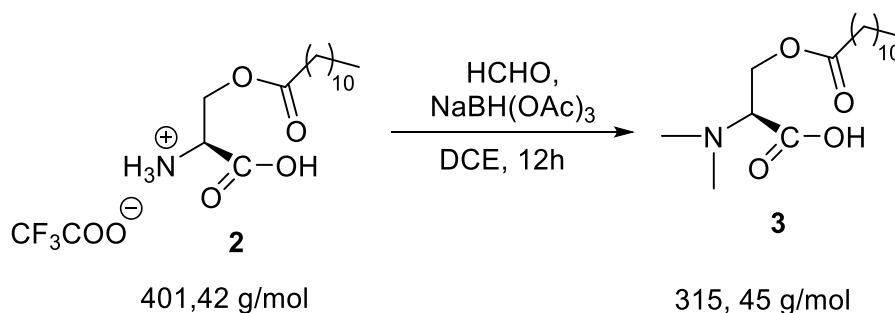
Serine (**1**) was dissolved in trifluoroacetic acid (0°C) under vigorous stirring. After 30 minutes, dodecyl chloride (1.5 eq) was added and the reaction mixture was stirred at room temperature for 4 hours. The reaction mixture was cooled in an ice bath and diethyl ether (20 mL) was added slowly. The white suspension was stirred for 15 more minutes at 0°C. The white solid was filtered under vacuum and washed several times with cold diethyl ether. The solid was allowed to dry overnight at r.t. The product was identified by <sup>1</sup>H-NMR as the desired product (**2**). All the reactions performed are summarized in **Table 4.3**.

**Table 4.3.** Synthesis of *O*-dodecanoylserine (**2**).

| <b>1</b>    | <b>Dodecanoyl chloride</b> | <b>CF<sub>3</sub>COOH</b> | <b>2a</b>   | <b>η (%)</b> |
|-------------|----------------------------|---------------------------|-------------|--------------|
| m/g (mmol)  | V/mL (mmol)                | V/mL (mmol)               | m/g (mmol)  |              |
| 2.50 (23.8) | 8.25 (35.7)                | 10.0 (130.7)              | 6.95 (17.3) | 73           |
| 2.51 (23.9) | 8.25 (35.7)                | 10.0 (130.7)              | 6.91 (17.2) | 72           |

*O*-dodecanoylserine (**2**); white solid; *R*<sub>f</sub> = 0.10 (DCM/MeOH 5:1). The <sup>1</sup>H-NMR obtained is according to the described in the literature. Adapted from literature <sup>18</sup>.

### B) Synthesis of *O*-dodecanoyl-*N,N*-dimethylserine (**3**)



To a solution of **3** in DCE (10 mL) triethylamine (2 eq.) was added, and the mixture was stirred for 15 min to liberate the amine from the salt. Formaldehyde (37%, 3.0 eq) was added and the mixture was stirred for 2h before addition of NaBH(OAc)<sub>3</sub> (3,5-4,0 eq). The mixture was stirred at rt for another 2h, and then quenched with HCl 1M (20 mL). The product was extracted with DCM (3 x 20 mL). The DCM extracts were dried (Na<sub>2</sub>SO<sub>4</sub>). The solvent was removed and the white solid obtained recrystallized from diethyl ether. The solid obtained was identified by <sup>1</sup>H NMR as compound **3**. All reactions performed are listed in **Table 4.4**.

**Table 4.4.** Synthesis of **3**.

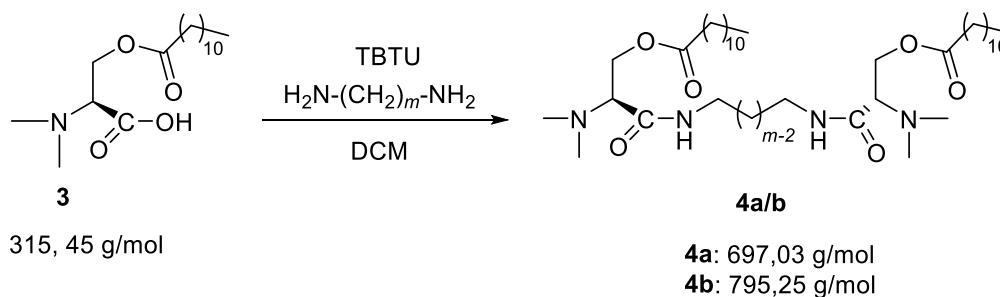
| <b>2a</b>   | <b>HCHO</b> | <b>NEt<sub>3</sub></b> | <b>NaBH(OAc)<sub>3</sub></b> | <b>3a</b>   | <b>η (%)</b> |
|-------------|-------------|------------------------|------------------------------|-------------|--------------|
| m/g (mmol)  | V/mL (mmol) | V/mL (mmol)            | m/g (mmol)                   | m/g (mmol)  |              |
| 2.09 (4.95) | 1.48 (15.0) | 1.37 (9.90)            | 4.20 (19.8)                  | 1.50 (4.75) | 96           |
| 1.99 (4.95) | 1.48 (15.0) | 1.37 (9.90)            | 4.20 (19.8)                  | 1.45 (4.59) | 93           |
| 1.51 (3.76) | 1.12 (11.2) | 1.30 (7.52)            | 3.18 (15,0)                  | 1.09 (3.46) | 92           |
| 1.16 (2.89) | 0.32 (8.67) | 0,80 (5.78)            | 2.45 (11.6)                  | 0.84 (2.97) | 92           |
| 2.02 (5.03) | 1.48 (15.0) | 1.39 (10,0)            | 4.26 (20.1)                  | 1.41 (4.47) | 89           |

The detailed description of  $^1\text{H}$ -NMR spectra of **3** is presented below:

O-dodecanoyl-*N,N*-dimethylserine (**3**); white solid;  $R_f = 0.44$  (DCM/MeOH 5:1).

$^1\text{H}$ -NMR (MeOD; 400MHz)  $\delta$  6.67-4.62 (m, 2H,  $-\text{CH}_2\text{OC(O)}(\text{CH}_2)_{10}\text{CH}_3$ ); 3.92-3.87 (m, 1H,  $\text{CH}-\text{CH}_2\text{OC(O)}(\text{CH}_2)_{10}\text{CH}_3$ ); 2.96 (bs, 6H,  $-\text{N}(\text{CH}_3)_2$ ); 2.40 (t, 2H,  $J = 7.4$  Hz  $-\text{CH}_2\text{OC(O)}\text{CH}_2\text{CH}_2-$ ); 1.70-1.53 (m, 2H,  $-\text{CH}_2\text{OCOCH}_2\text{CH}_2\text{CH}_2-$ ); 1.40-1.21 (bs, 16H,  $-\text{CH}_2(\text{CH}_2)_8\text{CH}_3$ ); 0.92 (t, 3H,  $J = 6.8$  Hz,  $\text{CH}_2-\text{CH}_2-\text{CH}_3$ ).

**C) Synthesis of (2*S*, 2'*S*)-*N,N'*-[(pentane-1,5-diyl)-bis(2-carbamoyl-2-dimethylamino)ethyl] didodecanoate (**4a**) and (2*S*, 2'*S*)-*N,N'*-[(dodecane-1,12-diyl)-bis(2-carbamoyl-2-dimethylamino)ethyl] didodecanoate (**4b**)**



O-dodecanoyl-*N,N*-dimethylserine (2 eq.) (**3**) and DIEA (2.0-2.5eq.) were dissolved in DCM (30 mL). The solution was cooled to  $0^\circ\text{C}$ , and TBTU (2 eq.) was added, followed by pentane-1,5-diamine or dodecane-1,12-diamine (1 eq.). The reaction was allowed to proceed at rt for 3 days. The reaction mixture was washed, successively, with an aqueous solution of 1% HCl (3 x 20 mL), saturated aqueous solution of  $\text{NaHCO}_3$  (3 x 20 mL) and water (3 x 20 mL). The organic layer was dried ( $\text{Na}_2\text{SO}_4$ ) and the solvent was evaporated. The crude product was submitted to column chromatography on silica gel (DCM/MeOH 20:1) The products **4a/b** were obtained as colorless oils (56-76% yields) and their structures were confirmed by  $^1\text{H}$  NMR. **Table 4.5.** summarizes all the experiments carried out as well as the results obtained.

Table 4.5.Synthesis of **4a/b**

| <b>3a</b>   | <b>Pentane-1,5-diamine</b> | <b>DIEA</b> | <b>TBTU</b> | <b>4a</b>   | $\eta$ (%) |
|-------------|----------------------------|-------------|-------------|-------------|------------|
| m/g (mmol)  | V/mL (mmol)                | V/mL (mmol) | m/g (mmol)  | m/g (mmol)  |            |
| 1.56 (4.95) | 0.29 (2.47)                | 1.33 (9.90) | 3.17 (9.90) | 1.07 (1.53) | 62         |
| 1.52 (4.81) | 0.28 (2.38)                | 1.30 (9.67) | 3.10 (9.68) | 0,97 (1.39) | 56         |

| <b>3a</b>   | <b>Dodecane-1,12-diamine</b> | <b>DIEA</b> | <b>TBTU</b> | <b>4b</b>   | $\eta$ (%) |
|-------------|------------------------------|-------------|-------------|-------------|------------|
| m/g (mmol)  | m/g (mmol)                   | V/mL (mmol) | m/g (mmol)  | m/g (mmol)  |            |
| 0.85 (2.70) | 0.27 (1.35)                  | 1.00 (5.84) | 1.75 (5.45) | 0.72 (0.90) | 66         |
| 0.53 (1.68) | 0.17 (0.85)                  | 0.60 (3.50) | 0.85 (2.65) | 0.46 (0.71) | 68         |

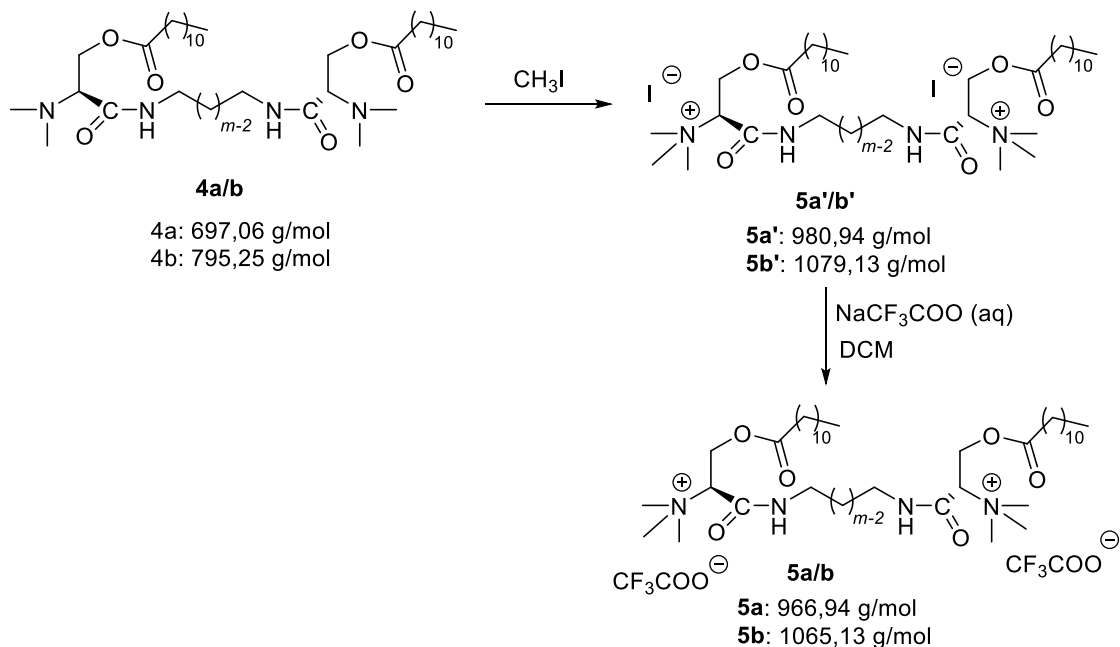
The detailed description of  $^1\text{H}$ -NMR spectra of **4a/b** is presented below:

(2S,2'S)-*N,N'*-[(pentane-1,5-diyl)-bis(2-carbamoyl-2-dimethyl-amino)ethyl] didodecanoate (**4a**); colorless oil; Rf 0,42 (DCM:MeOH 10:1)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz): 7.34-7.27 (m, 2H, 2x-NHCO); 4.54 (dd, 2H,  $J = 12.0, 4.0$  Hz, 2 x  $-\text{HCHOC}(\text{O})(\text{CH}_2)_{10}\text{CH}_3$ ); 4.37 (dd, 2H,  $J = 12.0, 6.8$  Hz, 2 x  $-\text{HCHOC}(\text{O})(\text{CH}_2)_{10}\text{CH}_3$ ); 3.30-3.20 (m, 6H, 2 x  $\text{C}^*\text{H} + \text{CONHCH}_2(\text{CH}_2)_3\text{CH}_2\text{NHCO}-$ ); 2.36 (bs, 6H, 2 x  $-\text{N}(\text{CH}_3)_2$ ); 2.28 (t, 4H,  $J = 7.6$ Hz, 2x  $\text{OC}(\text{O})\text{CH}_2(\text{CH}_2)_9\text{CH}_3$ ); 1.65-1.47 (m, 8H,  $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO} + 2 \times \text{OC}(\text{O})\text{CH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_3$ ); 1.40-1.16 (bs, 34H,  $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO}$ , 2 x  $\text{CH}_2(\text{CH}_2)_8\text{CH}_3$ ); 0.86 (t, 6H,  $J = 7.0$  Hz 2 x  $-\text{CH}_2\text{CH}_2\text{CH}_3$ )

(2S,2'S)-*N,N'*-[(dodecane-1,12-diyl)-bis(2-carbamoyl-2-dimethylamino)ethyl] didodecanoate (**4b**); colorless oil; Rf 0,45 (DCM:MeOH 10:1)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz): 7.30-7.26 (m, NHCO; overlapped with  $\text{CDCl}_3$  peak); 4.54 (dd, 2H,  $J = 12.0, 3.8$  Hz, 2 x  $-\text{HCHOC}(\text{O})(\text{CH}_2)_{10}\text{CH}_3$ ); 4.38 (dd, 2H,  $J = 12.0, 6.4$  Hz, 2 x  $-\text{HCHOC}(\text{O})(\text{CH}_2)_{10}\text{CH}_3$ ); 3.36-3.16 (m, 6H, 2 x  $\text{C}^*\text{H} + \text{CONHCH}_2(\text{CH}_2)_8\text{CH}_2\text{NHCO}-$ ); 2.38 (bs, 6H, 2 x  $-\text{N}(\text{CH}_3)_2$ ); 2.30 (t, 4H,  $J = 7.6$ Hz, 2 x  $-\text{OC}(\text{O})\text{CH}_2(\text{CH}_2)_9\text{CH}_3$ ) 1.64-1.56 and 1.54-1.44 (2m, 8H,  $\text{CONHCH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_2\text{CH}_2\text{NHCO} + 2 \times \text{COCH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_3$ ); 1.35-1.20 (bs, 48H,  $\text{CONHCH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_2\text{CH}_2\text{NHCO}$ , 2 x  $\text{CH}_2(\text{CH}_2)_8\text{CH}_3$ ); 0.87 (t, 6H,  $J = 6.8$  Hz, 2 x  $-\text{CH}_2\text{CH}_2\text{CH}_3$ ).

The spectra show traces of TMU peak at 2.80 ppm.

**D) Synthesis of N,N'-(pentane-1,5-diyl)-bis {[1-carbamoyl-2-(dodecanoyloxy)ethyl]-trimethylammonium} bis(trifluoroacetate) (5a) and N,N'-(dodecane-1,12-diyl)-bis{[1-carbamoyl-2-(dodecanoyloxy)ethyl]trimethylammonium} bis(trifluoroacetate) (5b)**



Compound **4a/b** was dissolved in CH<sub>3</sub>I (10-11eq.) and the mixture was stirred at room temperature for 48h until the reactants were consumed (TLC). The solvent was evaporated to give the crude ammonium salt, which was taken up in ethyl acetate (3 x 20 mL) and washed with sodium thiosulfate solution (10%; 3 x 20 mL). The ethyl acetate layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and, after removal of the solvent, product **5a/5b** was subjected to column chromatography on silica gel using DCM/MeOH (10:1) as eluent. All the reactions performed are listed in **Table 4.6**.

**Table 4.6.** Synthesis of **5a/b**.

| <b>4a</b>         | <b>CH<sub>3</sub>I</b> | <b>5a'</b>        | <b>η (%)</b> |
|-------------------|------------------------|-------------------|--------------|
| <b>m/g (mmol)</b> | <b>V/mL (mmol)</b>     | <b>m/g (mmol)</b> |              |
| 0.97 (1.39)       | 1.00 (16.1)            | 1.07 (1.09)       | 79           |
| 0.54 (0.77)       | 0.50 (8.03)            | 0.68 (0.69)       | 90           |
| <b>4b</b>         | <b>CH<sub>3</sub>I</b> | <b>5b'</b>        | <b>η (%)</b> |
| <b>m/g (mmol)</b> | <b>V/mL (mmol)</b>     | <b>m/g (mmol)</b> |              |
| 1.28 (1.83)       | 1.13 (18.3)            | 1.78 (1.64)       | 90           |

*N,N'*-(pentane-1,5-diyl)-bis{[1-carbamoyl-2-(dodecanoyloxy)ethyl]-trimethylammonium} diiodide (**5a'**) yellow oil; R<sub>f</sub> 0,42 (DCM:MeOH 10:1) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 9.09-9.03 (m, 2H, 2x-NHCO); 5.33-5.30 (m, 2H, 2 x -C\*H); 4.73 (dd, 2H, *J* = 13.2, 6.8 Hz, 2 x -HCHOC(O)(CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>); 4.63 (dd, 2H, *J* = 13.2, 4.4 Hz, 2 x -HCHOC(O)(CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>); 3.50 (bs, 18H, 2 x -N(CH<sub>3</sub>)<sub>3</sub>); 3.56-3.42 and 3.28-3.18 (2m, 4H, CONHCH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>NHCO-); 2.38 (t, 4H, *J* = 7.6 Hz, 2x-COCH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>); 1.68-1.56 (m, 8H, CONHCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>2</sub>CH<sub>2</sub>NHCO + 2 x OC(O)CH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>); 1.35-1.25 (bs, 34H, CONHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCO, 2 x CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>); 0.89 (t, 6H, *J* = 6.8 Hz 2 x -CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>)

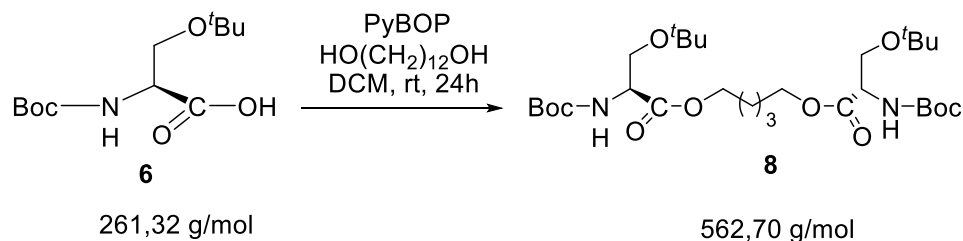
*N,N'*-(dodecane-1,12-diyl)-bis{[1-carbamoyl-2-(dodecanoyloxy)ethyl]trimethylammonium} diiodide (**5b'**) yellow oil; R<sub>f</sub> 0,42 (DCM:MeOH 10:1) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 9.14-9.08 (m, 2H, 2x-NHCO); 5.44 (m, 2H, 2 x -C\*H); 4.66 (dd, 2H, *J* = 12.8, 6.8 Hz, 2 x -HCHOC(O)(CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>); 4.50 (dd, 2H, *J* = 13.0, 5.4 Hz, 2 x -HCHOC(O)(CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>); 3.48 (bs, 6H, 2 x -N(CH<sub>3</sub>)<sub>3</sub>); 3.40-3.32 and 3.23-3.12 (2m, 4H, CONHCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>NHCO-); 2.35 (t, 4H, *J* = 7.6 Hz, 2 x -OC(O)CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>); 1.64-1.52 (m, 8H, CONHCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>2</sub>CH<sub>2</sub>NHCO + 2 x OC(O)CH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>); 1.40-1.16 (bs, 48H, CONHCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>2</sub>CH<sub>2</sub>NHCO, 2 x CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>); 0.87 (t, 6H, *J* = 6.8 Hz 2 x --CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>)

### **Conversion of 5a'/b' into the corresponding trifluoroacetate salts 5a/b**

Compound **5a'/b'** was dissolved in H<sub>2</sub>O and (10 eq) of NaHCO<sub>3</sub> and TFA were added. The mixture was stirred at room temperature for 3h. After this time, liquid-liquid extractions with DCM (2 x 20 mL) were performed. The DCM extracts were dried (Na<sub>2</sub>SO<sub>4</sub>) and the solvent evaporated. The products were analyzed by ESI-MS to confirm the counterion exchange (negative mode).

### 4.2.3. Synthesis of the ester series

#### A) Synthesis of (2*S*,2'*S*)-*O*,*O*'-Pentane-1,5-diyl-bis[3-*tert*-butyloxy-2-(*tert*-butyloxy carbonylamino)propanoate] (**8**)

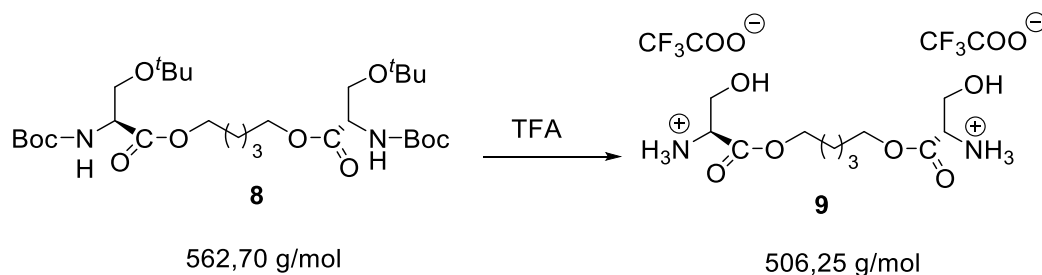


Serine derivative **6** (2,03 g; 7,7 mmol) was dissolved in DCM (30 mL) and DIEA (3,0 mL; 17 mmol) was added. The mixture was stirred 1h at room temperature. After this time, a solution of pentanodiol (0,39 g; 3,8 mmol) in DCM (20 mL) was added. The reaction was allowed to proceed at room temperature for 24h. The reaction mixture was washed, successively, with an aqueous solution of 1% HCl (2 x 10 mL), saturated solution of NaHCO<sub>3</sub> (2 x 10 mL) and water (10 mL). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and the solvent was evaporated. The crude product was submitted to column chromatography on silica gel (hexane/ethyl acetate 3:1) to yield the product (**8**) as a colorless oil (1,73 g; 3,07 mmol) with 81% yield.

The detailed description of <sup>1</sup>H-NMR spectrum of **8** is presented below:

(2*S*,2'*S*)-*O*,*O*'-Pentane-1,5-diyl-bis[3-*tert*-butyloxy-2-(*tert*-butyloxycarbonylamino)-propanoate] (**8**) colorless oil; R<sub>f</sub> 0,38 (Hexane/AcOEt 3:1) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): 5.37 (d, 2H, *J* = 8.8 Hz, 2x-NHCO); 4.38 (dt, 2H, *J* = 8.8, 2.8 Hz, -C\*H); 4.22-4.08 (m, 4H, -C(O)OCH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OC(O)-) 3.81 (dd, 2H, *J* = 8.8, 2.4 Hz, 2 x -HCHO<sup>t</sup>Bu); 3.58 (dd, 2H, *J* = 8.8, 3.2 Hz, 2 x -HCHO<sup>t</sup>Bu); 1.74-1.62 (m, 6H, -C(O)OCH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OC(O)-); 1.47 (bs, 18H, 2 x (CH<sub>3</sub>)<sub>3</sub>OC(O)NH-); 1.15 (bs, 18H, 2 x -OC(CH<sub>3</sub>)<sub>3</sub>). Spectrum shows traces of ethyl acetate and DCM.

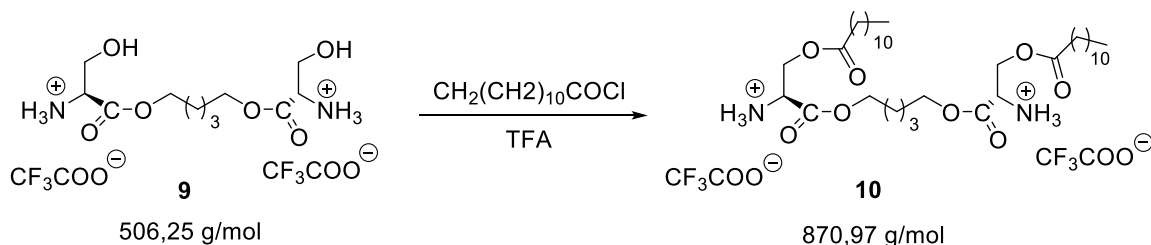
**B) Synthesis of (2S,2'S)-{O,O'-(Pentane-1,5-diyl)-bis[(2-hydroxy-1-oxycarbonyl)ethyl] trimethylammonium}} bistrifluoroacetate (9)**



The removal of both the *tert*-butyloxycarbonyl (Boc) and the *tert*-butyl group was accomplished by stirring compound **8** (1,70 g; 3.02 mmol) with TFA (3,0 mL) for 48h at room temperature. The mixture was treated with diethyl ether followed by evaporation until total removal of TFA. The product (1,4 g; 2,76 mmol) was obtained as a brown oil (93% yield) and identified by ESI-MS.

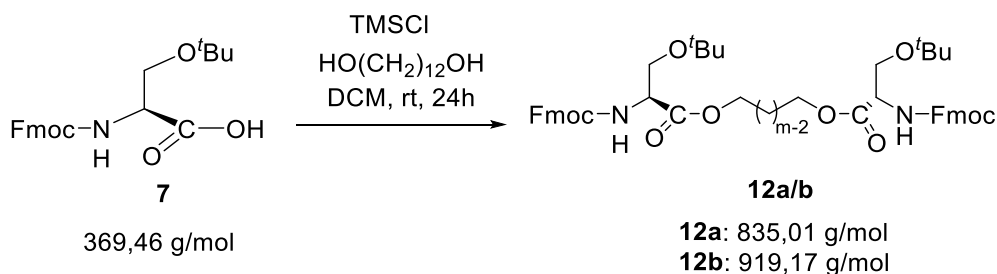
R<sub>f</sub> 0,10 (DCM/MeOH 10:1); **MS** (ESI, MeOH) calcd. 392.33 [M+CF<sub>3</sub>COO]<sup>+</sup>; found 392.27.

**C) Synthesis of (2S,2'S)-{O,O'-(Pentane-1,5-diyl)-bis[(2-dodecanoiloxy-1-oxycarbonyl)ethyl]trimethylammonium}} bistrifluoroacetate (10)**



Gemini precursor **9** (1.0 g; 1,98 mmol) was dissolved in TFA (1.5 mL; 20 mmol) and stirred for 15 minutos at 0°C. Then, dodecylchloride was added and the reaction was allowed to proceed for 4h. The mixture was treated with diethyl ether followed by evaporation until total removal of TFA. The residue was submitted to column chromatography on silica gel (DCM/MeOH 20:1) and two fractions, homogeneous by TLC, were isolated, and identified (<sup>1</sup>H-NMR) as the remaining dodecanoic acid and a mixture containing **10** and a by-product of the reaction ("amide" **11**, see **Scheme 4.8**; section 4.3.2).

**D) Synthesis (12b) of (2S,2'S)-O,O'-Pentane-1,5-diyl-bis[3-tert-butyloxy-2-(fluorenyloxy-carbonilamino)propanoate] (12a) and (2S,2'S)-O,O'-Dodecane-1,12-diyl-bis[3-tert-butyloxy-2-(fluorenyloxy-carbonilamino)propanoate]**



To a solution of **7** (1 mmol) in DCM (30 mL) was added TMSCl (1.8 mmol) and the mixture was stirred at room temperature for 1h. Then, pentane-1,5-diol or dodecane-1,12-diol (0.5 mmol) was added, and the mixture was allowed to react 48h. After this time, the solvent was evaporated and the crude subjected to a column chromatography using hexane/ethyl acetate (3:1) as eluent. The gemini precursors **12a** and **12b** were obtained as colorless oils. All the reactions performed are presented in **Table 4.7**.

**Table 4.7.** Synthesis of **12a/b**.

| 7          |        | Pentane-1,5-diol |        | TMSCl       |        | 12a        |        | $\eta$ (%) |
|------------|--------|------------------|--------|-------------|--------|------------|--------|------------|
| m/g (mmol) |        | V/mL (mmol)      |        | V/mL (mmol) |        | m/g (mmol) |        |            |
| 4,69       | (12,7) | 0,65             | (6,20) | 2,80        | (22,1) | 4,11       | (4,92) | 94         |
| 2,34       | (6,33) | 0,35             | (3,34) | 1,40        | (11,1) | 2,55       | (3,05) | 91         |

| 7          |        | Dodecane-1,12-diol |        | TMSCl       |        | 12b        |        | $\eta$ (%) |
|------------|--------|--------------------|--------|-------------|--------|------------|--------|------------|
| m/g (mmol) |        | m/g (mmol)         |        | V/mL (mmol) |        | m/g (mmol) |        |            |
| 5,25       | (14,2) | 1,39               | (6,89) | 3,15        | (24,9) | 3,11       | (3,38) | 53         |
| 4,00       | (10,8) | 1,07               | (5,27) | 2,40        | (19,0) | 3,48       | (3,79) | 78         |

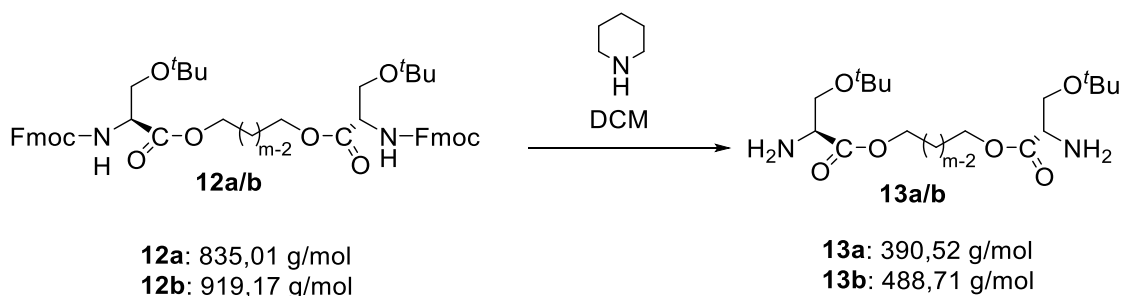
(2S,2'S)-O,O'-Pentane-1,5-diyl-bis[3-*tert*-butyloxy-2-(fluorenyloxy-carbonilamino)-propanoate] (**12a**) colorless oil;  $R_f$  0.30 (DCM/MeOH 10:1)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz): 7.79 (d, 2H,  $J = 7.6$  Hz, 2 x H6 + 2 x H6'); 7.64 (t, 4H,  $J = 7.0$  Hz, 2 x H3 + 2 x H3'); 7.42 (t, 4H,  $J = 7.4$  Hz, 2 x H5 + 2 x H5'); 7.34 (t, 4H,  $J = 7.4$  Hz, 2 x H4 + 2 x H4'); 5.69 (d, 2H,  $J = 8.8$  Hz, 2 x -NH-); 4.46-4.32 (m, 6H, 2 x H1 + 2 x H2); 4.31-4.16 (m, 6H, 2 x -C\*H + -C(O)OCH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OC(O)-); 3.90-3.80 (m, 2H, -HCHO<sup>*t*</sup>Bu); 3.68-3.60 (m, 2H, -HCHO<sup>*t*</sup>Bu); 1.75-1.68 (m, 2H, -COOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>OC(O)-); 1.51-1.43 (m, 2H, -COOCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>OC(O)-); 1.18 (bs, 18H, 2 x O<sup>*t*</sup>Bu).

The spectrum shows contamination with ethyl acetate.

2S,2'S)-O,O'-Dodecane-1,12-diyl-bis[3-*tert*-butyloxy-2-(fluorenyloxycarbonilamino)-propanoate] (**12b**) colorless oil;  $R_f$  0.30 (DCM/MeOH 10:1)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz): 7.67 (d, 2H,  $J = 7.2$  Hz, 2 x H6 + 2 x H6'); 7.53 (t, 4H,  $J = 7.2$  Hz, 2 x H3 + 2 x H3'); 7.30 (t, 4H,  $J = 7.4$  Hz, 2 x H5 + 2 x H5'); 7.22 (t, 4H,  $J = 7.4$  Hz, 2 x H4 + 2 x H4'); 5.69 (d, 2H,  $J = 8.8$  Hz, 2 x -NH-); 4.41-4.22 (m, 6H, 2 x H1 + 2 x H2); 4.20-4.04 (m, 6H, 2 x -C\*H + -COOCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>OC(O)-); 3.74 (dd, 2H,  $J = 9.0, 2.6$  Hz, -HCHO<sup>t</sup>Bu); 3.68-3.60 (m, 2H,  $J = 9.0, 2.6$  Hz -HCHO<sup>t</sup>Bu); 1.60-1.46 and 1.28-1.15 (m, 20H, -COOCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>OC(O)-); 1.18 (bs, 18H, 2 x O<sup>t</sup>Bu).

The spectrum shows contamination with ethyl acetate

**E) Synthesis of (2S,2'S)-O,O'-Pentane-1,5-diyl-bis[2-amino-3-(*tert*-butyloxy)-propanoate] (**13a**) and (2S,2'S)-O,O'-Dodecane-1,12-diyl-bis[2-amino-3-(*tert*-butyloxy)propanoate] (**13b**)**



Removal of the Fmoc group was accomplished by stirring gemini precursor **12a/b** with piperidine (5 eq.) in DCM for 24h at room temperature. The solvent was evaporated and the crude was submitted to column chromatography on silica gel (DCM/MeOH 20:1 to yield the product **13a/13b** as colorless a oil. All the reactions carried out are listed in **Table 4.8**.

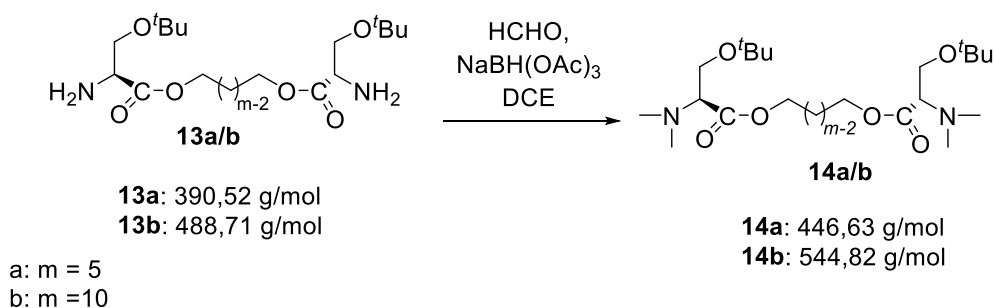
**Table 4.8.** Synthesis of **13a/13b**.

| <b>12a</b>  |  | <b>Piperidine</b> |  | <b>13a</b>  |  | $\eta$ (%) |
|-------------|--|-------------------|--|-------------|--|------------|
| m/g (mmol)  |  | V/mL (mmol)       |  | m/g (mmol)  |  |            |
| 1,53 (1,83) |  | 1,00 (10,1)       |  | 0,51 (1,30) |  | 71         |
| 2,57 (3,08) |  | 1,60 (16,2)       |  | 1,14 (2,93) |  | 95         |
| 2,85 (3,41) |  | 1,70 (17,2)       |  | 1,26 (3,23) |  | 76         |
| <b>12b</b>  |  | <b>Piperidine</b> |  | <b>13b</b>  |  | $\eta$ (%) |
| m/g (mmol)  |  | V/mL (mmol)       |  | m/g (mmol)  |  |            |
| 1,81 (1,94) |  | 1,00 (10,1)       |  | 0,58 (1,19) |  | 61         |
| 1,28 (1,37) |  | 0,70 (7,09)       |  | 0,65 (1,33) |  | 98         |
| 3,48 (3,73) |  | 1,90 (19,2)       |  | 1,33 (2,72) |  | 73         |

(2S,2'S)-O,O'-Pentane-1,5-diyl-bis[2-amino-3-(*tert*-butoxy)propanoate] (**13a**) colorless oil;  $R_f$  0.20 (DCM/MeOH 10:1)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz): 4.13 (dt, 4H,  $J = 6.8, 1.6$  Hz-  $\text{C}(\text{O})\text{OCH}_2(\text{CH}_2)_3\text{CH}_2\text{OC}(\text{O})$ ); 3.70-3.50 (m, 6H,  $2 \times -\text{CH}_2\text{O}^t\text{Bu} + 2 \times -\text{C}^*\text{H}$ ); 1.95-1.85 (bs, 4H,  $2 \times \text{NH}_2$ ); 1.70-1.60 (m, 4H and 1.50-1.40 (2m, 2H,  $-\text{COOCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OC}(\text{O})$ ); 1.14 (bs, 18H,  $2 \times \text{O}^t\text{Bu}$

(2S,2'S)-O,O'-Dodecane-1,12-diyl-bis[2-amino-3-(*tert*-butoxy)propanoate] (**13b**) colorless oil;  $R_f$  0.20 (DCM/MeOH 10:1)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz): 4.20-4.10 (m, 4H,  $-\text{C}(\text{O})\text{OCH}_2(\text{CH}_2)_{10}\text{CH}_2\text{OC}(\text{O})$ ); 3.68-3.57 (m, 6H,  $2 \times -\text{CH}_2\text{O}^t\text{Bu} + 2 \times -\text{C}^*\text{H}$ ); 1.96-1.90 (bs, 4H,  $2 \times \text{NH}_2$ ); 1.70-1.60 and 1.40-1.26 (2m, 20H,  $-\text{COOCH}_2(\text{CH}_2)_{10}\text{CH}_2\text{OC}(\text{O})$ ); 1.18 (bs, 18H,  $2 \times \text{O}^t\text{Bu}$ ).

**G) Synthesis of (2S,2'S)-O,O'-Pentane-1,5-diyl-bis[3-(*tert*-butoxy)-2-(dimethylamino)-propanoate] (**14a**) and (2S,2'S)-O,O'-Dodecane-1,12-diyl-bis[3-(*tert*-butoxy)-2-(dimethylamino)propanoate] (**14b**)**



Compound **13a/b** and formaldehyde (4 eq.) were dissolved in DCE and stirred at room temperature for 30 minutes, and then sodium triacetoxyborohydride (6 eq.) was added. The mixture was stirred at room temperature under an argon atmosphere for 12 h, quenched with water (10 mL) and the product was extracted with DCM (3 x 20 mL). The organic extracts were dried ( $\text{Na}_2\text{SO}_4$ ) and the solvent was evaporated to yield product **14a/b** (colorless oil). In **Table 4.9** are listed all the experiences performed.

Table 4.9. Synthesis of **14a/b**.

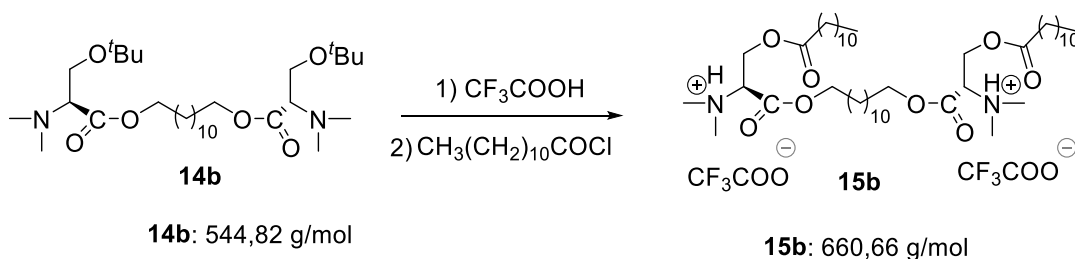
| 13a        |        | HCHO        |        | NEt <sub>3</sub> |        | NaBH(OAc) <sub>3</sub> |        | 14a        |        | η (%) |
|------------|--------|-------------|--------|------------------|--------|------------------------|--------|------------|--------|-------|
| m/g (mmol) |        | V/mL (mmol) |        | V/mL (mmol)      |        | m/g (mmol)             |        | m/g (mmol) |        |       |
| 0,51       | (1,30) | 0,60        | (21,8) | 1,00             | (7,12) | 1,88                   | (8,85) | 0,51       | (1,30) | 62    |
| 1,14       | (2,93) | 1,00        | (36,3) | 1,70             | (12,1) | 3,68                   | (17,3) | 1,04       | (2,32) | 80    |

| 13b        |        | HCHO        |        | NEt <sub>3</sub> |        | NaBH(OAc) <sub>3</sub> |        | 14b        |        | η (%) |
|------------|--------|-------------|--------|------------------|--------|------------------------|--------|------------|--------|-------|
| m/g (mmol) |        | V/mL (mmol) |        | V/mL (mmol)      |        | m/g (mmol)             |        | m/g (mmol) |        |       |
| 0,58       | (1,19) | 0,45        | (16,3) | 0,75             | (5,34) | 1,48                   | (6,97) | 0,580      | (1,19) | 90    |
| 1,33       | (2,72) | 1,00        | (36,3) | 2,00             | (14,2) | 3,27                   | (15,4) | 0,651      | (1,33) | 93    |

(2S,2'S)-O,O'-Pentane-1,5-diyl-bis[3-(*tert*-butyloxy)-2-(dimethylamino)propanoate] (**14a**), colorless oil; R<sub>f</sub> 0.30 (DCM/MeOH 10:1) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): 4.19-4.14 (m, 4H, -COOCH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OC(O)-); 3.76-3.70 (m, 2H, 2 x -C\*H); 3.63-3.57 (m, 2H, 2x -HCHO'Bu); 3.34-3.31 (m, 2H, 2x -HCHO'Bu) 2.41 (bs, 12H, 2 x -N(CH<sub>3</sub>)<sub>2</sub>) 1.76-1.68 and 1.62-1.44 (2 x m, 6H, -COOCH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OC(O)-); 1.19 (bs, 18H, 2 x O'Bu).

(2S,2'S)-O,O'-Dodecane-1,12-diyl-bis[3-(*tert*-butyloxy)-2-(dimethylamino)propanoate] (**14b**), colorless oil; R<sub>f</sub> 0.30 (DCM/MeOH 10:1) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): 4.18-4.11 (m, 4H, -COOCH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OC(O)-); 3.71 (dd, 2H, *J* = 8.6, 7.4 Hz, 2 x -C\*H); 3.60 (dd, 2H, *J* = 8.8, 5.6 Hz); 2x -HCHO'Bu); 3.32 (dd, 2H, *J* = 7.2, 5.6 Hz, 2x -HCHO'Bu) 2.40 (bs, 12H, 2 x -N(CH<sub>3</sub>)<sub>2</sub>) 1.70-1.61 and 1.40-1.24 (2 x m, 20H, -COOCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>OC(O)-); 1.19 (bs, 18H, 2 x O'Bu).

**I) Synthesis of (2S,2'S)-{O,O'-(dodecane-1,5-diyl)-bis[(2-dodecanoiloxy-1-oxy-carbonyl)ethyl]diethylammonium}] bistrifluoroacetate (**15b**)**



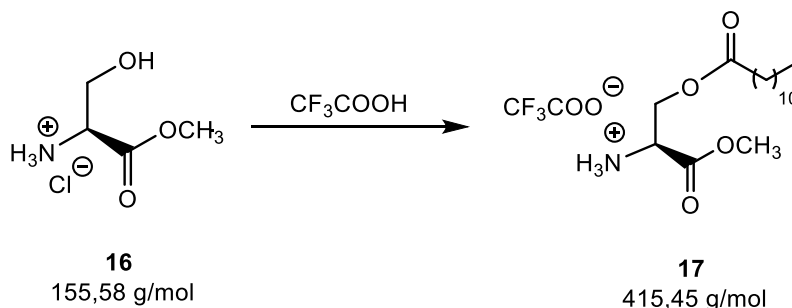
The removal of the *tert*-butyl groups was accomplished by stirring of gemini precursor **14b** (0,92 g; 1,7 mmol) with TFA (0,53 mL; 5eq.) for 24h at room temperature. The crude was treated with diethyl ether followed by evaporation until total removal of TFA.

The resulting product was dissolved in TFA (1,0 mL), dodecyl chloride was added (0,80 mL; 3,5 mmol), and the reaction mixture was stirred at room temperature for 4h. After successive washings with diethyl ether, the crude product was submitted to column chromatography on silica gel (DCM/MeOH 3:1) to yield the product (**15b**; 0,21 g; 0,32 mmol;  $\eta$  = 19 %) as a yellow oil.

(2S,2'S)-{O,O'-(dodecane-1,5-diyl)-bis[(2-dodecanoiloxy-1-oxy-carbonyl)ethyl]diimethyl-ammonium}} bistrifluoroacetate **15b**; colorless oil;  $R_f$  = 0.33 (DCM/MeOH 10:1) **<sup>1</sup>H NMR** (MeOD, 400 MHz): 4.18-4.11 (d, 4H,  $J$  = 6.4Hz, 2 x -CH<sub>2</sub>OCO(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>); 4.12-4.04 (m, 4H, C(O)OCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>OC(O)-), 4.45 (t, 2H,  $J$  = 6.6 Hz, 2 x -C\*H); 2.40 (bs, 12H, 2 x -N(CH<sub>3</sub>)<sub>2</sub>); 2.27 (t, 4H,  $J$  = 7.6 Hz, 2 x -OC(O)CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>); 1.66-1.51 and 1.40-1.24 (2 x m, 58H, -C(O)OCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>OC(O)- + OC(O)CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>); 0,84 (t, 6H,  $J$  = 6.8 Hz).

#### 4.2.4. Synthesis of the amine series

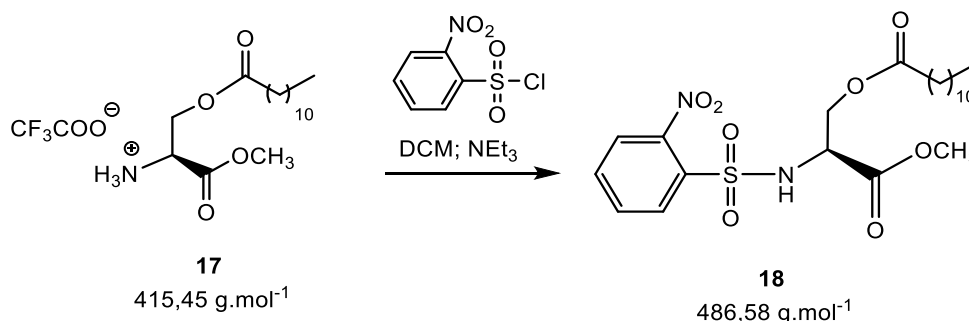
##### A) Synthesis of (S)-[2-(dodecanoyloxy)-(1-methyloxycarbonyl)]ethyl ammonium trifluoroacetate (**17**)



The introduction of the alkyl chain into the hydroxyl group of the amino acid residue (**1**) was performed according to the procedure previously described in section 4.1.1 The desired product was obtained after work-up as a white solid with 87% yield (2.44g; 5.87 mmol).

(S)-[2-(dodecanoyloxy)-(1-methyloxycarbonyl)ethyl] ammonium trifluoroacetate (**17**);  $R_f$  = 0,23 (DCM/MeOH 20:1) e 0,17 (Hex/AcOEt 3:1); white solid. **<sup>1</sup>H NMR**:  $\delta$  4.76 (dd, 1H,  $J$  = 12.2, 2.6 Hz, -HCH<sub>2</sub>OH), 4.61 [dd,  $J$  = 12.2, 4.2 Hz, 1H, -HCH<sub>2</sub>OH], 4.55-4.53 (m, 1H, -C\*H), 3.84 (s, 3H, -O(CH<sub>3</sub>)), 2.47-2.35 (m, 2H, -OC(O)CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 1.68-1.56 (m, 2H, OC(O)CH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>), 1.25 (s, 16H, COCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>), 0.88 (t, 3H,  $J$  = 8.0 Hz, -CH<sub>3</sub>).

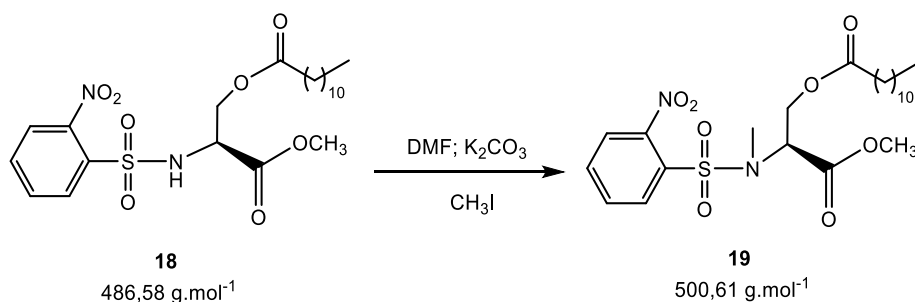
**B) Synthesis of (S)-[2-(2-nitrophenylsulfonamido)-3-(methyloxycarbonyl)ethyl] dodecanoate (18)**



O-acylated derivative **17** (2,52 g; 6,06 mmol) and triethylamine (2.10 mL; 15.0 mmol; 2.5 eq.) were dissolved in DCM (20 mL) and stirred for 15 minutes to liberate the amine from the salt. Then, 2-nitrobenzenesulfonyl chloride (1,48 g; 6.07 mmol; 1.1 eq.) was added and the mixture was allowed to proceed at room temperature for 20h. The mixture was washed with aq. HCl 10% (2 x 20 mL) and water (2 x 20 mL). After total removal of the solvent, the desired product (**18**) was obtained as a yellow oil with 97% yield (2.86 g; 5.88 mmol).

(S)-[2-(2-nitrophenylsulfonamido)-3-(methyloxycarbonyl)ethyl] dodecanoate (**18**);  $R_f = 0,24$  (DCM/MeOH 20:1) yellow oil.  $^1\text{H RMN}$  ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  8.12-8.06 (m, 1H, H3), 7.92-7.98 (m, 1H, H6), 7.78-7.72 [m, 2H, H4, H5], 6.42-6.32 (m, 1H, -NH-), 4.56-4.44 (m, 2H, -CH<sub>2</sub>OC(O)(CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>); 4.38-4.26 (m, 1H, -C\*H-), 3.59 (s, 3H, -OCH<sub>3</sub>), 2.27 (t, 2H,  $J = 6.0\text{Hz}$ , -OC(O)CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 1.66-1.50 (m, 2H, -OC(O)CH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>), 1.26 (sl, 16H, OC(O)CH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>), 0.88 (t, 3H,  $J = 6.9\text{Hz}$ , -CH<sub>3</sub>).

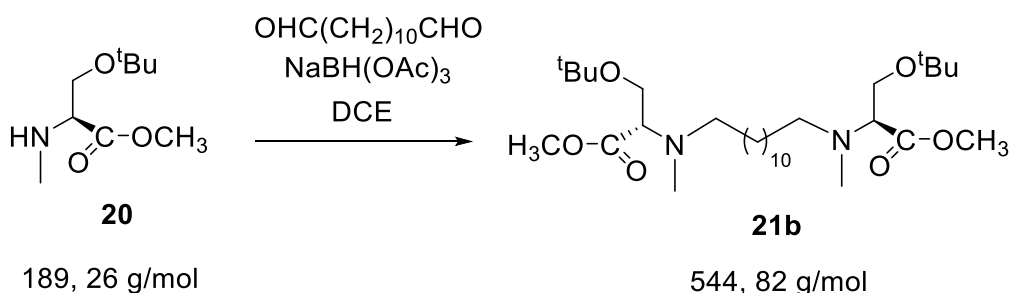
**C) Synthesis of (S)-[2-[N-methyl-(2-nitrophenylsulfonamido)]-3-(methyloxycarbonyl)ethyl] dodecanoate (19)**



To a solution of **18** (2.86 g; 5.88 mmol) in DMF was added sodium carbonate (0.97g; 7.05 mmol) and idomethane (2.20 mL; 35.3 mmol; 6.0 eq.), and the mixture was stirred at room temperature for 24h until the reactants were consumed (TLC). The solvent was evaporated, and the product was taken up in ethyl acetate (3 x 20 mL) and washed with sodium thiosulfate solution (10%; 3 x 20 mL). The ethyl acetate layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and, after removal of the solvent, product **19** was obtained as a yellow oil (1.41 g; 2.82 mmol;  $\eta$  = 48%).

(S)-{2-[N-methyl-(2-nitrophenylsulfonamido)]-3-(methyloxy-carbonyl)ethyl} dodecanoate (**19**);  $R_f$  = 0,47 (DCM/MeOH 20:1); yellow oil. <sup>1</sup>H RMN (CDCl<sub>3</sub>, 400 MHz):  $\delta$  8.11-8.06 (m, 1H, H3), 7.75-7.69 [m, 2H, H6, H4 (or H5)], 7.66-7.60 [m, 2H, H5 (or H4)], 4.81 (dd,  $J$  = 4.8, 7.6 Hz, -CH-), 4.07 (dd, 1H,  $J$  = 12.0, 4.8 Hz, -CH(H)OH), 3.97 (dd, 1H,  $J$  = 12.0, 8.0Hz, -CH(H)OH), 3.67 (s, 3H, -OCH<sub>3</sub>), 3.04 (s, 3H, -N(CH<sub>3</sub>)).

**D) Synthesis of methyl (2S,2'S)-N,N'-(pentane-1,5-diyl)-bis[(2-tert-butyloxy)-1-(methylamino)propanoate] 21b**



To a solution of **20** (previously synthesized in our group) in dichloroethane (5 mL) was added triethylamine and the mixture was stirred for 5 minutes. Dodecane-1,12-dial was added. The mixture remained under magnetic stirring at room temperature for 70 h and was then washed with water (20 mL). The organic phase was dried with anhydrous sodium sulphate. After evaporation of the solvent, the compound was identified by <sup>1</sup>H-NMR as the target compound (**21b**).

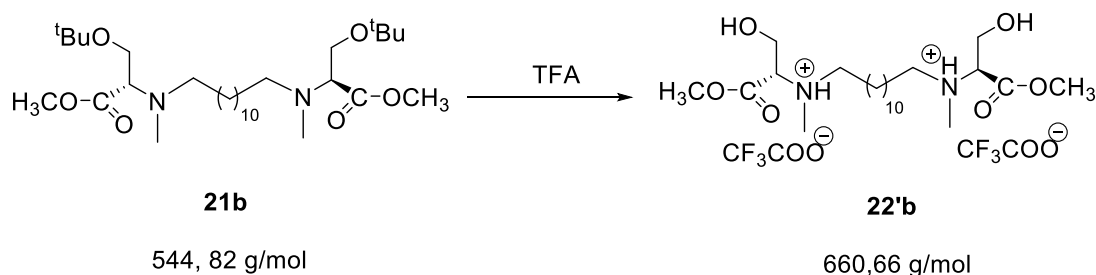
**Table 4.10.** summarizes all the experiments carried out as well as the results obtained.

Table 4.10. Synthesis of **21b**.

| 20           |      | Dodecane-1,12-dial |      | NaBH(OAc) <sub>3</sub> |      | 21b        |      | $\eta$ (%) |
|--------------|------|--------------------|------|------------------------|------|------------|------|------------|
| m/g (mmol)   |      | m/g (mmol)         |      | m/g (mmol)             |      | m/g (mmol) |      |            |
| <b>0,730</b> | 3,86 | 0,389              | 1,96 | 1,09                   | 5,14 | 0,698      | 1,28 | 66         |
| <b>0,806</b> | 4,26 | 0,453              | 2,29 | 1,12                   | 5,26 | 1,205      | 1,90 | 89         |
| <b>0,820</b> | 4,31 | 0,429              | 2,16 | 1,12                   | 5,27 | 0,934      | 1,71 | 80         |

Methyl (2S,2'S)-N,N'-(pentane-1,5-diyl)-bis[(2-*tert*-butoxy)-1-(methylamino)propanoate] **21b**;  $R_f$  = 0,25 Hex/AcOEt (3:1); yellow oil. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  3.72-3.68 (m + s, 8H, 2x -CH + 2 x -OCH<sub>3</sub>), 3.54 (dd, 2H,  $J$  = 8.4, 5.2Hz 2 x -CH(H)OtBu), 3.44 (dd, 1H,  $J$  = 8.0, 5.2Hz, 2x -CH(H)OtBu), 2.56-2.42 [m, 4H, 2 x N(CH<sub>3</sub>) CH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>N(CH<sub>3</sub>)], 2.33 [s, 6H, 2 x -NH(CH<sub>3</sub>)], 1.50-1.38 [m, 4H, 2 x N(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>2</sub>CH<sub>2</sub>N(CH<sub>3</sub>)], 1.26 [bs, 16H, N(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>2</sub>CH<sub>2</sub>N(CH<sub>3</sub>)], 1.17 (s, 9H, -C(CH<sub>3</sub>)<sub>3</sub>).

**E) Synthesis of (2S,2S')-N,N'-dodecane-1,12-diyl-bis{[2-hydroxy-1-(methyloxycarbonyl)ethyl]methylammonium trifluoroacetate}**



The procedure for removing *tert*-butyl groups of **21b** was analogous to the procedure described in section 4.12 - I. The mixture of **21b** with trifluoroacetic acid was stirred at room temperature during several hours (see **table 4.11**). The product obtained after work-up was chromatographically homogeneous and was identified by <sup>1</sup>H-NMR as the expected product.

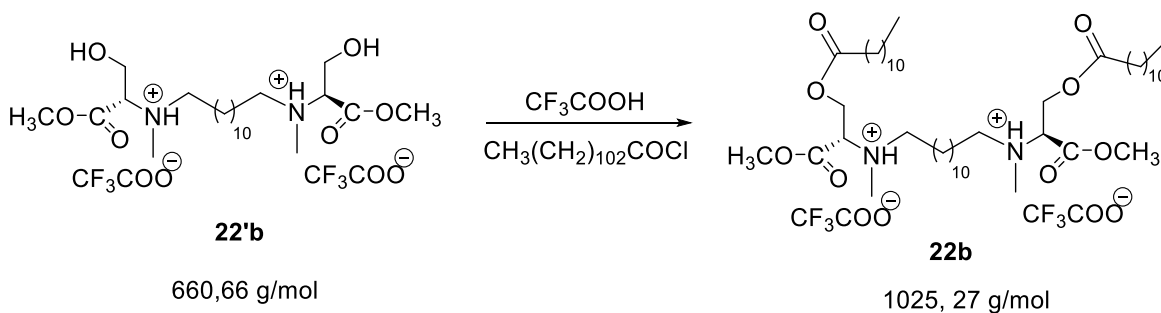
**Table 4.11** presents a summary of the reactions performed.

**Table 4.11.** Deprotection of compound **21b**.

| T <sub>reaction</sub> (hour) | 21b        |      | CF <sub>3</sub> COOH |      | 22'b       |      | η (%) |
|------------------------------|------------|------|----------------------|------|------------|------|-------|
|                              | m/g (mmol) |      | V/mL (mmol)          |      | m/g (mmol) |      |       |
| 65                           | 0,261      | 0,48 | 2,00                 | 26,1 | 0,373      | 0,56 | 97    |
| 105                          | 0,698      | 1,28 | 1,00                 | 13,1 | 0,843      | 1,28 | 92    |
| 40                           | 1,20       | 2,21 | 1,70                 | 22,2 | 1,73       | 2,62 | 95    |
| 49                           | 0,934      | 1,71 | 1,50                 | 19,6 | 1,37       | 2,07 | 94    |

(2*S*,2*S'*)-*N,N'*-dodecane-1,12-diyl-bis{[2-hydroxy-1-(methyloxycarbonyl)ethyl]methylammonium trifluoroacetate} **22b**; brown oil. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  4.30-4.14 (m, 2H,  $J = 8.4, 5.2\text{ Hz}$  2 x  $-\text{CH}_2\text{O}t\text{Bu}$ ), 4.12-4.02 (m, 1H,  $-\text{CH}$ ) 3.85 (s, 8H, 2x  $-\text{CH}$  + 2 x  $-\text{OCH}_3$ ), 3.36-3.26 [m, 4H, 2 x  $\text{N}(\text{CH}_3)\text{CH}_2(\text{CH}_2)_{10}\text{CH}_2\text{N}(\text{CH}_3)$ ], 3.04 (s, 6H, 2 x  $-\text{NH}(\text{CH}_3)$ ), 1.70-1.68 [m, 4H, 2 x  $\text{N}(\text{CH}_3)\text{CH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)$ ], 1.40-1.24 [m, 16H,  $\text{N}(\text{CH}_3)\text{CH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)$ ].

**F) Synthesis of (2*S*,2*S'*)-*N,N'*-dodecane-1,12-diyl-bis{[2-(dodecanoyloxy)-1-(methyloxycarbonyl)ethyl]methylammonium trifluoroacetate} **22b****



Compound **22'b** (0.971 g; 1.47 mmol) was dissolved in trifluoroacetic acid (0.60 mL; 7.84 mmol), the reaction mixture was placed in an ice bath and dodecanoyl chloride (0.70; 3.0 mmol) was added. The reaction was monitored by TLC and stopped when the starting reagent was no longer observed. After successive washings of the product with ethyl ether (to ensure total elimination of trifluoroacetic acid) liquid-liquid extractions were carried out. The organic phase was washed with saturated NaHCO<sub>3</sub> solution (2 x 20 mL) and dried over anhydrous sodium sulfate. After evaporation of the solvent a brown oil was obtained which was subjected to column chromatography using DCM/MeOH 20:1 as eluent.

Three distinct fractions were obtained:

F1: yellow oil, homogeneous by TLC [ $R_f = 0.64$  DCM/MeOH (20:1)], identified (TLC) as the desired compound, **22b**.

F2: white solid, homogeneous by TLC [ $R_f = 0.31$  DCM/MeOH (20:1)], identified (TLC) as dodecanoic acid.

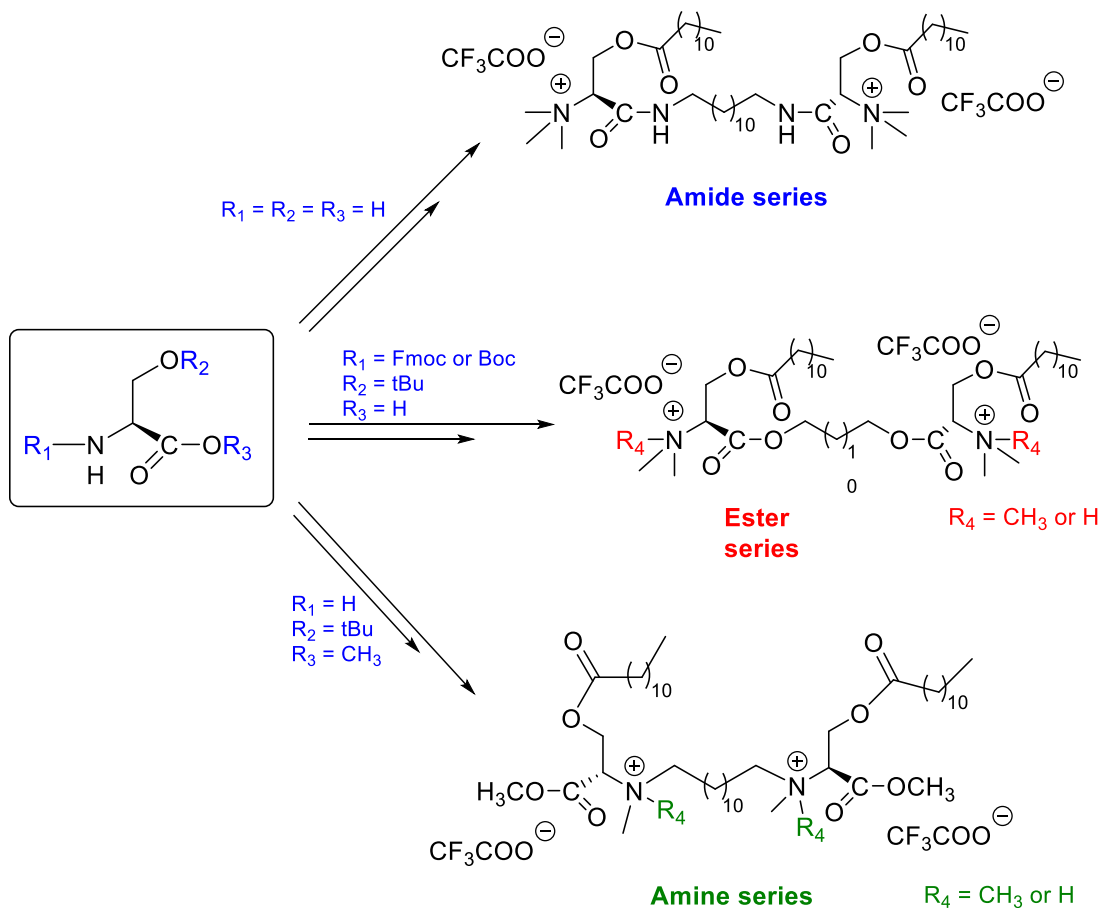
F3: yellow solid, homogeneous by TLC [ $R_f = 0.21$  DCM/MeOH (20:1)], identified by  $^1\text{H}$  NMR as the desired compound, **22b**.

The total mass of **22b** obtained was 0.881 g (0.859 mmol;  $\eta = 58\%$ ).

(2*S*,2*S'*)-*N,N'*-dodecane-1,12-diyl-bis{[2-(dodecanoyloxy)-1-(methyloxycarbonyl)ethyl]-methylammonium trifluoroacetate} **22b**;  $R_f = 0,27$  (DCM/MeOH 20:1); orange oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  4.77 (dd, 2H,  $J = 13.6$  Hz,  $J = 6.4$  Hz, 2 x  $-\text{CH}$ ), 4.62-4.56 (m, 4H, 2 x  $-\text{CH}_2\text{O}^t\text{Bu}$ ), 3.82 (s, 6H, 2 x  $-\text{OCH}_3$ ), 3.33-3.17 [m, 4H, 2 x  $\text{N}(\text{CH}_3)\text{CH}_2(\text{CH}_2)_{10}\text{CH}_2\text{N}(\text{CH}_3)$ ], 3.02 [s, 6H, 2 x  $-\text{NH}(\text{CH}_3)$ ], 2.32 (t, 4H,  $J = 7.6$  Hz, 2 x  $-\text{COCH}_2(\text{CH}_2)_{10}\text{CH}_3$ ), 1.80-1.70 e 1.64-1.54 (2m, 4H, 2 x  $\text{N}(\text{CH}_3)\text{CH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)$  + 2 x  $-\text{COCH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$ ), 1.26 [s, 52H,  $\text{N}(\text{CH}_3)\text{CH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)$  + 2 x  $-\text{COCH}_2\text{CH}_2(\text{CH}_2)_8\text{CH}_3$ ], 0.88 (t, 6H,  $J = 6.8\text{Hz}$ , 2 x  $-\text{CH}_3$ )

### 4.3. Results and discussion

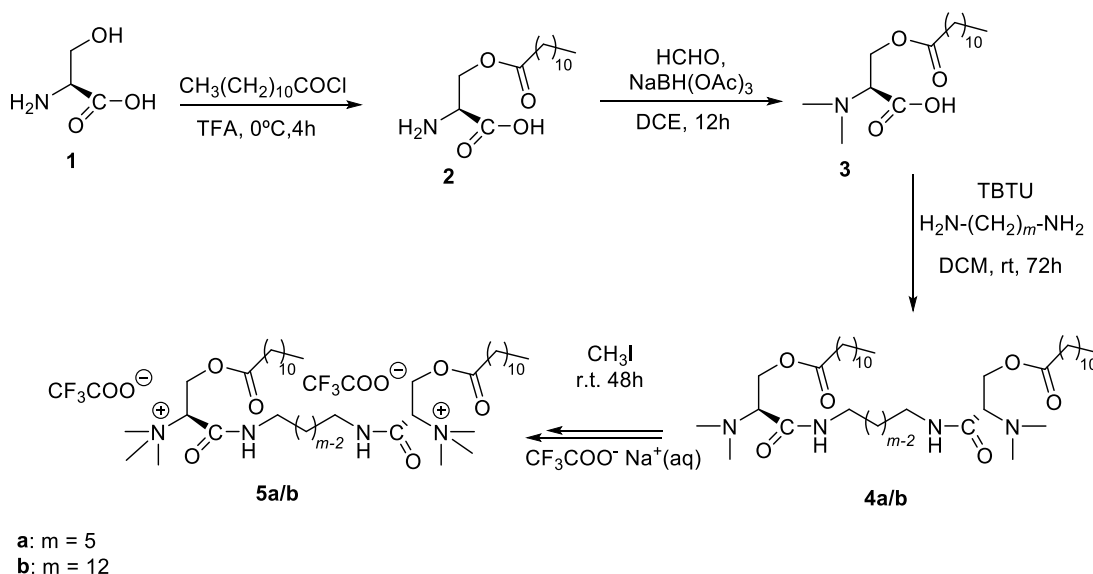
The objective for obtaining gemini surfactants was to synthesize three different series of novel amino acid-based surfactants to allow a systematic study of the influence of (i) the spacer length and (ii) the nature of the bond between the spacer and polar headgroup on their interfacial and aggregation properties, in order to assess their potential use as antimicrobial agents. More specifically, our objective was to synthesize serine-based ammonium salts with an alkyl chain linked to the hydroxyl group (side chain of the serine residue) through an ester bond and a spacer linked either at the carboxylic acid group (amide and ester series) or at the amino group (amine series). In this chapter, the methodologies of synthesis of the surfactants will be described and discussed.



**Scheme 4.4.** Obtention of the desired compounds using the appropriate serine derivatives.  
(Created using ChemDraw® 20.0).

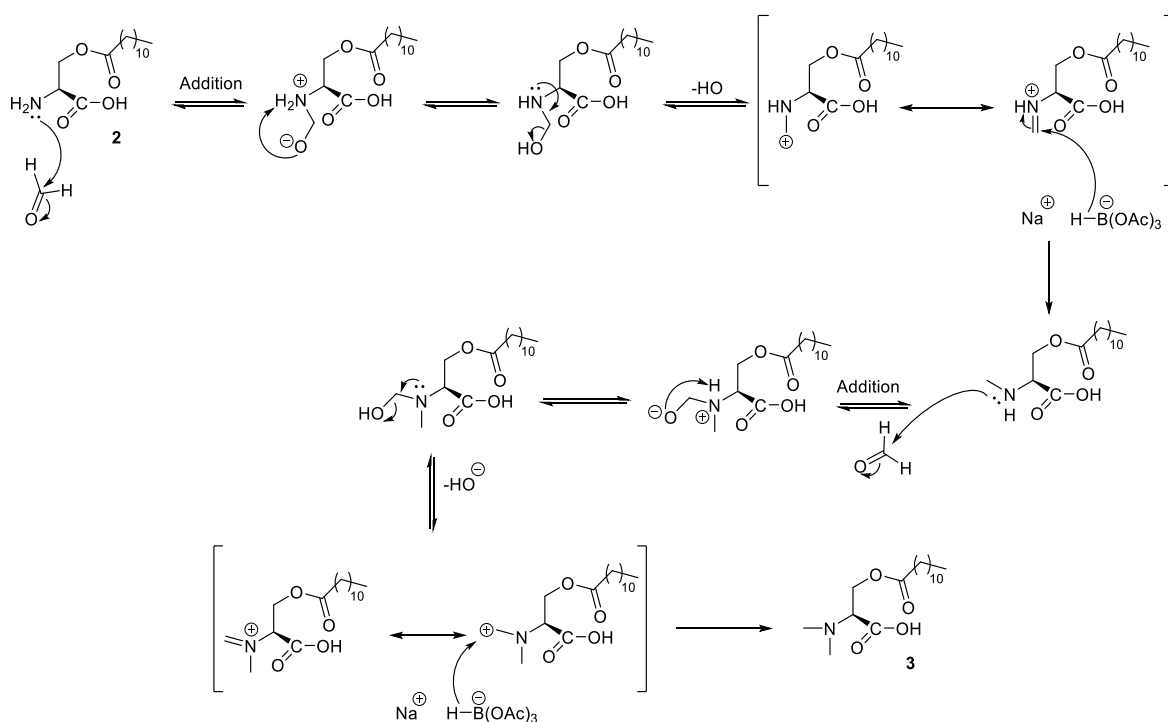
### 4.3.1. Synthesis of the amide series

A total of two serine-based cationic gemini surfactants have been synthesized. The most efficient synthetic methodology for the obtention of this surfactant series is outlined in **Scheme 4.5**.



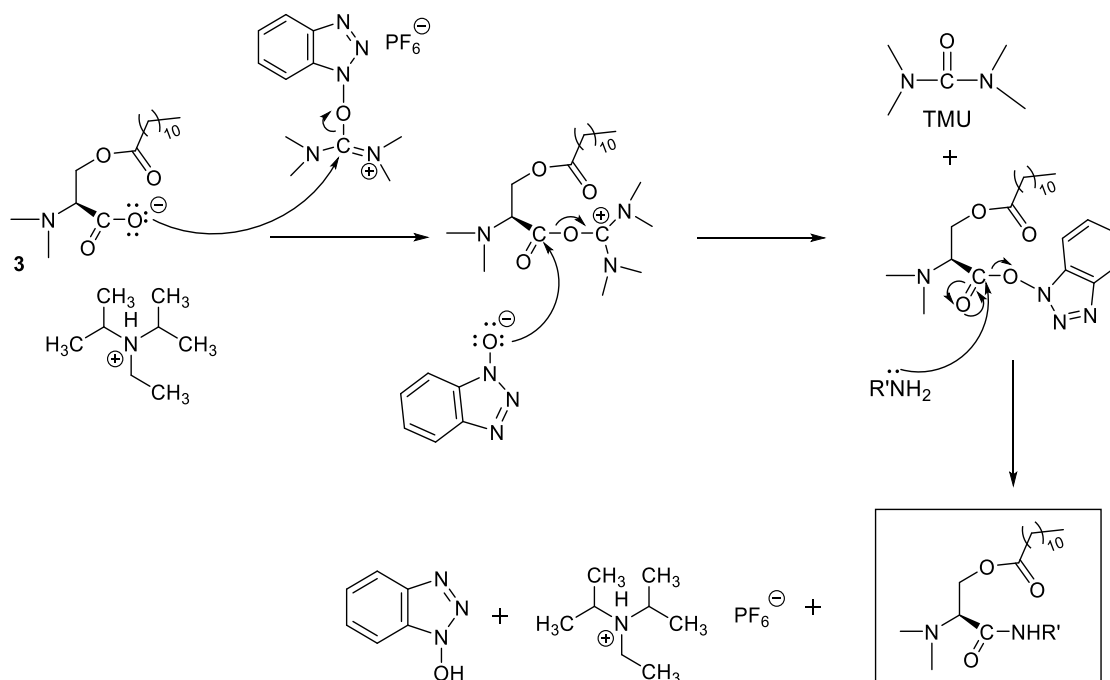
**Scheme 4.5.** Synthesis of the surfactants of the amide series. (Created using ChemDraw® 20.0).

For the synthesis of the gemini surfactants of the amide series, serine (**1**) was used as starting material. The aliphatic chain was introduced into the amino acid residue by acylation with dodecanoyl chloride. This reaction was performed according to a procedure described in the literature.<sup>18</sup> The acid conditions prevent side-reactions, namely *N*- and *O*-alkylation of the amino and carboxylate group since it ensures protonation of the amino and carboxylic acid groups of serine. After 4 hours of reaction compound **2** was precipitated from the reaction mixture using diethyl ether. After filtration, the product (**2**) was allowed to dry at room temperature (overnight). The yields of this reaction were in general high (~73%). The next step consisted in the introduction of the two methyl groups at the nitrogen atom of the amino group of **2**, *via* reductive amination using formaldehyde. The mechanism of this reaction is presented in **Scheme 4.6**.



**Scheme 4.6.** Dimethylation reaction mechanism by reductive amination. (Created using ChemDraw® 20.0).

After 12h of reaction *N*, *N*-dimethyl-*O*-dodecanoylserine (**3**) was isolated from the reaction mixture, through liquid-liquid extractions, using DCM and an aqueous solution of HCl 1M. The product was recrystallized from diethyl ether ( $\eta = 96\%$ ). Gemini precursors **4a/b** were obtained by reaction of **3** with pentane-1,5-diamine and dodecane-1,12-diamine, respectively. The reactions were performed according to conventional peptide condensation methods, using TBTU as coupling agent. The reaction mechanism is presented in **Scheme 4.7**. The yields of the condensations were, in general, low ( $\approx 60\%$ ), mainly due to the extension of the reactions, which could not be driven to completeness as none of the reagents could be used in excess (the diamines in order to avoid monocondensation, and the monomeric precursors due to their high cost of preparation). Moreover, it was necessary to purify compounds **4a/b** several times, by flash column chromatography, to ensure total removal of the tetramethylurea (TMU) formed, which also contributed to the low yields obtained.



**Scheme 4.7.** Mechanism of TBTU-mediated coupling. (Created using ChemDraw® 20.0).

The production of the final cationic gemini surfactants (**5a/b**) was achieved by methylation of the dimeric precursors (**4a/b**) according to a procedure already described in the literature; methylation was carried out in DMF using 2 equiv. of iodomethane. The resulting compounds were then converted into the corresponding trifluoroacetate salts (**5a/b**) by treatment with a solution of CF<sub>3</sub>COONa (1.0 eq.).

#### 4.3.2. Synthesis of the ester series

The first synthetic approach performed by the research group where this work has been carried out consisted in the obtention of the dimeric precursors of the ester series through the reaction of **3** with diols (**Figure 4.1.**) in the presence of a coupling reagent. Several coupling reagents were used; however, in none of the reactions performed the desired compound was obtained. The NMR analysis revealed the absence of the signals of the amino acid moiety, and confirmed the formation of an ester resulting from the attack of the diol at the carbonyl group of the side chain of **3**.

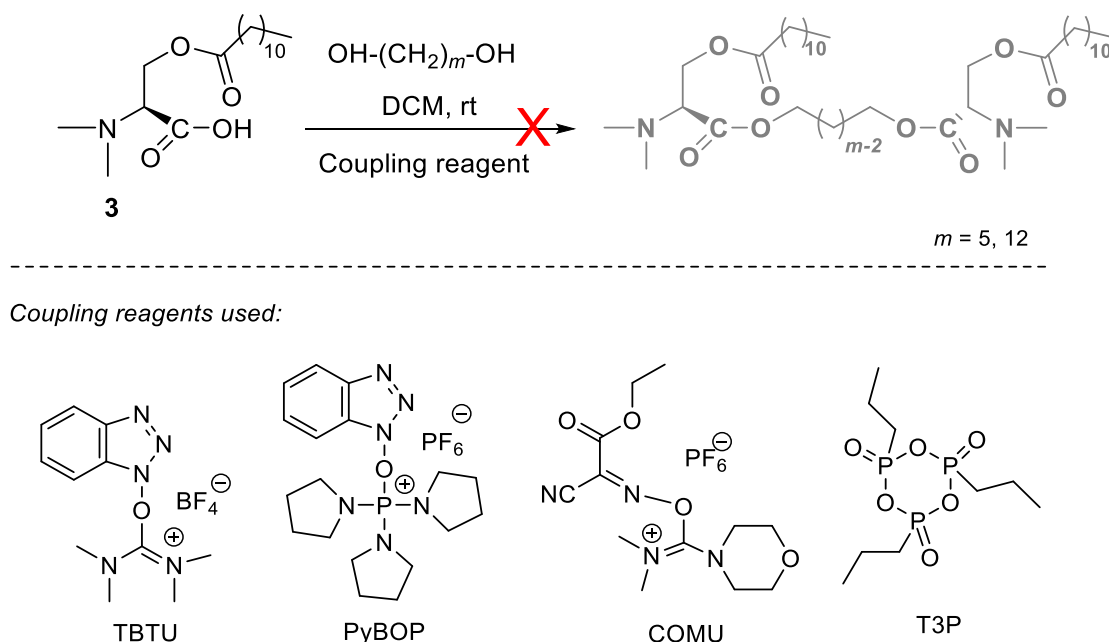
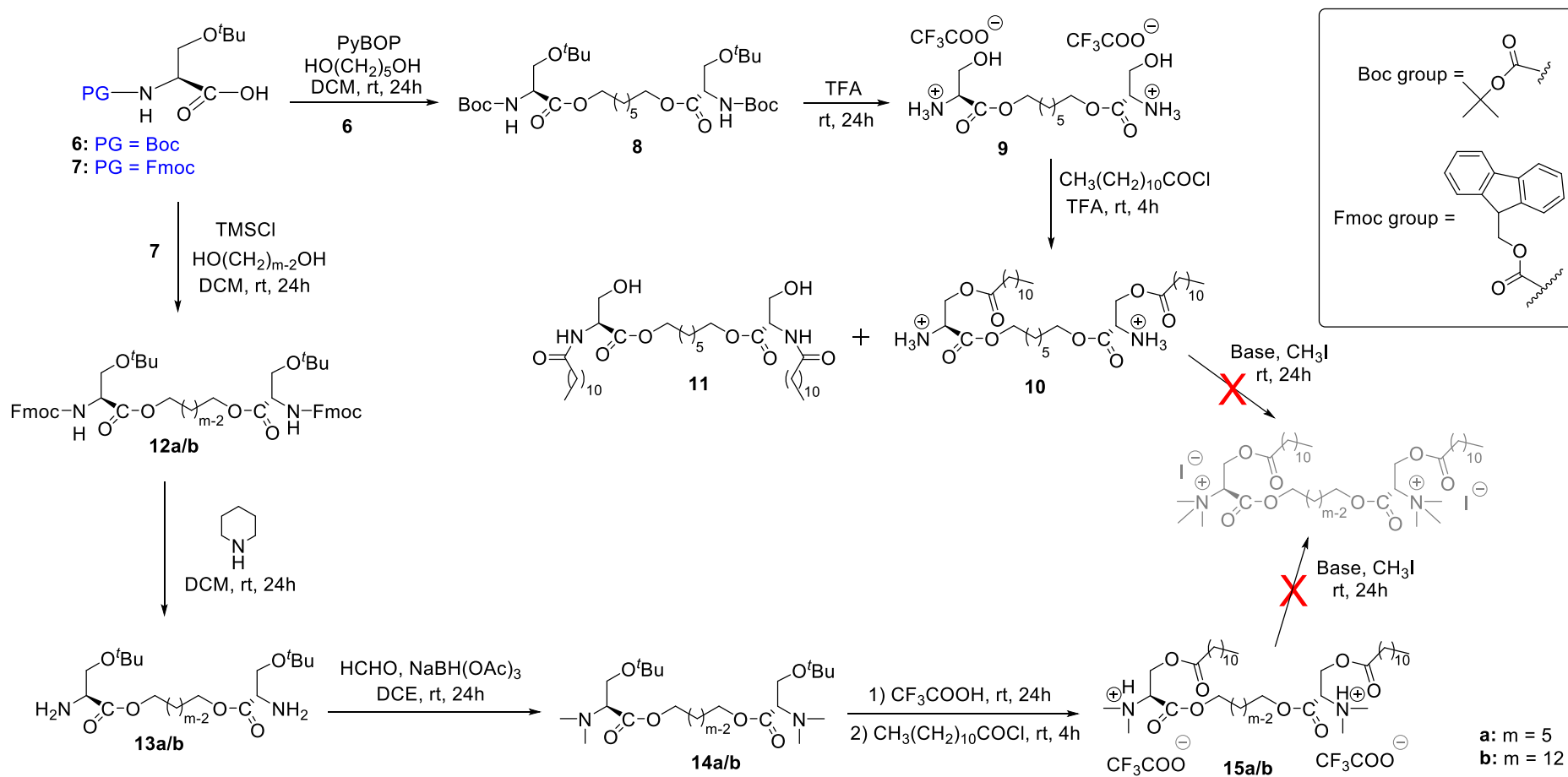


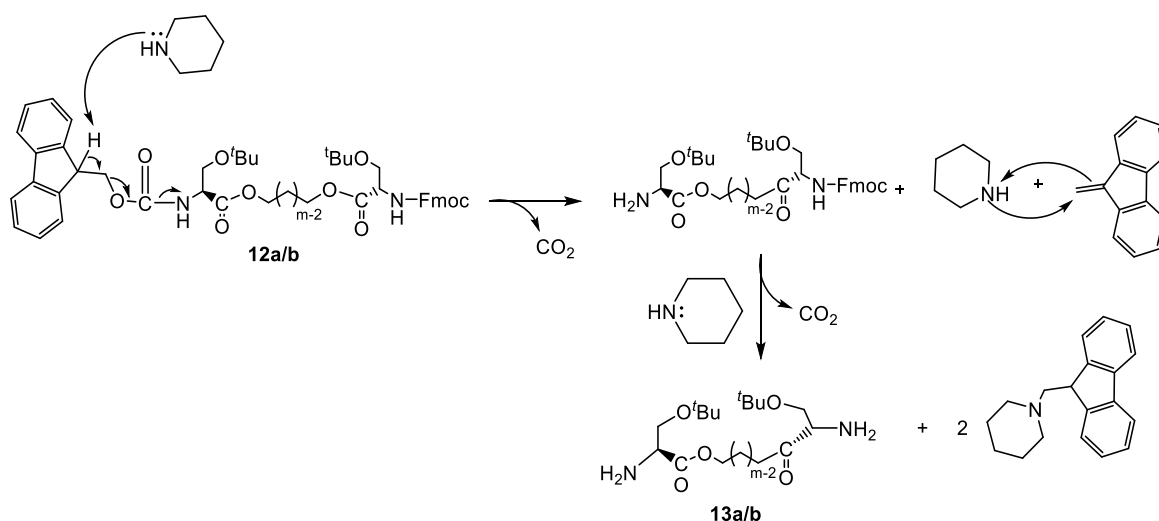
**Figure 4.1.** Coupling reagents used in the attempts to obtain the dimeric precursor of the ester series. (Created using ChemDraw® 20.0).

Taking this result into account, in the present work alternative synthetic pathways were explored, involving the use of *N*-protected derivatives (**6**, **7**). The two synthetic routes followed are presented in **Scheme 4.8**. The first synthetic approach consisted of the introduction the spacer between the carboxylic acid groups of *N*-Boc serine (**6**) by condensation using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) as a coupling agent. After 12h of reaction, the formation of a new product, with higher  $R_f$  than the starting materials, was observed by TLC. However, as reagents were still present in the mixture, it was allowed to react for more 12h. Dimeric precursor **8** was obtained after purification by column chromatography with 81% yield. The subsequent reaction consisted of the removal of the Boc group using trifluoroacetic acid. This procedure lead also to the removal of the *tert*-butyl group on the oxygen of the serine side chain, giving rise to the deprotected gemini precursor **9**. Acylation of the hydroxyl groups of **9** was performed by reaction with dodecanoyl chloride under acidic conditions. Analysis of the reaction mixture revealed the presence of the desired compound **10** but also of the amide **11**, formed through acylation at the nitrogen atom of the serine moiety. This outcome was not expected, since in the acidic conditions used the amino group would most probably be protonated and thus be non-nucleophilic towards the acyl chloride.



**Scheme 4.8.** Synthetic routes for the obtention of ester derivatives. (Created using ChemDraw® 20.0).

To circumvent the problems resulting from the concomitant removal of the two protecting groups (*N*-Boc and *O*-*tert*-butyl) which led to a non-selective acylation, another synthetic pathway using an amino acid with orthogonal protecting groups (*N*-Fmoc and *O*-*tert*-butyl, **7**) as starting material was explored. The reaction of the *N*-Fmoc protected amino acid **7** with a diol using TMSCl as activating agent gave rise to dimeric precursor **12a/b** which was isolated from the reaction mixture by column chromatography with a 50% yield. (**Scheme 4.8.**) The removal of the Fmoc-protecting group, to yield **13a/b**, was performed using piperidine and did not affect the stability of the *O*-*tert*-butyl group, which requires acidic conditions to be cleaved. The product was isolated by column chromatography in moderate yields since it was very difficult to separate **13a/b** from the *N*-Fmoc piperidine byproduct, which presented similar retention behaviour in all the eluents tested. The mechanism of deprotection is outlined in **Scheme 4.9**.

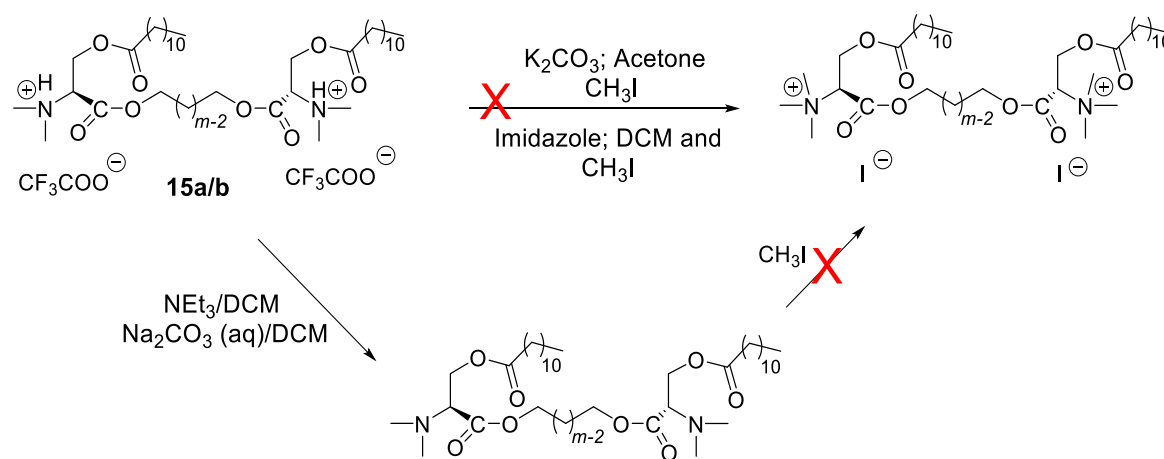


**Scheme 4.9.** Removal of the Fmoc group using piperidine. (Created using ChemDraw® 20.0).

Subsequent dimethylation of the free amino group *via* reductive amination using formaldehyde yielded gemini **14a/b**. The gemini precursor **15a/b** was obtained after removal of the *tert*-butyl group followed by acylation using dodecanoyl chloride. As already mentioned, the *tert*-butyl group was removed in acidic media, using an excess of trifluoroacetic acid. After 48h of reaction, the mixture was treated with diethyl ether followed by evaporation until total removal of TFA. The acylation was also performed in acidic conditions (TFA) using 2.1 eq. of dodecanoyl chloride. After 4h of reaction, the presence of a new product with a R<sub>f</sub> higher than the starting material was visible by TLC. However, starting material was also present in the reaction mixture and therefore it was allowed to react for more than 8h. After purification by column chromatography, the isolated compound was analyzed by NMR and identified as being the desired precursor (**15b**). The yields of the

acylations were low ( $\approx 19\%$ ), mainly due to the purification process (both dodecanoic acid and product have similar  $R_f$  values).

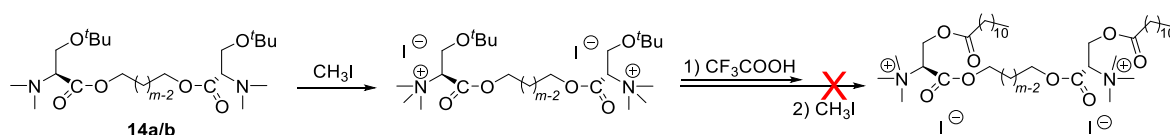
The obtention of the desired cationic surfactant would be easily achieved by exhaustive methylation of **15b** with e.g. iodomethane. However, this reaction revealed to be very difficult. The several attempts performed are depicted in **Scheme 4.10**.



**Scheme 4.10.** Attempts were performed for the obtention of the final cationic surfactants of the ester series. (Created using ChemDraw® 20.0).

As compound **15b** was present as ammonium trifluoroacetate it was necessary to deprotonate the amino group prior to methylation. Our first attempt consisted in the use of triethylamine as base. Compound **15b** was dissolved in DCM and stirred for 30 minutes with 2.1 eq. of triethylamine. After this time, the disappearance of starting material and the formation of a new compound was observed (TLC). The product was isolated from the reaction mixture by liquid-liquid extraction, and subjected to NMR analysis, where a shift of the signals to lower ppm values, consistent with the non-protonated compound, was observed. We proceeded to the methylation using 4. eq of iodomethane; however, after 72h of reaction, no visible changes were evident by TLC. The reaction mixture was analyzed by NMR and only the peaks corresponding to the protons of the alkyl chain were observed. Due to this result, the reaction conditions were changed. Experiments using (i) potassium carbonate and iodomethane (*in situ* methylation); imidazole and iodomethane (*in situ* methylation) and (ii) trimethylamine/sodium carbonate and then iodomethane were performed but in none of these reactions, the desired product could be identified.

Yet another approach consisted in the methylation of **14a/b** before acylation (**Scheme 4.11**).



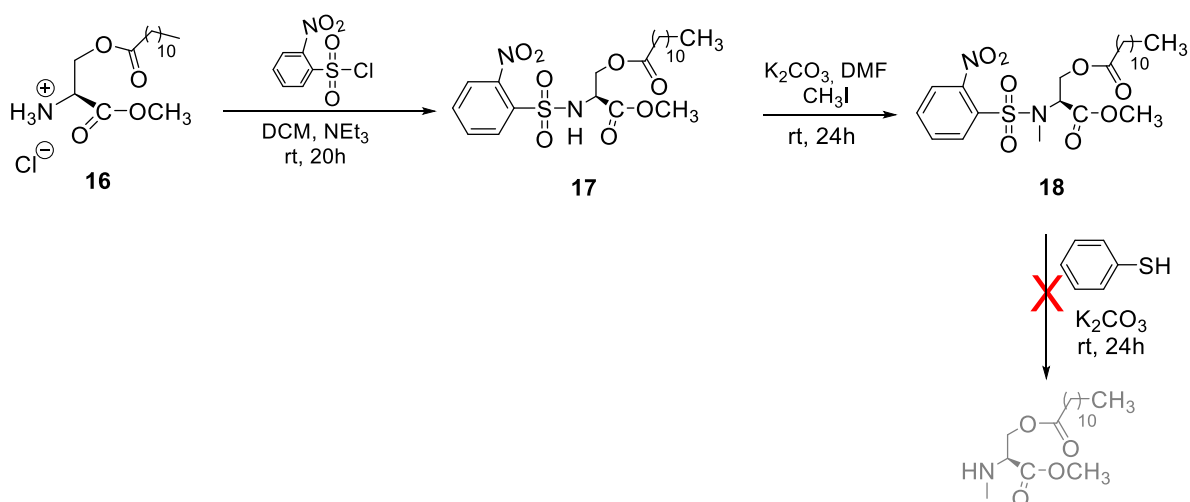
**Scheme 4.11.** Attempted synthesis of the cationic surfactant from **14a/b**. (Created using ChemDraw® 20.0).

Iodomethane was used as methylating agent and the reaction was complete after 30 minutes. Removal of the *tert*-butyl groups was accomplished, as usually, with TFA. Acylation of the hydroxyl groups was performed in DCM/TFA (1:1) using 2.1 eq. of dodecanoyl chloride. After 2h a white solid started to precipitate from the reaction mixture. The two fractions isolated were analyzed by NMR. From the NMR spectra analysis it was possible to identify the white solid as starting material (**14a/b**) and the other fraction as dodecanoic acid resulting from the hydrolysis of dodecanoyl chloride.

As to conclude, the cationic surfactants **15a/b** have been successfully synthesized in moderate to good yields. As they are ammonium salts, their charge can be modulated by varying the pH. Several attempts were made to introduce the third methyl group at the nitrogen atom to obtain the corresponding ammonium salts, however without success.

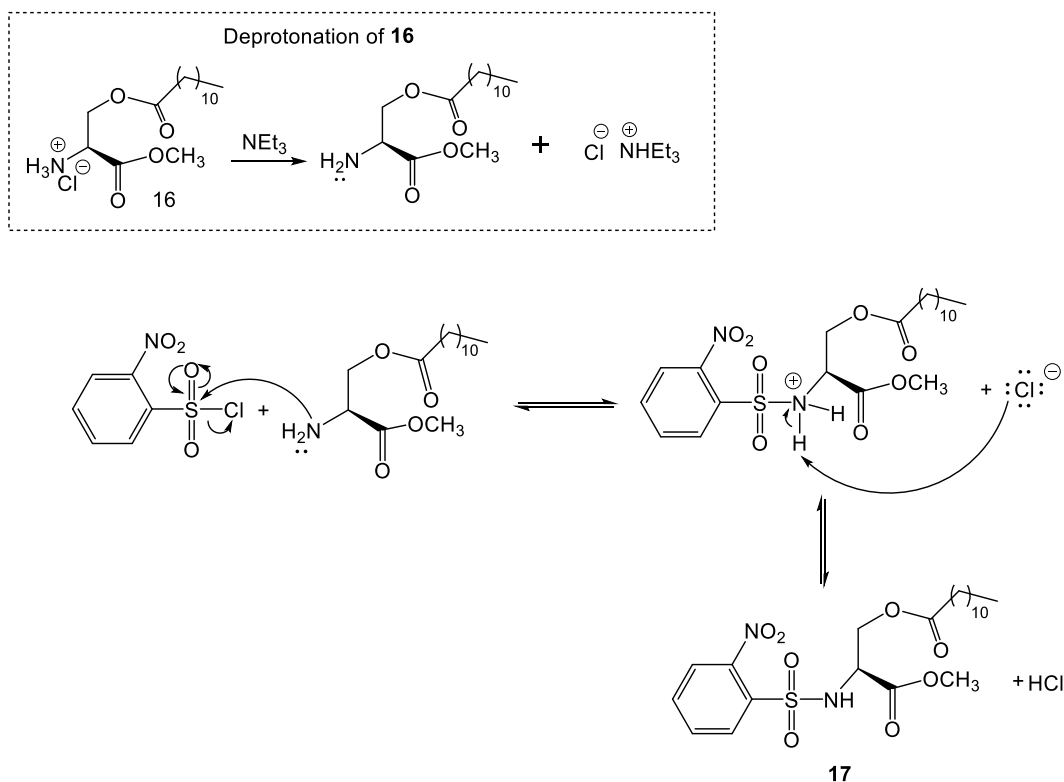
#### 4.3.3. Synthesis of the amine series

In the synthesis of gemini surfactants of the amine series it was necessary to first synthesize the *N*-monomethylated serine derivative *N*-methyl-*O*-dodecanoyl methyl serinate (**16**, previously obtained by *O*-acylation using dodecanoyl chloride), through a sulfonation, methylation, desulfonation reaction sequence (**Scheme 4.12**).<sup>18</sup> This procedure was necessary, to avoid the the formation of cyclic compounds ( $m = 5$ ) when introducing the spacer by reductive amination as well as to avoid the introduction of two methyl groups at the nitrogen atom through reductive amination of formaldehyde.



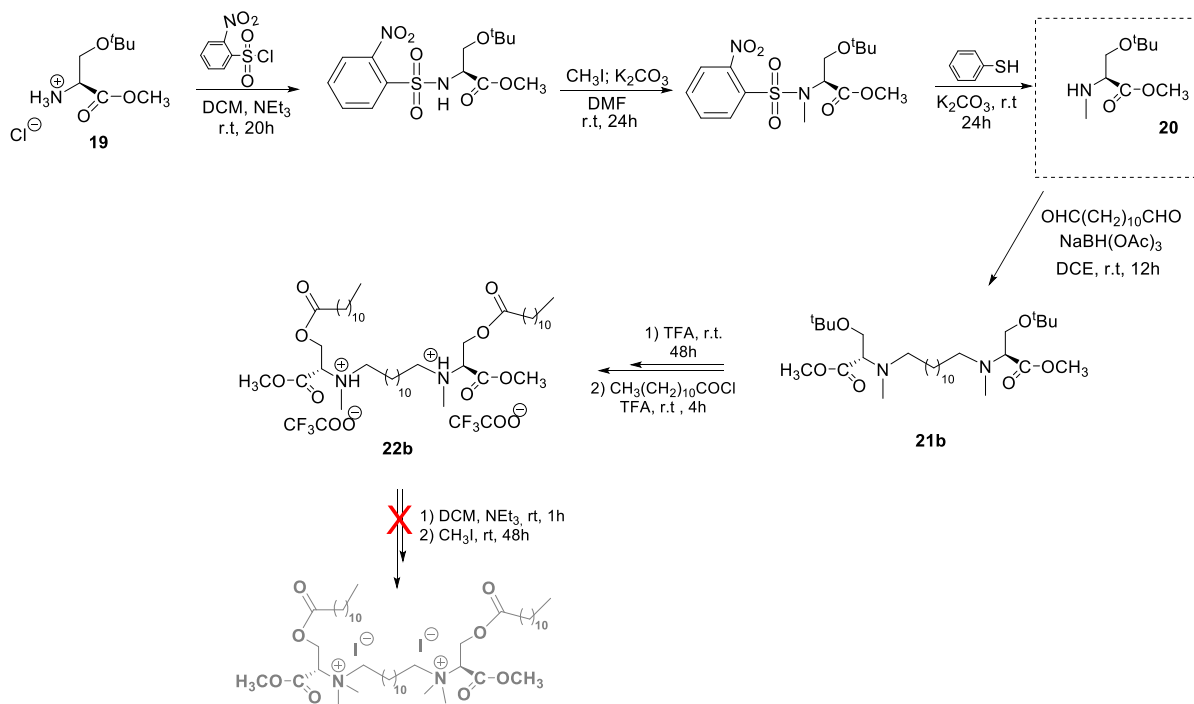
**Scheme 4.12.** Synthetic pathway for the obtention of *N*-methyl-*O*-dodecanoylmethyl serinate (Created using ChemDraw® 20.0).

The sulfonation of the amino group was performed using 2-nitrobenzenesulfonyl chloride. After 20 hours of reaction, total consumption of **16** and the formation of a new product with a higher  $R_f$  were observed. The workup of the reaction mixture consisted in liquid-liquid extractions, using  $\text{HCl}$  (aq.) 1M, to ensure the total removal of the remaining triethylamine. The product obtained was analyzed by NMR and identified as the corresponding sulfonamide **17** ( $\eta = 87\%$ ). The sulfonation mechanism is outlined in **Scheme 4.13**.



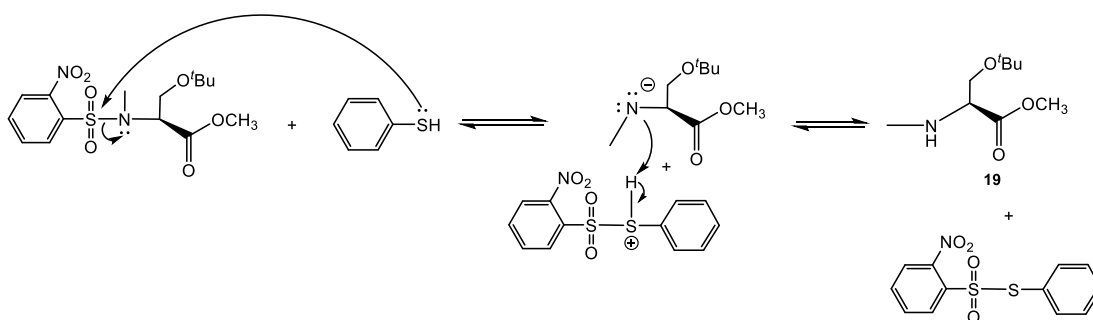
**Scheme 4.13** Sulfonation mechanism using 2-nitrobenzenesulfonyl chloride. (Created using ChemDraw® 20.0).

Methylation of **17** was performed using iodomethane, in the presence of potassium carbonate and DMF (catalytic amount). After 24h, the formation of a new product and the total disappearance of **17** was observed by TLC. The product was isolated by liquid-liquid extraction, and identified by NMR as being compound **18** ( $\eta = 97\%$ ). The subsequent step consisted in the desulfonation, which was performed using thiophenol. After 24h of reaction the presence of a new compound with a  $R_f$  superior than that of the starting material was observed by TLC. Following aqueous workup, the main product was isolated by flash chromatography and analyzed by NMR. It was identified as mainly alkyl chain since (dodecanoic acid) since all the signals corresponding to the amino acid residue were absent. This reaction with thiophenol seems to decompose the sulfonamide through some reaction at the side chain ester group. Therefore we changed the starting material and performed the same reaction sequence using aminoacid **19** as starting material. The premise was that the *O*-*tert*-butyl group being less reactive than the ester group should not be prone to decomposition upon reaction with thiophenol. The methodology used to obtain the target final surfactants is outlined in **Scheme 4.14**.



**Scheme 4.14.** Synthetic route followed for the obtention of gemini surfactants of the amine series. (Created using ChemDraw® 20.0).

Sulfonation of **19** and methylation of the resulting sulfonamide proceeded as described above for acylated derivative **16**. However, in this case the desulfonation with thiophenol gave the desired compound with a yield of 50 %. The mechanism of this reaction is presented in **Scheme 4.15**.



**Scheme 4.15.** Desulfonation mechanism using thiophenol. (Created using ChemDraw® 20.0).

The introduction of the spacers between the amino groups of **20** was achieved by reductive aminations either of glutaraldehyde or of dodecane-1,12-dial. As dodecane-1,12-dial is not commercially available it was synthesized from the corresponding diol using tetrapropylammonium perruthenate (TPAP)/Methyl morpholine N-oxide (NMO), according to a well established procedure. Gemini precursor **21b** was obtained in good yields after purification by flash chromatography. The *tert*-butyl group was then removed using TFA and the two alkanoyl chains were introduced by acylation. Once again the low yield obtained in

the diacylation reaction is mainly due to significant losses in the purification process. The resulting salt (**22b**) was neutralized using triethylamine and then methylated to obtain the final compound. After 48h of reaction, all the iodomethane was eliminated in the rotatory evaporator and the crude product analyzed by NMR. The NMR showed total absence of the desired compound, the only signals observed were those corresponding to the alkyl chains.

Methylations using the same conditions as described for the ester series were also tested, but none of the attempts lead to the obtention of the desired compound (the quaternary ammonium salt). For this series of surfactants we succeeded to obtain the cationic gemini ammonium trifluoroacetates **22b** in moderate to good yields.

#### 4.3.4. Determination of the basic interfacial parameters

The evaluation of the physicochemical properties of the synthesized gemini surfactants had the purpose of providing the information necessary for the execution of the subsequent experiments on antimicrobial activity and cytotoxicity. Specifically, the determination of the *critical aggregation concentration* (*cac*) is essential for understanding the concentration range at which these compounds can be tested in upcoming experiments in order to successfully evaluate the influence of their organization in solution (monomer or aggregate) in their biological/biophysical properties. To predict the phase behaviour of the surfactants synthesized, a phase-scanning study was also conducted. All these experiments were performed at Prof. Eduardo Marques Lab (CIQUP), by Dr(s) Sandra Silva and Isabel Oliveira.

##### **Critical aggregation concentration**

The gemini surfactants are soluble in water at 25 °C. The interfacial behaviour of (SerOC12)<sub>2</sub>CON5 and (SerOC12)<sub>2</sub>CON12 in an aqueous solution was thus studied by tensiometry at this temperature. Stock solutions of 1mM were prepared in MiliQ water. The surface tension curves obtained are shown in **Figure 4.2**.

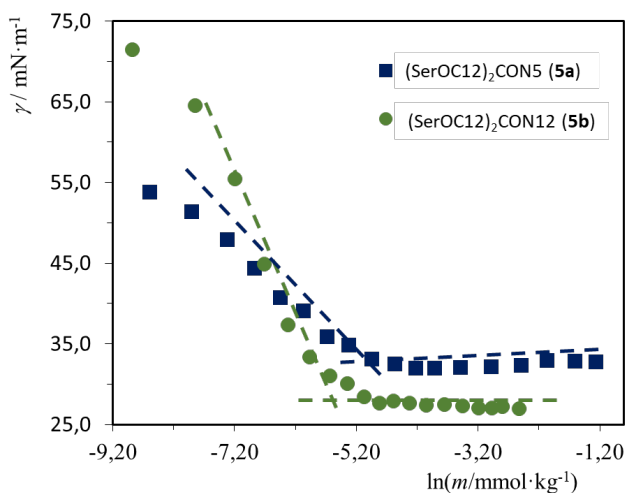


**Figure 4.2.** Surface tension versus  $\ln(\text{concentration})$  curves for the  $(\text{SerOC12})_2\text{CON5}$  and  $(\text{SerOC12})_2\text{CON12}$  at 25 °C.

**Table 4.12** shows the interfacial parameters determined from the surface tension curves, namely the *critical aggregation concentration* (*cac*) and the surface tension at *cac*. The *cac* values were determined as the break points in the  $\gamma$  versus  $\ln(c)$  curves as illustrated in **Figure 4.2**.

**Table 4.12.** Interfacial properties of serine-based gemini surfactants of the amide series.

| Surfactant                                     | $cac / \mu\text{mol}\cdot\text{kg}^{-1}$ | $\gamma_{cac} / \text{mN}\cdot\text{m}^{-1}$ |
|------------------------------------------------|------------------------------------------|----------------------------------------------|
| $(\text{SerOC12})_2\text{CON5}$ ( <b>5a</b> )  | $6.1 \pm 0.3$                            | $31.6 \pm 0.2$                               |
| $(\text{SerOC12})_2\text{CON12}$ ( <b>5b</b> ) | $3.3 \pm 0.3$                            | $28.2 \pm 0.4$                               |

Both surfactants present low *cac* values; however, they present a distinct phase behaviour. While compound **5a** forms micelles in solution, compound **5b** form vesicles (or liposomes). The global phase behavior of both amphiphiles was determined through phase scanning. This technique is a simple and qualitative method that gives helpful information on the self-assembly pattern of the surfactant under study. For compound **5a** a dilute isotropic solution phase (L) was observed (**Figure 4.3 A**) which is consistent with micellar-forming surfactants. The derivative **5b** (**Figure 4.3 B**) also shows a dilute L phase, followed by a  $L_\alpha$  phase, characterized by oily streaks and myelin figures (**Figure 4.3 B<sub>1</sub>**). This type of phase behavior is typical for double-chained surfactants and is dominated by a lamellar phase (the only liquid-crystalline phase present) and, at low concentrations, by a dispersion of vesicular structures.

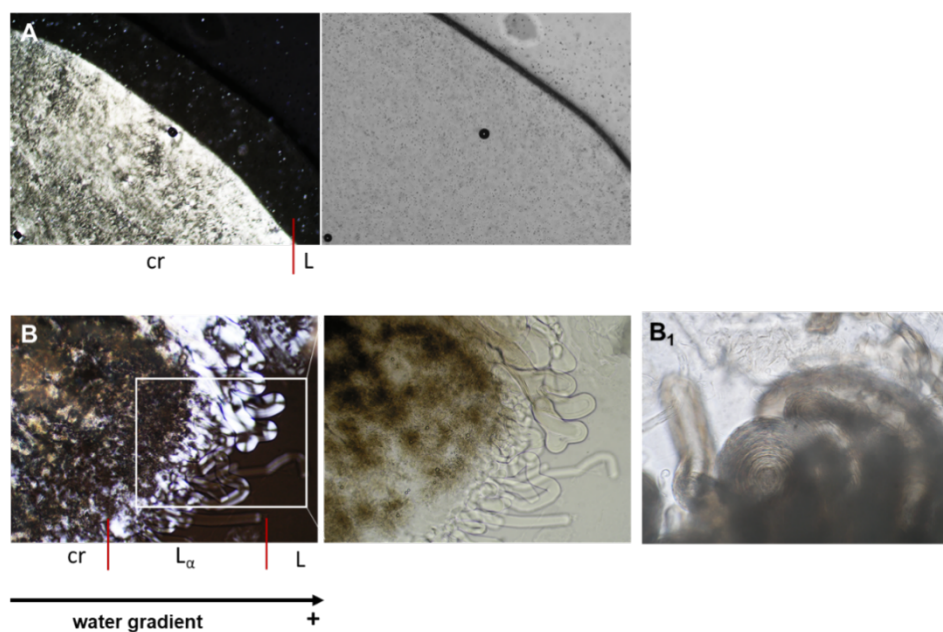


**Figure 4.3.** Phase penetration scans: (A)  $(\text{Ser OC12})_2\text{CON5}$  and (B)  $(\text{SerOC12})_2\text{CON12}$ , at 25 °C. Legend: L, isotropic solution phase,  $L_\alpha$ , lamellar phase, cr, hydrated crystals. Water is diffusing from left to right into the surfactant crystalline film.

#### 4.4. Conclusion

In this work, a new family of serine-based gemini surfactants has been synthesized. Our aim was to synthesize surfactants of three different series, designated as amine, amide or ester, according to the bond “spacer-residue”. All the surfactants possess twelve-carbon chains and spacers with five or twelve methylene groups.

The synthesis of the amide series was successfully accomplished. A total of four compounds of this series has been obtained: two tertiary amine derivatives (**4a**, five carbon spacer; **4b**, twelve carbon spacer), which can be transformed into the corresponding ammonium surfactants in acidic media, and two quaternary ammonium salts (**5a/b**). The most efficient synthetic pathway has been established and consisted in the introduction of the spacers between the carboxylic acid groups of *N*, *N*-dimethyl-*O*-dodecanoylserine (**3**) through peptide condensation methods using TBTU as coupling agent. The final quaternary ammonium salts were obtained by exhaustive methylation of the ammonium precursors.

In the series of esters, alternative synthetic pathways were explored, involving the use of *N*-protected derivatives as starting material. To get around the problems resulting from the concomitant removal of the two protecting groups (*N*-Boc and *O*-tert-butyl) of the amino acid initially used, which led to a non-selective acylation, another synthetic route using an amino acid with orthogonal protecting groups (*N*-Fmoc and *O*-tert-butyl, **7**) as starting material was explored. Obtaining the desired cationic surfactant would be easily accomplished by exhaustive methylation with iodomethane. Several attempts have been made to introduce the third methyl group on the nitrogen atom to obtain the corresponding ammonium salts but without success. Thus, in the case of the ester series, the final cationic surfactants obtained were the corresponding ammonium salts. These compounds are also very interesting as their charge can be modulated by pH variation.

For the amine series, we also succeeded in the obtention of the cationic gemini ammonium trifluoroacetates, but were not able to transform them into the corresponding quaternary ammonium salts., despite the various attempts made. Other methylating agents, such as dimethylsulfate, maybe tested. This was not done due to lack of time.

The evaluation of the physicochemical properties of the synthesized gemini surfactants aimed to provide the necessary information to carry out subsequent experiments on antimicrobial activity and cytotoxicity.

Both surfactants show lower *cac* values relative to conventional bis-quats, which can be of great interest for biomedical applications, as lower amounts of compound may achieve the same therapeutic effect. Furthermore, these surfactants are also very

interesting as they exhibit distinct phase behavior: while the amide gemini with a five carbon spacer forms micelles in solution, the one with the twelve carbon spacer forms vesicles (or liposomes). Such a behavior had already been observed for another series of gemini surfactants synthesized in our laboratories and had been assigned to the conformational rigidity at the headgroup level, characteristic of the amide bond. The twelve carbon methylene group is expected to bend inwards the core and the surfactants adopt a cylindrical geometry at the interface, which favors the formation of liposomes. This does not occur for shorter spacers.

Overall, the objectives of the project were achieved. Some of the reactions have been optimized resulting in higher yields of products. Six new compounds were successfully obtained.

## 4.5. References

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## 5. Antimicrobial and anti-biofilm serine-derived gemini surfactant to fight *S. aureus* strains

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### Abstract

As existing antibiotics are becoming less effective, it is urgent to develop new compounds with antimicrobial potential. Among the existing bacteria, *Staphylococcus aureus* is the one that has been showing high resistance to antibiotics, being the target of studies. *S. aureus* causes several infections mainly in catheters and transplants due to its ability to form biofilms. So, given the history of infections and the worsening resistance to conventional antibiotics, we looked for a viable alternative.

The study carried out to evaluate the activity of amide 5C and amide 12C was carried out in three different strains of *Staphylococcus aureus* (*S. aureus* DSM 2569, MRSA ATCC 33592, *S. aureus* ATCC 6538 - biofilm formation) in the planktonic state and in one biofilm structure. The two surfactants proved to be bactericidal against the three strains of *S. aureus*, as after 24 hours the bacterium was dead. As for anti-biofilm activity, both surfactants caused a decrease in biofilm viability, although amide 5C was more effective than amide 12C in planktonic bacteria, the difference between the two compounds was not significant. Thus, both compounds demonstrated *S. aureus* activity in the planktonic state and in the biofilm structure.

After these promising results, studies on the effect of these compounds on hemolytic and cytotoxic activities were carried out. Both compounds have shown promising properties for use as antimicrobial agents.

### 5.1. Introduction

Bacteria resistance to antibiotics has increasingly become a problem for society and the antibiotics are becoming ineffective, there is an urgent need to develop new compounds with bactericidal potential.<sup>1</sup>

Recently, with the increase in the number of organ and bone marrow transplants, in addition to invasive diagnostic tests, sepsis events have increased. *S. aureus* ranks first among bacteria that cause bloodstream infections and is the leading cause of nosocomial pneumonia.<sup>2</sup> Members of the *Staphylococcus* genus inhabit the skin, skin glands, and mucous membranes of humans, animals, and birds. They are Gram-positive cocci with sizes ranging from 0.5 to 1.5  $\mu\text{m}$  in diameter.<sup>3</sup>

Amino acid-based surfactants have been seen as a promising alternative as antibacterial and antifungal agents. Some reports of cationic gemini surfactants derived from lysine, arginine, alanine and cysteine, amongst others, can also be found in the literature.<sup>24, 27-31</sup> In **Table 5.1** are listed the applications in which these surfactants as promising.

**Table 5.1.** Gemini surfactants are described in the literature and their biological applications.

| Amino acid | Applications                         | Ref. |
|------------|--------------------------------------|------|
| Alanine    | Antibacterial and antifungal agent   | 4, 5 |
| Arginine   | Antimicrobial agent                  | 6    |
| Cysteine   | Transfection agent                   | 7    |
| Lysine     | Antimicrobial and transfection agent | 8, 9 |

These compounds can acquire different modes of action and different targets just by changing their structure, thus becoming more valuable compared to existing conventional antibiotics.<sup>10</sup> This new class of gemini surfactants possess two alkyl chains that interact with cell membranes, leading to depolarization, lysis and cell death, probably resulting from an advantageous incorporation in the phospholipid bilayer, which hinders bacterial resistance.<sup>10</sup> However, some studies suggest a more complex mechanism of action on membranes.<sup>11, 12</sup> Gemini surfactants are thought to be able to disturb the membrane structure, but not necessarily to break it. It is suggested that these compounds penetrate the plasma membrane and that they are able to enter the cell, interact with the mitochondrial membrane and inhibit respiratory enzymes. It was also thought about the possibility of surfactants to be incorporated in the plasma membrane forming pores for influx.<sup>11, 12</sup>

Several structural features affect the antimicrobial behaviour of amino acid-derived gemini surfactants, such as the length of the spacer chain, the length of the alkyl chain, the location where the cationic charge is located, and the counterion, which are generally halides.<sup>13, 14</sup>

This study focuses on amide series cationic gemini surfactants (**Figure 5.1**) with different sizes between the two polar heads, amide 5C corresponds to the 5-carbon spacer, while amide 12C corresponds to the 12-carbon spacer.

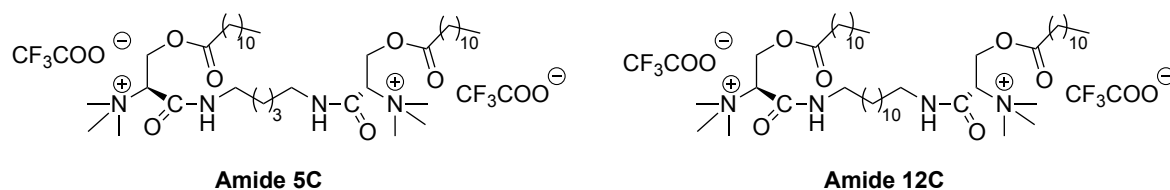


**Figure 5.1.** Structures of the serine-derived gemini surfactants belonging to the amide series used in this study.

This study aimed to evaluate the ability to fight the *S. aureus* strains biofilms without interfering with the cytocompatibility of fibroblasts and red blood cells. The physico properties were also assessed in terms of size, concentration and stability of Gemini surfactant with two different spacers (amide 5C and amide 12C).

## 5.2. Materials and methods

### 5.2.1. Physical characterization

#### 5.2.1.1. Size and concentration by Nanoparticle Tracking Analysis

Nanoparticle size and concentration were analyzed using a NanoSight® N300 (Malvern Instruments, UK), which uses Nanoparticle Tracking Analysis (NTA) technology. Briefly, amide 5C and amide 12C surfactants were diluted in type I water at a concentration of 10.09 µg/mL and 21.06 µg/mL, respectively.

#### 5.2.1.2. Surface charge by Electrophoretic Light Scattering

The surface charge of the surfactants was determined using a Zeta-Potential Analyzer (ZetaPals, Brookhaven Instruments Corporation, Software: PALS Zeta Potential Analyser v.5, Brookhaven Instruments; Holtsville, NY, USA). This equipment performs Electrophoretic Light Scattering (ELS) analysis to measure the zeta-potential (ZP). The surfactants were diluted in type I water with a concentration of 1300 µg/mL.

### 5.2.2. *In vitro* susceptibility of gemini surfactants against *S. aureus* strains

#### 5.2.2.1. Bacteria culture

Three strains of *S. aureus* were used to performed the antimicrobial and anti-biofilm assays, namely DSM 2569 (equivalent to ATCC 29213) purchased from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Methicillin-Resistant *S. aureus* (MRSA) ATCC 33592 and *S. aureus* ATCC 6538 purchased from American Type Culture Collection. *S. aureus* strains grown on solid culture medium (Trypticase Soy Agar, TSA). The bacterium is thawed and made a streaked spread on a plate with a solid medium. The plate was incubated at 37°C, for 24h, in an aerobic atmosphere. Afterwards, 1 to 2 individual colonies were collected and resuspended in Trypticase Soy Broth medium (TSB, Merck Millipore, Germany) and incubated at 37°C, under at 150 revolutions per minute (rpm) for 18-20 hours.

#### 5.2.2.2. Evaluation of minimal inhibitory and bactericidal concentrations

After bacteria growth, bacteria were collected by centrifugation using 2300g, for 10 minutes at 4°C. The bacteria pellet was washed twice using phosphate-buffered saline (PBS, 1M, pH 7.4). Bacteria strains were resuspended in Mueller-Hinton Broth medium (MHB, Sigma-Aldrich, USA) and optical density was measured at  $\lambda = 600$  nm. The antibacterial activity of the gemini surfactants was evaluated by the determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) using the microdilution method according to <sup>15</sup>. Briefly, bacteria inoculum was placed in a 96-well round-bottom plate (Falcon®, USA) at concentration  $1 \times 10^5$  CFU/mL and surfactant was placed at ratio 1:1. Serial concentrations were performed from 325.0 µg/mL to 2.54 µg/mL. The 96-well plate was incubated at 37°C, under aerobic conditions, for 24h. The MIC was determined considering the first well without visual pellet formation at the bottom of the well. The MBC was determined by plating the contents of the next three wells where no visible growth was observed.

#### 5.2.3. *In vitro* efficiency of gemini surfactant against *S. aureus* biofilms

##### 5.2.3.1. *S. aureus* biofilm growth

For biofilm preparation, an inoculum of *S. aureus* strains (DSM 2569, ATCC 33592 and ATCC 6538) was prepared as described in section 5.2.2.1. Bacteria were collected by centrifugation under 2500g, for 10 min at 4°C and their concentration was adjusted at  $1 \times 10^5$  CFU/mL in TSB medium. Bacteria were seeded in 96-well flat-bottom tissue culture plates (Falcon®, USA) and incubated for 90 minutes for the adhesion of bacteria. Then, the non-adherent bacteria were removed and replaced with a fresh TSB medium and incubated until 24h, under aerobic conditions, at 37°C

##### 5.2.3.2. Gemini surfactants susceptibility assays

The minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were evaluated using three strains of *S. aureus*: DSM 2569, ATCC 33592 and ATCC 6538. Biofilm growth was performed as described in section 5.2.2.4. After 24h of biofilm formation, gemini surfactant (amide 5C and amide 12C) were prepared in different concentrations between 2.5 to 650 µg/mL. The supernatant was removed and biofilm was washed once with PBS (1M, pH 7.4) and then, the gemini

surfactants were added to biofilms in MHB medium and incubated at 37°C under aerobic conditions over 24h.

The effect of Gemini surfactants against *S. aureus* biofilms was evaluated based on biofilm viability and biomass using resazurin and crystal violet staining assays.

### Biofilm viability

After exposure of *S. aureus* biofilms to Gemini surfactants, the supernatant was removed and the well was washed once with PBS (1M, pH 7.4). Then, the biofilm viability was assessed using 20µM of resazurin sodium solution (Sigma-Aldrich, USA) and incubated for 30 min at 37°C, protected from light. Afterwards, 100µL of suspension was transferred to the black-well plate for fluorescence (Falcon®, USA) and the fluorescence was detected using a FlexStation 3 Microplate Reader (Molecular Devices, Germany) at  $\lambda_{ex}=560$  nm and  $\lambda_{em}=590$  nm.

The reduction of biofilm viability was measured according to the following equation (Eq. 3), knowing that 100% is the initial biofilm viability before gemini surfactant treatment.

$$\text{Reduction of biofilm viability (\%)} = 100 - \frac{\text{Experimental value}}{\text{Initial Biofilm}} \times 100 \quad (3)$$

### Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC)

After exposure of *S. aureus* biofilms to gemini surfactants, the supernatant was removed and the well was washed once with PBS (1M, pH 7.4).

The anti-biofilm activity of the gemini surfactants was evaluated by the determination of the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) using the microdilution method.

After incubation, the MBIC was determined considering the first well without visual pellet formation at the bottom of the well. The MBEC was determined by plating the contents of all wells where no visible growth was observed.

### Quantification of biofilm biomass

Briefly, the supernatant was carefully removed. Then, *S. aureus* biofilm was washed twice with PBS (1M, pH 7.4). Biofilm was fixed with methanol (MeOH, 99.8%, LabKem, Spain) for 15 minutes. Methanol was removed, biofilm dried and 0.1% v/v of crystal violet (CV, Sigma-Aldrich, USA) was added. Staining was performed for 5 min. Excess crystal violet was removed and the biofilms were rinsed five times with type II water. The remaining stain was dissolved using 200 µL of dimethylsulfoxide (DMSO 99.9% v/v; Fisher Scientific,

UK), and incubated at RT for 5 min before measuring absorbance ( $\lambda=570$  nm) using a Multiskan GO - Microplate Reader (Thermo Fisher Scientific, USA).

#### 5.2.3.3. Bioimaging evaluation

##### **Biofilm staining**

For the imaging of *S. aureus* biofilms, biofilms were grown in  $\mu$ -Slide 8-well glass bottom (Ibidi®, Germany) at a concentration of  $1 \times 10^5$  CFU/mL and biofilm were treated with Gemini surfactant at a concentration of 325  $\mu$ g/mL, as described in section 5.2.2.5. As a positive control, untreated biofilms were used, incubating with an MHB medium. To stain biofilms, the Filmtracer® LIVE/DEAD Biofilm viability kit (Invitrogen®, USA) was used for the staining following the manufacturer's instructions. Briefly, a mixture of SYTO 9 and propidium iodide (PI) (ratio 1:1) were performed in type I water and added to *S. aureus* biofilms. The  $\mu$ -Slide was incubated for 30 min at RT, protected from light. Then, the stain was removed and the biofilms were washed with type I water. To prevent drying, the stained biofilms were covered with PBS (1M, pH 7.4).

##### **Confocal laser scanning microscopy**

Confocal laser scanning microscopy (CLSM) was used for the imaging of the *S. aureus* biofilms. The bacteria stained with SYTO 9 were observed with excitation at 488 nm and bacteria stained with PI excitation at 561 nm. Images were recorded on a Leica Stellaris 8 confocal microscope (Leica Microsystems Wetzlar, Germany) equipped with the Leica Application Suite X package (LAS X). Images were acquired with a resolution of 1024x1024 using a 63x/1.4 Leica oil immersion objective and biofilm stacks were obtained with a step-size of 0.30  $\mu$ m.

#### 5.2.4. Cytocompatibility studies

##### 5.2.4.1. Cell culture conditions

To assess the cytocompatibility of the gemini surfactants, cytotoxicity studies were performed using the murine fibroblast cell line (L929) purchased from the European Collection of Authenticated Cell Cultures (ECACC), according to <sup>16</sup>. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco, USA) supplemented with 10% v/v

inactivated fetal bovine serum (FBS, Gibco, USA) and 1% v/v Penicillin-Streptomycin (Gibco, USA) at 37°C and under 5% CO<sub>2</sub> in humidified air. Cells were cultured for 24h of incubation and trypsinized when 80-90% confluence was reached, followed by counting in a Neubauer chamber with trypan blue solution (0.4% w/v, Sigma-Aldrich, USA) at ratio 1:1. Cells were seeded at a concentration of  $1 \times 10^5$  cells/mL to tissue culture 96-well plate (Falcon®, USA) and incubated 37°C, 5% CO<sub>2</sub>, for 24 hours. Then, the culture medium was removed and gemini surfactant was added to the first well at ratio 1:1, and serial dilutions were performed from a concentration of 325.0 µg/mL to 2.54 µg/mL). The plates were incubated for 24h at 37°C, and under 5% CO<sub>2</sub> in humidified air.

#### 5.2.4.2. Resazurin assay

The metabolic activity of cells in the presence of surfactants was evaluated using the fluorometric assay, resazurin reduction assay. This test assesses the metabolic activity of living cells by measuring the fluorescence caused by the conversion of resazurin to resofurin.

After exposure with Gemini surfactants, the supernatant was removed, and 10 µg/mL of resazurin was added to cells, and incubated for 2h, at 37°C, 5% CO<sub>2</sub>. Afterwards, the supernatant was transferred to a black 96-well plate (Falcon®, USA) and the fluorescence was detected using a Microplate Reader (Cytation 3, Biotek®, Bedfordshire, UK) at  $\lambda_{\text{ex}}$ =560 nm and  $\lambda_{\text{em}}$ =590 nm. A positive control (cells growing in fresh culture medium) and negative control (cells treated with 2% v/v Triton™ X-100; Sigma-Aldrich, USA) were included to normalize the results. Metabolic activity was determined according to the following equation (Eq.4).

$$\text{Metabolic activity (\%)} = \frac{\text{Experimental value} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100 \quad (4)$$

#### 5.2.4.3. Cell lysis

Cell death was evaluated through the LDH assay, that is, by quantifying the lactate dehydrogenase (LDH) activity that was released into the cell culture medium after cell lysis. After 24h of exposure to gemini surfactants, the plates were centrifuged (250g, for 10 minutes at RT) and the supernatant was collected and placed in a 96-well plate with the LDH Cytotoxicity Detection Kit (Takara Bio Inc, Japan) in a ratio 1:1. After 15 minutes of incubation, at RT and protected from light, the absorbance was measured at 490 and 690 nm using the microplate reader (Cytation 3, Biotek®, Bedfordshire, UK). The cytotoxicity

of the surfactants was determined according to the following equation (Eq. 5), in which the positive control was cells treated with a 2% v/v Triton™ X-100 solution and the negative control was untreated cells.

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental value} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100 \quad (5)$$

#### 5.2.4.4. Hemocompatibility study

Fresh human blood samples were obtained from healthy volunteers (Centro Hospitalar Universitário do Porto, Porto, Portugal) and processed to obtain human red blood cells (hRBC) by centrifugation at 995g at 4°C, for 5 minutes. After removal of the plasma upper layer, the lower layer containing hRBC was washed three times with 0.85% NaCl (w/v) (Sigma-Aldrich, USA). hRBC were dilute to 4% (v/v) in NaCl and hRBC were seed in a 96-well plate. Gemini surfactants were added and serially diluted and the range of gemini surfactants tested went from 325 to 1.27 µg/mL. After 1h of incubation at 37°C, the supernatant was carefully transferred to a 96-well plate and the absorbance values of the resealed haemoglobin were measured at 540 nm using a Microplate Reader (Cytation 3, Biotek®, Bedfordshire, UK). Untreated hRBCs were used as a negative control and hRBCs were treated with 1% (v/v) Triton™-X 100 (Sigma-Aldrich, USA). The percentage hemolysis was calculated according to the following equation (Eq.6).

$$\text{Hemolysis (\%)} = \frac{\text{Experimental value} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100 \quad (6)$$

#### 5.2.5. Statistics

Statistical analysis was performed with Graph Pad Prism 9.0 (Graph-Pad Software, La Jolla, USA). Results are presented as the mean ± standard deviation (SD). A p-value of <0.05 was considered statistically significant. Statistical significance was determined using the one-way ANOVA non-parametric test (Dunnett's multiple comparisons test) was used for comparisons of treated and untreated.

### 5.3. Results and discussion

The characterization of surfactants (size, surface charge, among other properties) is very important for their development and application. Surfactant characterization and quantification measures are a huge challenge, as they are affected by particle size and nature, sample concentration, solution properties and the fundamental principles of the applied methods.<sup>17</sup>

Among the various existing characterization methods, the following methods were addressed: the nanoparticle tracking analysis (NTA) method used to calculate the particle population concentration and particle size and the Zeta Potential (used to determine the charged surface of the particles). The behaviour in an aqueous solution of the surfactants gemini amide 5C and amide 12C was studied.

**Table 5.2** presents, the results obtained in relation to the value of size, zeta potential and concentration.

**Table 5.2.** Characterization of nanoparticles. The nanoparticles were characterized in terms of size and concentration using NanoSight® N300 and zeta potential using Zeta-Potential Analyzer (ZetaPals, Brookhaven Instruments Corporation, Software: PALS Zeta Potential Analyser v.5, Brookhaven Instruments; Holtsville, NY, USA). Results are presented as mean  $\pm$  standard deviation (SD).

| Samples                         | Amide 5C                               | Amide 12C                              |
|---------------------------------|----------------------------------------|----------------------------------------|
| Size (nm)                       | 124.1 $\pm$ 2.0                        | 155.9 $\pm$ 1.9                        |
| Zeta potential (mV)             | 45.5 $\pm$ 1.9                         | 66.9 $\pm$ 1.4                         |
| Concentration<br>(particles/mL) | 1.02 $\times 10^8 \pm 4.2 \times 10^6$ | 8.37 $\times 10^7 \pm 2.1 \times 10^7$ |

These compounds are known to aggregate into different structures, the aggregates formed by the amide 5C surfactants form a micellar structure and the amide 12C surfactants form vesicles. This difference can be explained by the fact that the structure of these compounds differentiates the spacer size, that is, the increase in spacer size leads to a greater distance between the two hydrophobic parts of the molecule. This difference only influences the aggregation, since when compared in terms of the size of the hydrodynamic diameter the difference is relatively small, the amide 5C measures 124.1 nm while the amide 12C measures 155.9 nm.

To determine the concentration of nanoparticles, nanoparticle tracking analysis (NTA) was used.

Two representative figures from NTA videos are shown in **Figure 5.2** and the determined concentrations are shown in **Table 5.2**.

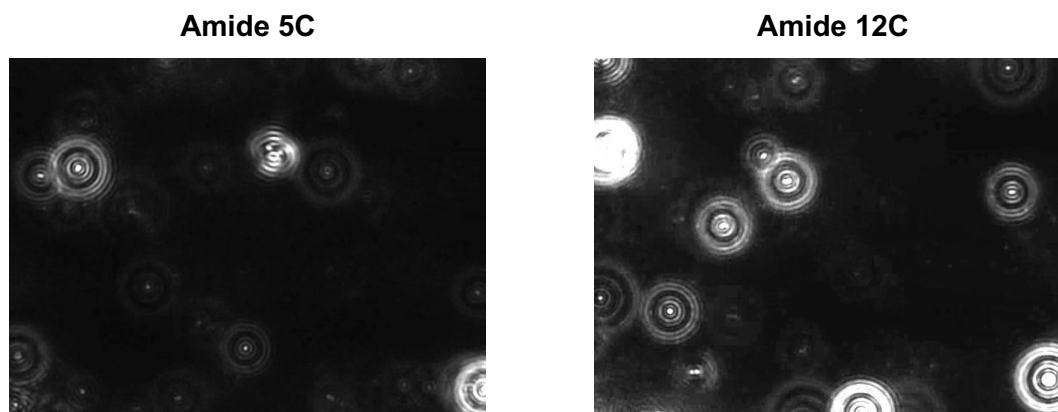


**Figure 5.2.** Representative frames of the video produced for NTA by NanoSight® N300. The images show the light diffracted by the nanoparticles. NTA determines the size and concentration of nanoparticles by tracking their movement.

In terms of surface charge, the structures of the surfactants proved to be stable, as they presented values greater than 30 mV and did not demonstrate to form aggregates, as it was possible to observe in the videos obtained for NTA during the determination of the concentration of nanoparticles (**Figure 5.2**).

Particles that have a zeta potential greater than or equal to 30 mV (negative or positive) are considered physically stable.<sup>18</sup>

In terms of size and zeta potential, the results obtained for amide 5C and amide 12C are similar (100-200 nm and + 40mV) to the previous results obtained by Silva *et al.* in another family of serine-derived gemini surfactants.<sup>19</sup>

**Figure 5.3** shows the profile of size and concentration stability of gemini surfactants over 70 days. This study allowed us to understand how the concentration and size of nanoparticles changed over time.

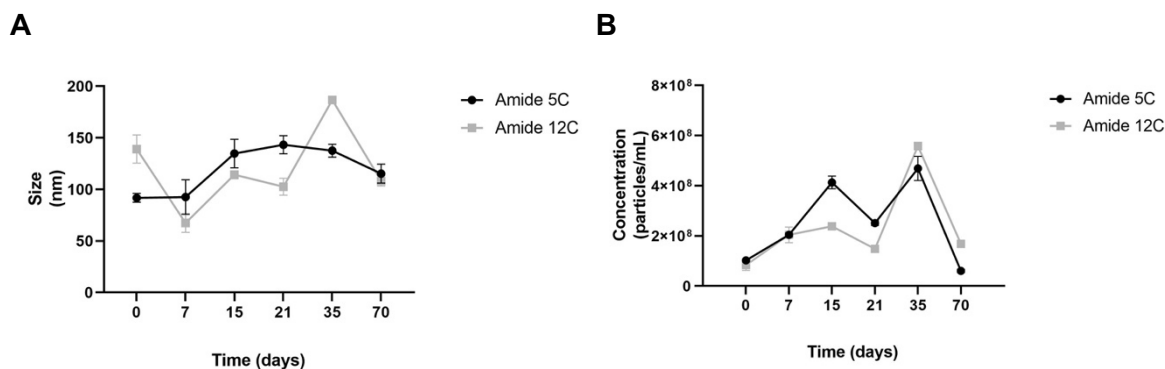


**Figure 5.3.** Stability study. The size (**A**) and concentration (**B**) of the nanoparticles were analyzed over a range of days using NTA by NanoSight N300 for 70 days. Results are expressed as mode  $\pm$  standard deviation (SD) (**A**) and mean  $\pm$  standard deviation (SD) (**B**). The surfactants amide 5C and amide 12C surfactants were diluted in type I water at a concentration of 10.09  $\mu\text{g/mL}$  and 21.06  $\mu\text{g/mL}$ , respectively.

As mentioned before, the surfactants through zeta potential values demonstrated high stability, which was proven since, after 70 days, the solutions still presented the surfactants in the form of aggregates with concentrations similar to the initial ones. Furthermore, the sizes of the hydrodynamic diameters of the nanoparticles also remained similar, which indicates, once again, the great stability of these compounds.

The compounds seem to have good physical properties and that is why you were evaluating the potential of being applied to eliminate *S. aureus*, the effect of amide 5C and amide 12C surfactants on their antimicrobial and antibiofilm activity on *S. aureus* strains was explored.

The bactericidal activity of the developed nanoparticles was evaluated by the minimum inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC).

The minimum inhibitory concentration (MIC) is considered one of the most important parameters in determining the antimicrobial activity of a compound. This defines the lowest concentration at which visible bacterial growth does not occur. The minimal bactericidal concentration (MBC) is complementary to the MIC, is defined as the lowest concentration of drug needed to kill 99.9% of the bacteria.

**Table 5.3.** shows the results obtained for the two gemini surfactants (amide 5C and amide 12C) under study, in the different strains of *S. aureus*.

**Table 5.3.** Antibacterial activity of nanoparticles on different bacteria. Numerical data represents MIC and MBC of gemini surfactants concentration against *S. aureus* strains ( $\mu\text{g/mL}$ ).

| Strain                     | Amide 5C |       | Amide 12C |       |
|----------------------------|----------|-------|-----------|-------|
|                            | MIC      | MBC   | MIC       | MBC   |
| <i>S. aureus</i> DSM 2565  | 5.08     | 10.16 | 10.16     | 10.16 |
| MRSA ATCC 33592            | 5.08     | 5.08  | 10.16     | 10.16 |
| <i>S. aureus</i> ATCC 6538 | 5.08     | 10.16 | 10.16     | 10.16 |

The two surfactants were shown to be bactericidal against the three strains of *S. aureus*, since the MBC of both compounds was found. Only the amide 5C, in the MRSA ATCC 33592 strain, was shown to have better activity, as only 5.08  $\mu\text{g/mL}$  is needed to obtain the minimum bactericidal concentration. In the other strains for the two compounds, a concentration of 10.16  $\mu\text{g/mL}$  was required.

The fact that  $cac$  is 5.98  $\mu\text{g/mL}$  ( $6.10 \mu\text{mol}\cdot\text{kg}^{-1}$ ) and 3.56  $\mu\text{g/mL}$  ( $3.30 \mu\text{mol}\cdot\text{kg}^{-1}$ ), for amide 5C and amide 12C, respectively, indicates that these compounds do not have bactericidal activity even in the form of monomers, they only have activity when they are in the form of aggregates. How these gemini surfactants interact with the bacterial membrane is thought to depend on the aggregates they form. This may explain the differences between the values obtained for the two surfactants. It is known that for cationic surfactants there are three different ways to interact with the bacterial membrane and that they can reach the bacterial surface electrostatically; accumulate on the bacterial surface until reaching the effective concentration, inserting and breaking the bacterial membrane.<sup>20</sup> Furthermore, the stability of the surfactant aggregates also affects its activity against Gram-positive and Gram-negative bacteria. When aggregated structures interact with bacteria, two balances occur simultaneously, that is, there is a disaggregation balance between aggregates and monomers and an aggregation balance between monomer molecules and the bacterial lipid membrane.<sup>20</sup> These two aspects may indicate the reason why amide 5C achieves bactericidal activity at lower concentrations of surfactant.

Therefore, the antimicrobial activity of surfactants depends on the structure of the aggregates, where micellar aggregates have superior antimicrobial potency.<sup>20</sup>

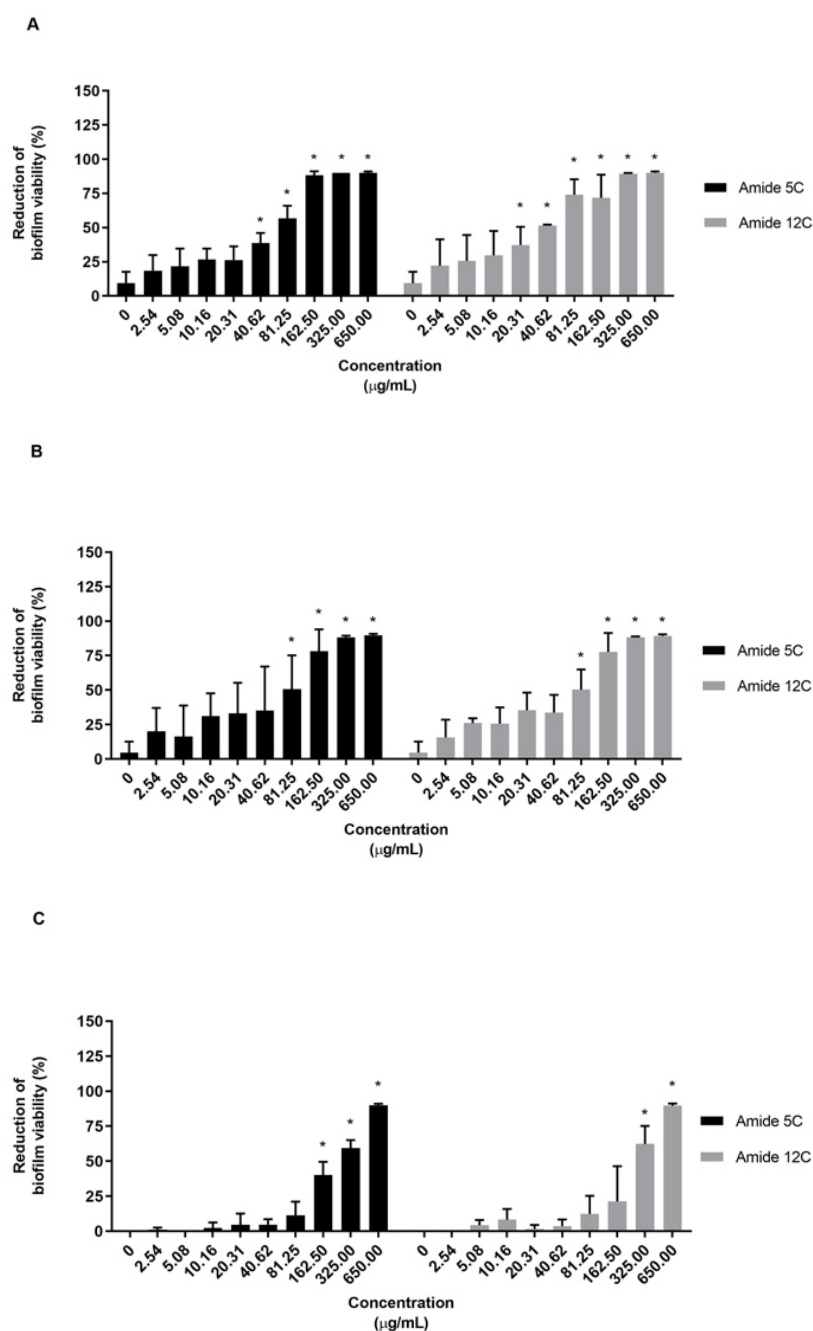
Once proven the activity of these surfactants in *S. aureus* in its planktonic form. The study of the activity of the gemini surfactants amide 5C and amide 12C in *S. aureus* biofilms was carried out.

Biofilm is associated with life-threatening infections due to its excellent resistance to antibiotics.<sup>21</sup> In this study, the anti-biofilm activity of the surfactants was evaluated. In terms

of biofilm, the minimum biofilm inhibitory concentration (MBIC) is defined as the lowest concentration at which there is no time-dependent increase in the number of viable bacteria in biofilm and the minimum biofilm eradication concentration (MBEC) is the lowest concentration needed to completely eradicate the biofilm.<sup>22</sup>

In **Figure 5.4.** the results obtained from the quantification of the reduction in biofilm viability through the resazurin, MBIC and MBEC tests obtained in the susceptibility assays and the quantification of the total biomass are shown.

I

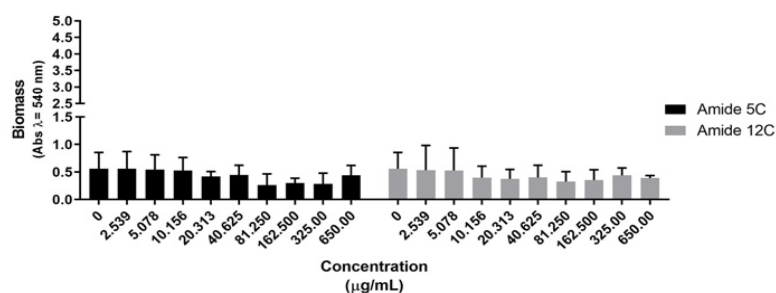


II

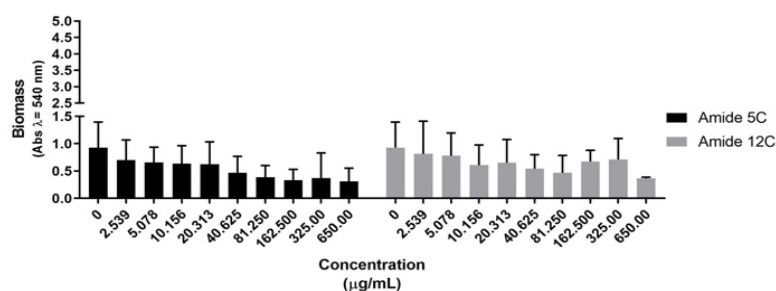
| Strain                     | Amide 5C |       | Amide 12C |       |
|----------------------------|----------|-------|-----------|-------|
|                            | MBIC     | MBEC  | MBIC      | MBEC  |
| <i>S. aureus</i> DSM 2569  | 162.5    | 325.0 | 162.5     | 325.0 |
| MRSA ATCC 33592            | 162.5    | 325.0 | 162.5     | 325.0 |
| <i>S. aureus</i> ATCC 6538 | 325.0    | 650.0 | 325.0     | 650.0 |

III

A



B



C

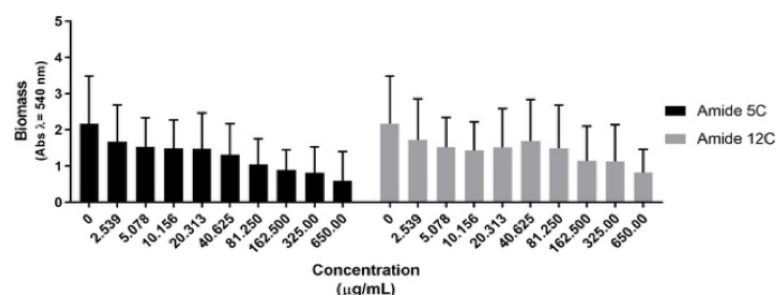


**Figure 5.4.** Anti-biofilm evaluation. I. Reduction in biofilm viability by biofilm viability assessment using resazurin. II. Correlation between MBEC and MBIC and the concentration of gemini surfactants. Numerical data are presented in surfactant concentration ( $\mu\text{g/mL}$ ). III. Quantification of total biofilm biomass, quantified by the CV assay. The letters **A**, **B** and **C** correspond to *S. aureus* strains: DSM 2569, MRSA ATCC 33592 and *S. aureus* ATCC 6538, respectively. \*  $p < 0.05$ , significantly different compared to untreated.

Both surfactants caused a decrease in biofilm viability, although amide 5C was more effective than amide 12C in planktonic bacteria, here the difference between the two compounds was not significant.

However, at lower surfactant concentrations, the effectiveness of compounds in the biofilm is not high, it is believed that the biofilm matrix is negatively charged due to polysaccharides and attracts the positively charged compounds to protect bacterial cells.<sup>23</sup> It is known that the polysaccharide matrix plays an important role in biofilm maintenance and antibiotic resistance.<sup>11</sup>

In addition to this characteristic of biofilms, the size of gemini surfactants is essential for them to penetrate the matrix, it is indicated that nanoparticles with diameters between 100 and 200 nm can effectively penetrate the biofilm matrix.<sup>24</sup> In addition, the activity of surfactants in the biofilm is dependent on the type of amino acid and the length of the alkyl chain.<sup>11</sup>

In addition to the results shown in the viability reduction assay and biomass quantification, the potency of this class of compounds as anti-biofilm agents was investigated by performing MBIC and MBEC. A MBIC was found at 325 µg/mL for *S. aureus* DSM 2569 and MRSA ATCC 33592, and at 650 µg/mL for *S. aureus* ATCC 6538, suggesting that this class of compounds is shown to have antimicrobial activity. Therefore, this class of compounds has great potential to be developed as selective therapeutic agents to fight infections associated with bacteria and biofilms formed by *S. aureus*.

At the same concentration as MBIC, confocal microscopy also revealed a significant disruption of the biofilm, as shown in **Figure 5.5**. Although the biofilm decrease mechanism is not yet concrete, it is believed that there are strong electrostatic and hydrogen bond interactions with the biofilm extracellular matrix components.

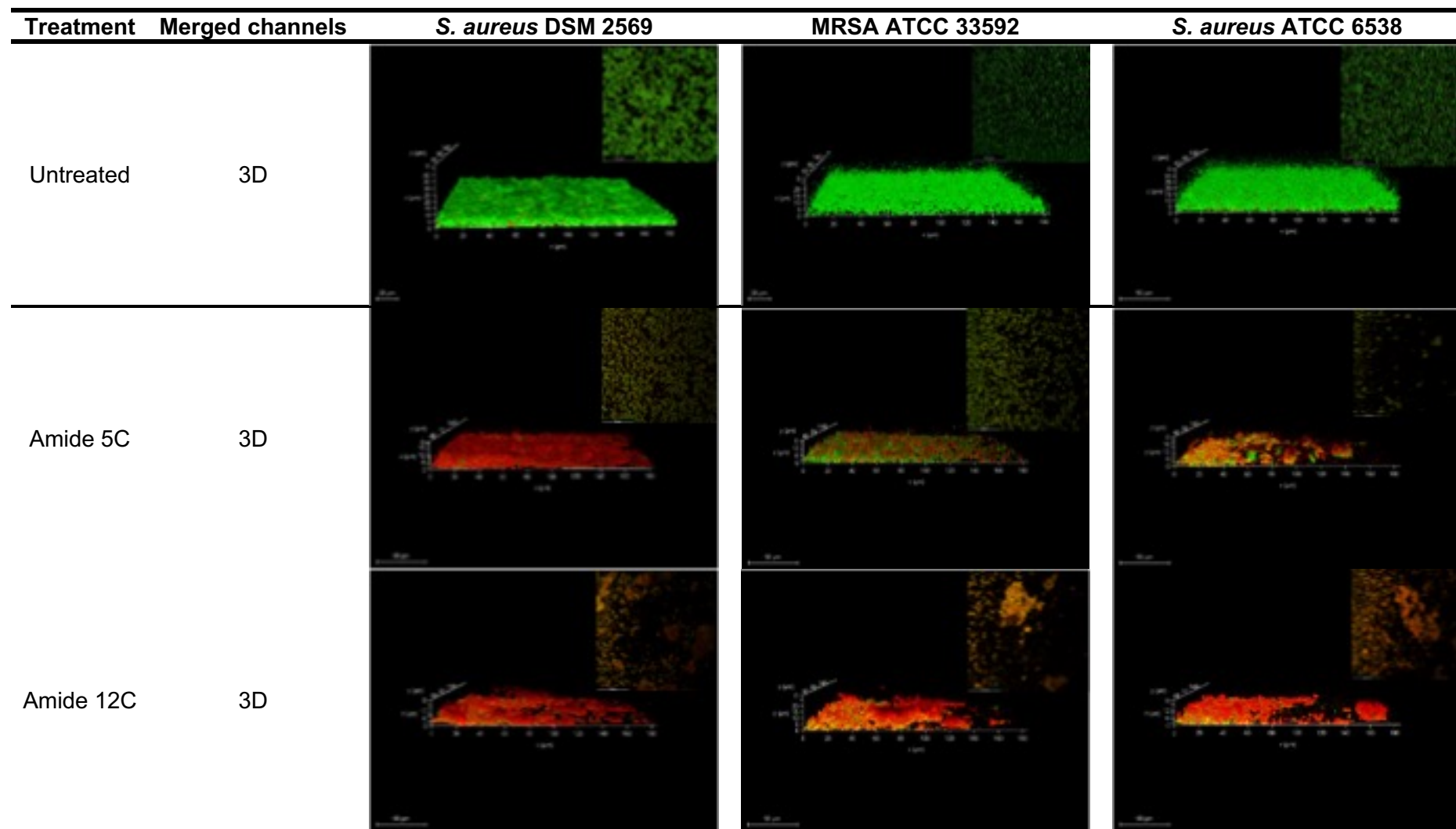


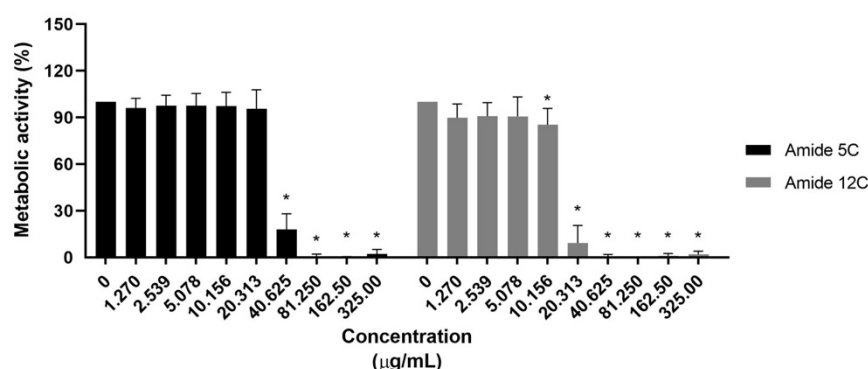
**Figure 5.5.** CLSM images of *S. aureus* biofilms untreated and treated with amide 5C and amide 12C. Images 2D and 3D are a merge of two channels: live bacteria are shown in green and dead bacteria are shown in red. Images were acquired with a resolution of 1024x1024 using a 63x/1.4 Leica oil immersion objective and biofilm stacks were obtained with a step-size of 0.30  $\mu\text{m}$ .

Biofilm analysis using CLSM provides qualitative information about the viability and organization of bacteria in the biofilm. The images obtained for the biofilms show live bacteria (in green). In both surfactants it is possible to visualize an extent of biofilm breakage compared to the untreated control. With the analysis of biofilms using CLSM, it was possible to corroborate the information previously obtained during the MIBC tests, concluding that the compounds have antimicrobial activity.

In addition to antimicrobial activity, cytotoxicity is also an important evaluation aspect for the development of antimicrobial surfactants. Since cytotoxicity is one of the limiting parameters of conventional surfactants, since they have high cytotoxicity.<sup>20</sup> Thus, it is also important to study amide 5C and amide 12C.

The evaluation of the cytotoxicity of the gemini surfactants was carried out in L929 fibroblasts, after 24 hours of exposure, in order to obtain results regarding cell lysis and the metabolic activity of the cells, respectively. In **Figure 5.6.** are the results obtained.

**A**



**B**

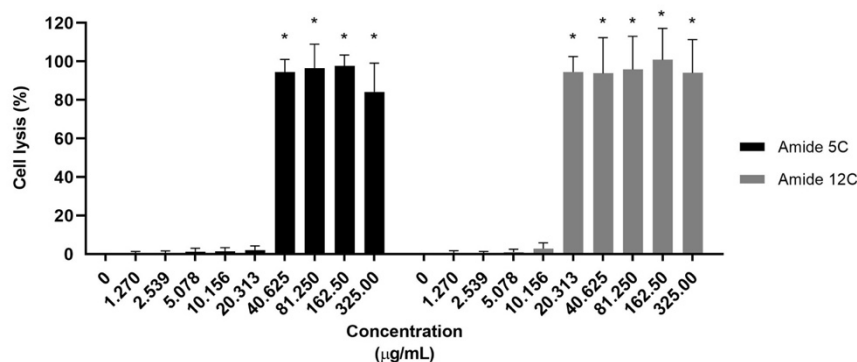


**Figure 5.6.** Cytocompatibility evaluation by **(A)** metabolic activity assessment by resazurin test and **(B)** cell lysis analysis by LDH assay. **(A)** shows the mean and standard deviation of the results for percent cell viability, and **B** shows the mean and standard deviation of the results for percent cell lysis. \*  $p < 0.05$ , significantly different compared to untreated.

The results obtained indicate a notable difference between the two surfactants, when the compounds are in the form of aggregates, amide 12C presents greater cytotoxicity compared to amide 5C. According to the ISO 19903-5 standard, from 70% the compounds are considered toxic.<sup>16</sup> When the concentration is below the *cac*, none of the compounds showed cytotoxicity.

When surfactants are in the form of aggregates, the main mechanism associated with cytotoxicity is solubilization, that is, after the surfactant electrostatically binds to the cell surface, the surfactant aggregates solubilize cell membrane lipids and/or proteins, forming mixed aggregates. This effect causes cell lysis.<sup>20</sup>

The difference in the concentrations of the two surfactants amide 12C and amide 5C that cause cytotoxicity is due to the increase in the spacer, making the amide 12C more hydrophobic, that is, more cytotoxic.<sup>20</sup>

Furthermore, the stability of the aggregates also influences the cytotoxicity of the surfactants. The more stable the aggregate, the lower the cytotoxicity caused.<sup>20</sup>

In addition to cytotoxicity assays, a preliminary study of hemolysis was performed. The results obtained are shown in the **Figure 5.7.**

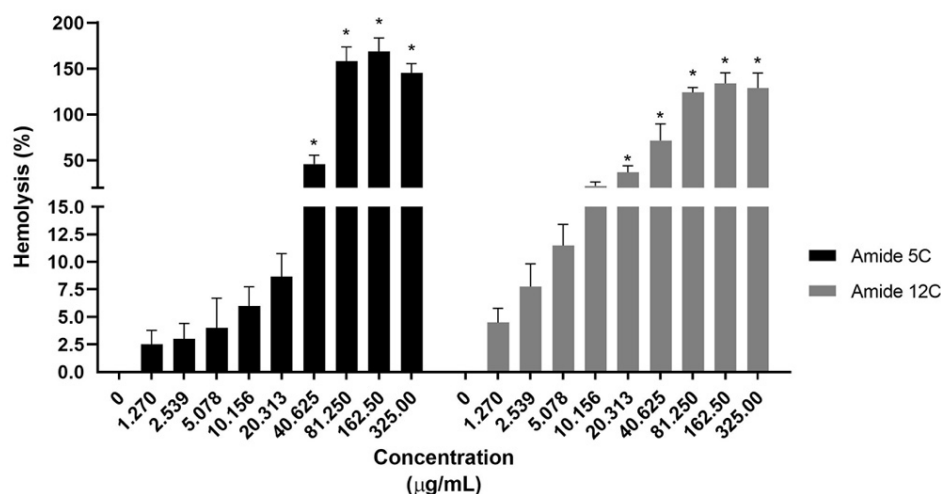


**Figure 5.7.** Hemocompatibility study. Shows the mean and standard deviation of the results for percent haemoglobin release. \*  $p < 0.05$ , significantly different from untreated.

From the values obtained, it can be seen that there is a great effect of surfactants on the destruction of the plasma membrane of human red blood cells. According to the ASTM F756-13 standard<sup>25</sup>, the two surfactants have a hemolysis effect starting at 5%, so both amide 5C and amide 12C have a high release of haemoglobin. Hemolysis is usually related to hydrophobicity, in which case it is correlated with spacer size. The larger the spacer size, the greater the hemolysis effect.<sup>23</sup>

## 5.4. Conclusion

The study of the biological and physical characteristics of serine-derived gemini surfactants only focused on the amide series of gemini surfactants with spacer carbon numbers equal to 5 and 12.

Both surfactants have low *cac* values; however, they exhibit distinct phase behavior. While compound amide 5C forms micelles in solution, compound amide 12C forms vesicles. In terms of the size of the hydrodynamic diameter the difference is relatively small, the amide 5C measures 124.1 nm while the amide 12C measures 155.9 nm.

Since amino acid based surfactants have been seen as a promising alternative as antibacterial and antifungal agents, the effect of amide 5C and amide 12C surfactants on their antimicrobial and antibiofilm activity in *S. aureus* strains was explored. The two surfactants proved to be bactericidal against the three virulent strains of *S. aureus*, as after 24 hours the bacterium was dead. These compounds only show activity when they are in the form of aggregates, and amide 5C showed better activity when compared to amide 12C.

Both surfactants caused a decrease in biofilm viability, although amide 5C was more effective than amide 12C on planktonic bacteria, here the difference between the two compounds was not significant.

Therefore, this class of compounds has great potential to be developed as selective therapeutic agents to combat infections associated with bacteria and biofilms formed by *S. aureus*.

In addition to antimicrobial activity, cytotoxicity is also an important evaluation aspect for the development of antimicrobial surfactants. Since cytotoxicity is one of the limiting parameters of conventional surfactants, as they present high cytotoxicity.

The results obtained indicate a notable difference between the two surfactants, when the compounds are in the form of aggregates, amide 12C presents greater cytotoxicity compared to amide 5C. When the concentration is below *cac*, none of the compounds showed cytotoxicity.

The difference in the concentrations of the two surfactants amide 12C and amide 5C that cause cytotoxicity is due to the increase in the spacer, making the amide 12C more hydrophobic, that is, more cytotoxic.

In addition to cytotoxicity assays, a preliminary hemolysis study was performed. From the values obtained, it can be seen that there is a great effect of surfactants on the destruction of the plasma membrane of human red blood cells.

Thus, the tested gemini surfactants can be used in the future as effective bactericidal agents against *S. aureus* in its planktonic and biofilm form.

It is noteworthy that both compounds, due to their strong antimicrobial activity, are promising candidates for new systematic studies on how different chain lengths can affect biological and physical characteristics.

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## 6. Conclusion and future perspectives

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The aim of the project was to synthesize three different series of novel amino acid-based surfactants to allow a systematic study of the influence of (i) the spacer length and (ii) the nature of the bond between spacer and polar headgroup on their interfacial and aggregation properties, in order to assess their potential use as antimicrobial agents.

In this work, a new family of serine-based gemini surfactants has been synthesized. Our aim was to synthesize surfactants of three different series, designated as amine, amide or ester, according to the bond “spacer-residue”. All the surfactants possess twelve-carbon chains and spacers with five or twelve methylene groups.

The synthesis of the amide series was successfully accomplished. A total of four compounds of this series has been obtained: two tertiary amine derivatives (**4a**, five carbon spacer; **4b**, twelve carbon spacer), which can be transformed into the corresponding ammonium surfactants in acidic media, and two quaternary ammonium salts (**5a/b**). The most efficient synthetic pathway has been established and consisted in the introduction of the spacers between the carboxylic acid groups of *N*, *N*-dimethyl-*O*-dodecanoylserine (**3**) through peptide condensation methods using TBTU as coupling agent. The final quaternary ammonium salts were obtained by exhaustive methylation of the ammonium precursors.

In the case of amine and ester series, the synthesis of the ammonium salts was also successfully accomplished. Two compounds, one of each series, with twelve-carbon spacers were obtained (**15b**, ester; **22b**, amine). In both cases the most efficient synthetic pathway consisted in the introduction of the spacer into the amino acid derivatives followed by *O*-acylation. The transformation of these compounds into the corresponding ammonium surfactants has not yet been achieved, despite the many attempts made. The major difficulty experienced was the deprotonation of the ammonium salts (previous to methylation), eventually due to the presence of the trifluoroacetate anion. For these reasons, a future approach may consist in the conversion of the trifluoroacetates into the corresponding chlorides before methylation.

Overall, the objectives of the project were achieved. Some of the reactions have been optimized resulting in higher yields of products. Six new compounds were successfully obtained.

The evaluation of the physicochemical properties of the synthesized gemini surfactants aimed to provide the necessary information to carry out subsequent experiments on antimicrobial activity and cytotoxicity.

The study of the biological and physical characteristics of serine-derived gemini surfactants only focused on the amide series of gemini surfactants with spacer carbon numbers equal to 5 and 12.

Both surfactants showed *cac* values much lower than those of homologous conventional analogs. More interestingly, they exhibit distinct phase behavior: while the amide gemini with a five carbon spacer forms micelles in solution, the one with the twelve carbon spacer forms vesicles (or liposomes). Such a behavior had already been observed for another series of gemini surfactants synthesized in our laboratories and had been assigned to the conformational rigidity at the headgroup level, resulting from the rigidity of the amide bond. The twelve carbon methylene group is expected to bend inwards the core and the surfactants adopt a cylindrical geometry at the interface, which favors the formation of liposomes. This does not occur for shorter spacers, and hence this distinct behavior of the two geminis.

In terms of the size of the hydrodynamic diameter of the aggregates, the difference is relatively small, the amide 5C measures 124.1 nm while the amide 12C measures 155.9 nm.

Since amino acid based surfactants have been seen as a promising alternative as antibacterial and antifungal agents, the effect of amide 5C and amide 12C surfactants on their antimicrobial and antibiofilm activity in *S. aureus* strains was explored. The two surfactants proved to be bactericidal against the three strains of *S. aureus*, as after 24 hours the bacterium was dead. Only amide 5C, in the MRSA ATCC 33592 strain, was shown to have better activity, as only 5.08 µg/mL are needed to obtain the minimum bactericidal concentration. In the other strains for the two compounds, a concentration of 10.16 µg/mL was required.

The fact that *cac* is 5.98 µg/mL and 3.56 µg/mL, for amide 5C and amide 12C, respectively, indicates that these compounds do not have bactericidal activity even in the form of monomers, they only have activity when they are in the form of aggregates.

Both surfactants caused a decrease in biofilm viability, although amide 5C was more effective than amide 12C on planktonic bacteria, here the difference between the two compounds was not significant.

In addition to the results presented in the viability reduction assay and biomass quantification, the potency of this class of compounds as anti-biofilm agents was investigated by performing MBIC and MBEC. An MBIC was found at 325 µg/mL for *S. aureus* DSM 2569 and MRSA ATCC 33592, and at 650 µg/mL for *S. aureus* ATCC 6538.

Therefore, this class of compounds has great potential to be developed as selective therapeutic agents to combat infections associated with bacteria and biofilms formed by *S. aureus*.

In addition to antimicrobial activity, cytotoxicity is also an important evaluation aspect for the development of antimicrobial surfactants. Since cytotoxicity is one of the limiting parameters of conventional surfactants, as they present high cytotoxicity.

The evaluation of gemini surfactants cytotoxicity was performed in L929 fibroblasts, after 24 hours of exposure, in order to obtain results regarding cell lysis and metabolic activity of cells, respectively.

The results obtained indicate a notable difference between the two surfactants, when the compounds are in the form of aggregates, amide 12C presents greater cytotoxicity compared to amide 5C. When the concentration is below *cac*, none of the compounds showed cytotoxicity.

The difference in the concentrations of the two surfactants amide 12C and amide 5C that cause cytotoxicity is due to the increase in the spacer, making the amide 12C more hydrophobic, that is, more cytotoxic.<sup>1</sup>

In addition to cytotoxicity assays, a preliminary hemolysis study was performed. From the values obtained, it can be seen that there is a great effect of surfactants on the destruction of the plasma membrane of human red blood cells.

Thus, the tested gemini surfactants can be used in the future as effective bactericidal agents against *S. aureus* in its planktonic and biofilm form. Using these compounds in a hospital setting to prevent microbial adhesion can help prevent the spread of difficult-to-fight infections.

It is noteworthy that both compounds, due to their strong antimicrobial activity, are promising candidates for new systematic studies on how different chain lengths can affect biological and physical characteristics.

In addition to the existing application possibilities for the two surfactants tested regarding their biological activity, there are still other complementary studies to be carried out, in order to try to reduce their cytotoxicity.

In the future, the objective will be to complete the synthesis of the other two series in order to be able to carry out an activity structure study. In addition to these two series (ester and amine) there is also the possibility of altering the alkyl chain of these compounds, in order to understand how its size will influence the activity of the compounds.

## Supplementary material

