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SYNTHESIS OF SMALL PEPTIDES FOR BIOMEDICAL APPLICATIONS

FINAL RESEARCH PROJECT BY LUIS GUTIERREZ CADUCH

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ABBREVIATIONS

AA = Amino acid
DCC = N, N'-dicyclohexylcarbodiimide
DCU = Dicyclohexylurea
DMAP = 4-Dimethylaminopyridine
DME = Dimethoxyethane
DMF= N,N-Dimethylformamide
DMSO = Dimethylsulfoxide
EDCl = N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
ES-QTOF= Electrospray quadrupol time of flight
Etc = Etcetera
EtOH= Ethanol
F = Phenylalanine
FF = Diphenylalanine
HOSu= N-Hydroxysuccinimide
LMW = Low molecular weight
MeOH= Methanol
MGC = Minimum gel concentration
N = Amino end
NMR= Nuclear magnetic resonance
SEM = Scanning electron microscopy
TEM = Transmission electron microscopy
THF = Tetrahydrofuran
Z = Bencyloxycarbonyl

1. INTRODUCTION

Gelation

Stimuli-responsive hydrogels are intriguing biomaterials useful for controlled release of drugs, cell engineering and medical diagnosis. To date, many physical and chemical stimuli-responsive polymer hydrogels have been developed by chemical modification of polymer chains and cross-linking points. In particular, conjugation with biomolecules to polymers produced promising biomolecule-responsive hydrogels what indicates high potentials of stimuli-responsive hydrogels as promising biomaterials. In addition to polymer hydrogels, supramolecular hydrogels formed by the assembly of small molecules (hydrogelators) via non-covalent interactions have also been regarded as unique and promising soft materials due to their flexible programmability in rendering them stimuli-responsive with the larger macroscopic change (i.e., gel−sol transition) (Figure 1).

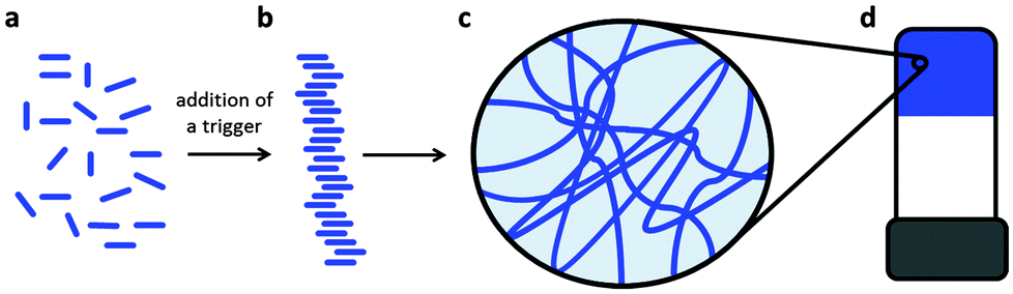


Figure 1. General process of self-assembly of supramolecular gels.
a)Gelator molecules b)Self-assembly by non-covalent interactions c)crosslinking 3D structure d)Supramolecular gel.

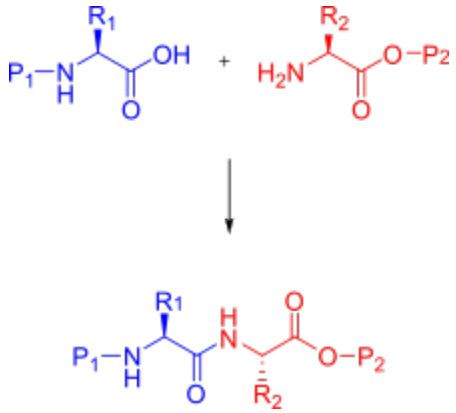
Such biomolecule-responsive hydrogels are a potentially promising tool for user-friendly early diagnostics and on-demand drug-releasing soft materials.[1]

Thanks to its structure and chemical conditions, and the great knowledge of the proteins, peptides have become an important field of study within the science of biomaterials. Our study focuses on the formation of small peptides, low molecular weight (LMW), able to form dynamic supramolecular hydrogels that may reverse the assembly process due to different stimuli such as temperature, concentration, pH changes, etc. [2][3]

Peptides

A peptide is a molecule that results from the union of two or more amino acids (AA) by amide bonds, which are called peptide bonds and are the result of the reaction of the carboxyl group of one AA with the amino group of another, with elimination of a molecule of water. This a priori simple reaction requires the activation of the

carboxylic acid of one of the AAs and the protection of any nucleophilic group that may compete with the peptide bond formation (Scheme 1).



Scheme 1. Scheme of reaction of two protected amino acids.

As a general rule, the peptides are not bigger than 50 AAs, but regardless of the length of the polypeptide chain, there will always be two unreacted ends, an NH_2 group (the amino terminus) and a COOH group (the carboxyl terminus). Small self-assembling peptides follow the same hierarchical structural organization of self-assembly as proteins (Figure 2).

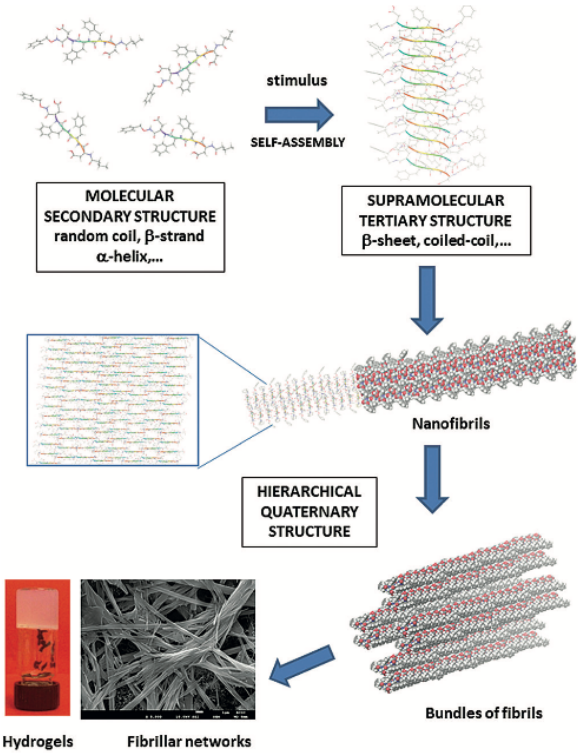


Figure 2. Parallels between proteins and gel in assembling.

Small peptides in solution present a secondary structure, so the peptide backbone is programmed to self-assembly, by non-covalent intermolecular interactions, into a supramolecular tertiary structure, which can be combined to form a quaternary structure in order to form a gel by crosslinking between them.[2]
LMW peptides are much easier to synthesize, purify or be tuned for specific applications. The simplest compounds that form gels are N-protected amino acids in which polar fragments of the amino acid backbone are combined with hydrophobic groups either as side groups or as N-capping residues. Several examples of N-protected amino acids bearing aromatic groups[19], such as fluorenylmethyloxycarbonyl (Fmoc), have been reported as efficient hydrogelators and π-stacking has been revealed to be a crucial interaction for the formation of fibrillar aggregates and gels.[4]

However, upon increasing the size of the molecule with extra amino acids (AAs), the design opportunities also increase. Therefore, the design of dipeptide hydrogelators and longer analogues are frequently based on the molecular secondary structure. This structure should be pre-organized, at least partially, to arrange in an ordered 1-D packing, which can be seen as a supramolecular tertiary structure **(Figure 2)**. The secondary structure preferences are determined by the rigidity of the amide group, as well as the size and polarity of the side chains. However, within the different secondary structures that can present, the β-sheet predominates in LMW peptides.[5]

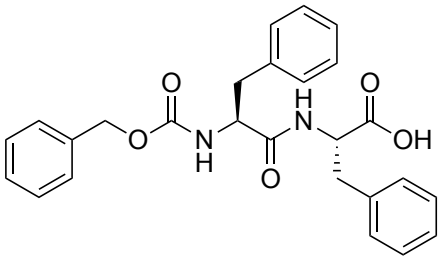


Figure 3. ZFFOH

Our molecular design is based on the previous studies that FF bearing protecting groups at the N-terminus, such Z-FF-OH, can self-assemble into fibrous nanostructures, thereby giving rise to hydrogel formation.[6] A base- and slightly acid- tolerant Z-protected dipeptide is chosen to being added to an hydrazide. [7]

Hidrazide

The carboxylic acid end of FF will be converted into a hydrazide, by an amide bond.[8]

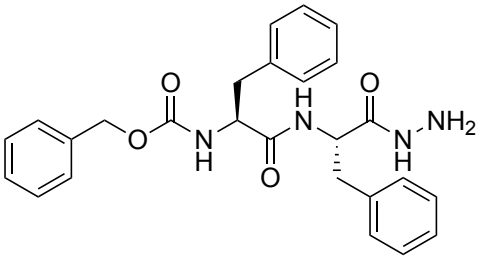


Figure 4. ZFFNHNH$_2$

The presence of hydrazide will allow us to form hydrazone bonds, sensitive to pH changes, with drugs that present carboxyl C = O, of ketone [9] or aldehyde groups [10][11] as well as N-glycoside bond.[7]

Skin layers and the pH

The skin is an organ made up of different tissues, joined together to perform specific activities that is formed by three different layers that protect the body from the external factors. It consists firstly the layer called epidermis, that prevents from the environment, continued by a secondary layer called dermis, that prevents from traumatisms and finally the hypodermis, which holds the adipose tissue that keeps the body warm and gives mobility to the skin.

There are enough differences but we are going to focus on the pH of them. The hypodermis is known as “acid mantle” because it has a pH slightly acidic, unlike the other layers which have a neutral pH. The average pH levels are 4.85 for men and 5 for women, although this difference is not that significant to create different cosmetics claiming differences in pH levels. The pH also varies according to the area of the body, less acid in the armpits and between the fingers, or the age, since the pH values are more alkaline from birth to puberty and in old age. [20]

This change in acidity is the most important point on which we focus because this allows us to apply a gel topically, releasing the drug by gel degradation when detect the external stimuli caused by that pH change between the external and internal skin, thus providing treatment to the inner area damaged.

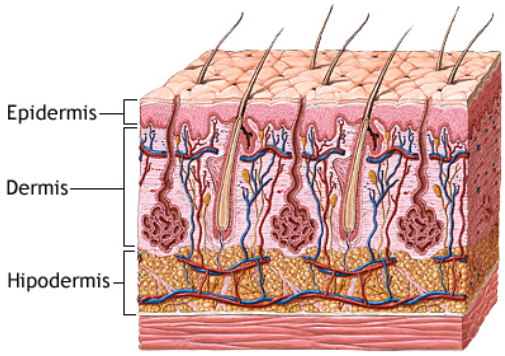


Figure 5. Skin layers.

Though the synthesis of a hydrogel, we can apply treatments focused on skin diseases and also treatments on skin wounds. It allows us for example to heal infected wounds or help the healing of wounds in people suffering diabetes, ... [12]

Nanoparticles have an ability to deliver a sustained and controlled release of therapeutics that result in an accelerated healing process. An increasing number of innovative nanotherapies have emerged in the field of wound healing and are currently under clinical investigation. There are two main categories of nanomaterials

used in wound healing, nanomaterials that exhibit intrinsic beneficial properties for the treatment of wounds and nanomaterials used as delivery vehicles for therapeutic agents, which is our case.

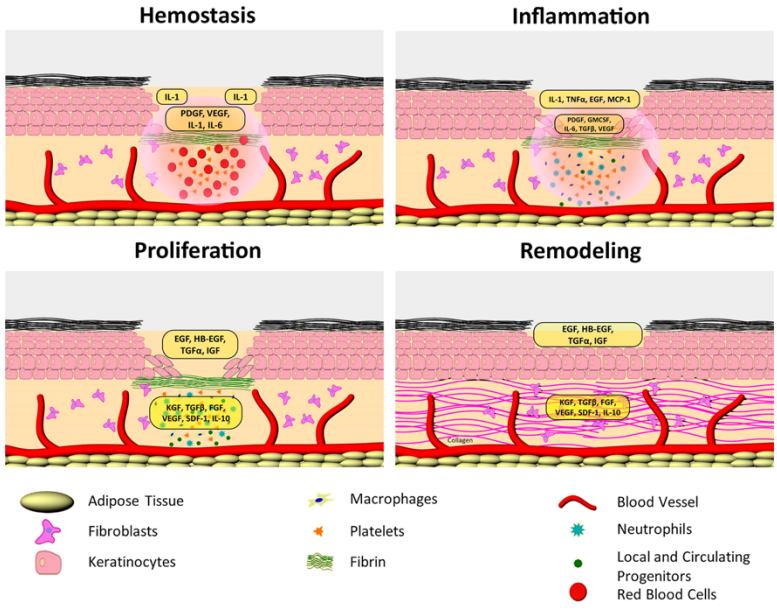
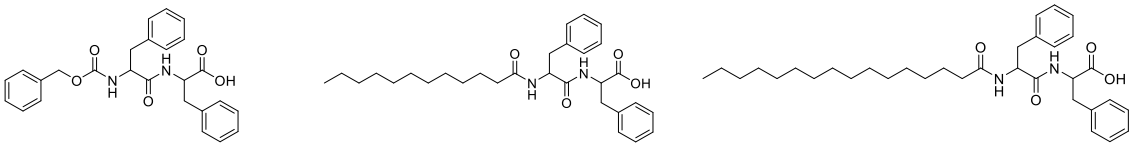


Figure 6. Phases of cutaneous wound healing depicting the cells and molecules responsible for the regaining of a healthy barrier.

2. OBJECTIVE

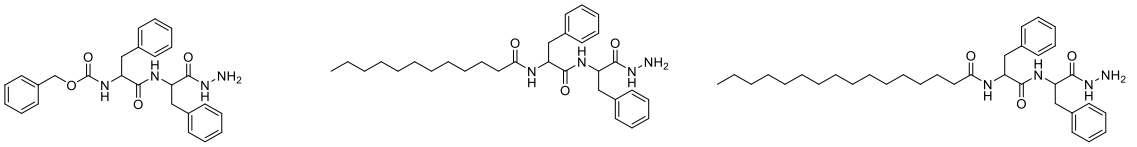
The objective of this study is the design of small peptides for biomedical applications, constructing biocompatible hydrogels able to respond to an external stimulus and form gel in a different pH. Mainly, we focus on the creation of a small peptide that forms a gel able to release the drug in response to a pH change similar to that found between the external and internal skin that could be useful for the treatment of skin wounds.



ZFFOH C_{12}FFOH C_{16}FFOH

Figure 7. Good gelators peptides.

As we know because of the previous studies done in the Dr. Beatriu Escuder research group, these three dipeptides are good gelators.[13]

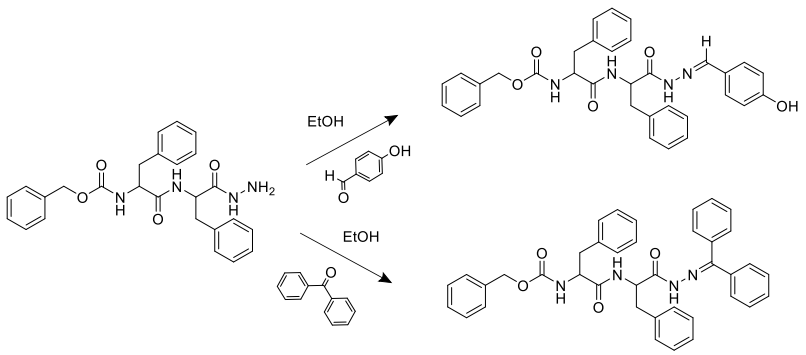


ZFFNHNH_2 C_{12}FFNHNH_2 C_{16}FFNHNH_2

Figure 8. Peptides bound to hydrazide.

We will try to synthesize these three dipeptides and then characterize them.
The only commercial product from them is ZFFOH, so we decide to use it like model compound to determine the final methodology to assembly the hydrazine that allow us to form bonds with drugs that contain carboxyl group, like ketone and aldehyde group.

Once characterized, we will proceed to the assembly of the hydrazide with the ketone and aldehyde groups. We will have to check once purified that they can form gels, and do drug release tests. This could make possible the controlled release of drugs, for example in skin wounds to help the healing of it, which would be a long term objective.



Scheme 2. ZFFNHNH_2 assembly with ketone and aldehyde group.

3. RESULTS AND DISCUSSION

Synthesis of peptide-hydrazides.

Our studies focus on the production of three peptides that could be good gelators which could contain the drug and release it after a pH change.

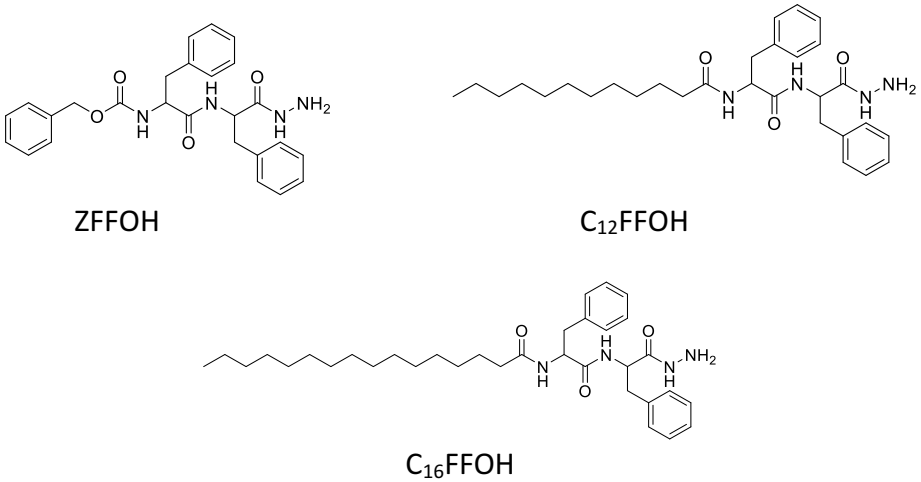
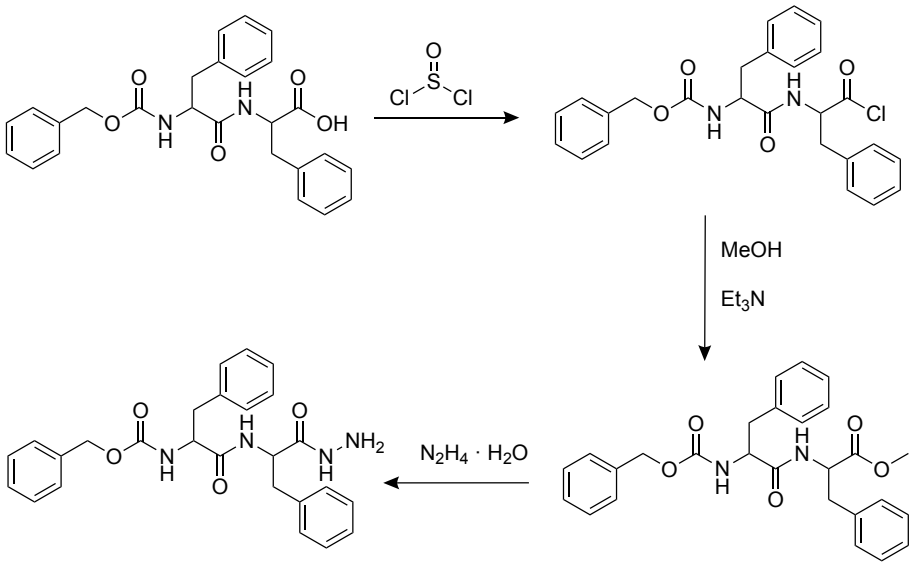


Figure 9. Peptides bound to hydrazide.

To choose the correct methodology we took the ZFFOH as model product, because it was the only one commercial product from the three of them.

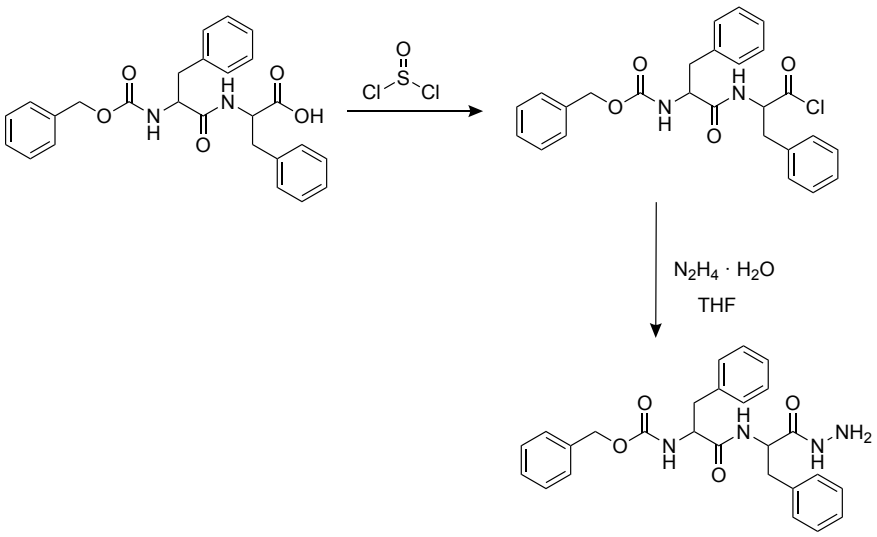
Based on the article by Luo and co-workers [14] we tried to apply a methodology of methyl ester formation that reacts later with hydrazine to bind amines to carboxylic acid:



Scheme 3. Methodology using methyl ester formation.

The results were not satisfactory because the products were obtained with very low yield, impossible to purify completely afterwards.

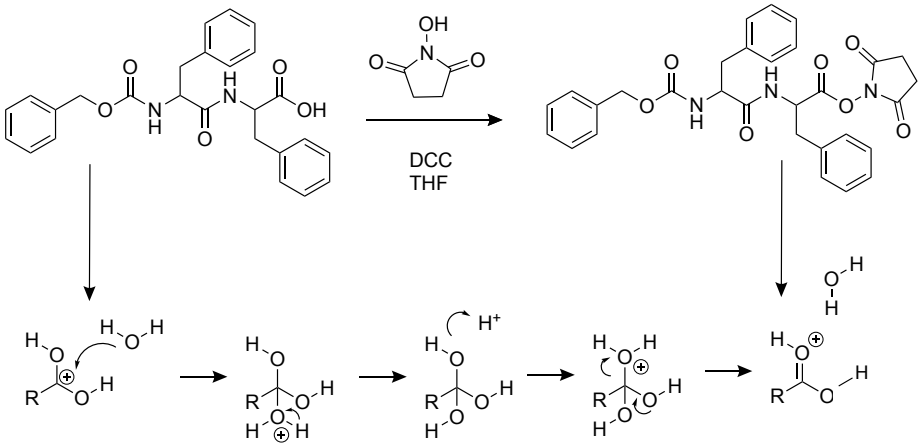
We decided then to take another route based on the paper by Okesola and Smith [15], starting from the ZFFOCl obtained by the first part of the previous study and react it directly with hydrazine in THF.



Scheme 4. Methodology reacting directly with N_2H_4.

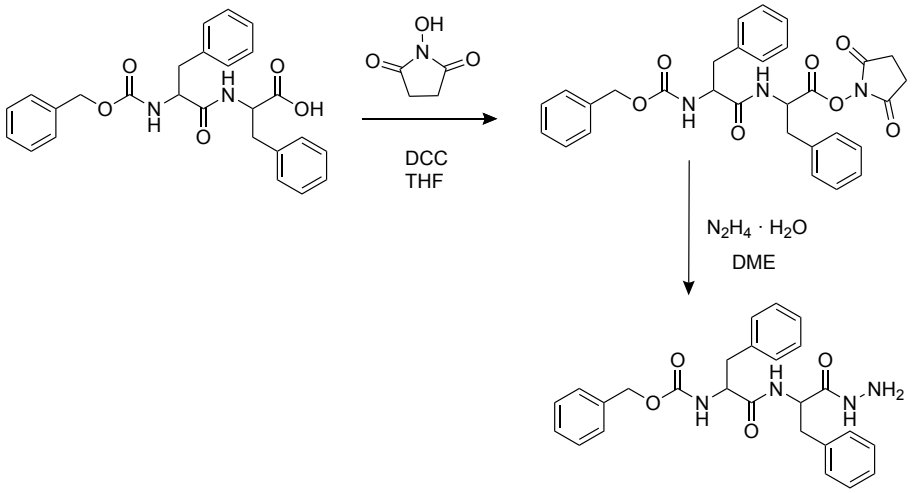
The yield was not the expected and the compound was not pure, so we changed the action line.

We decided to directly react our dipeptide with hydrazine in the presence of HOSu. It forms an excellent leaving group that increases reactivity and does not form sub-products, so if our product is not soluble in the reaction media it allows a fantastic separation.



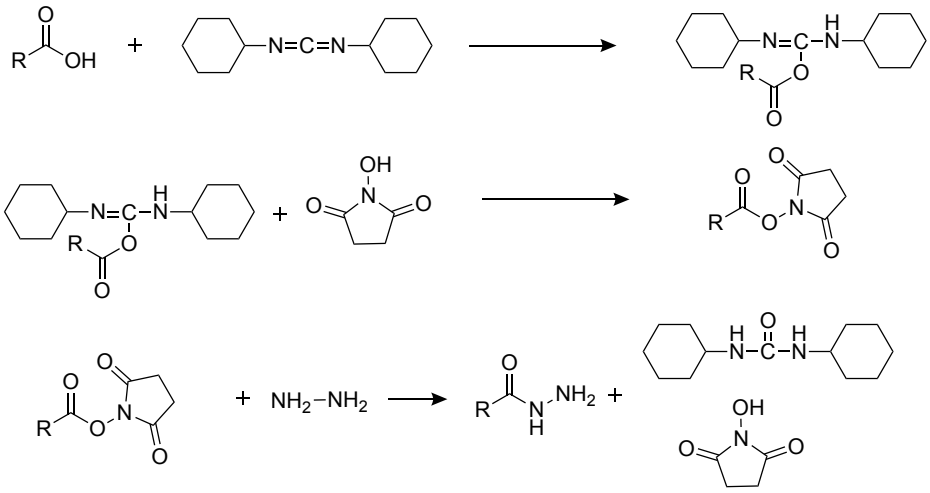
Scheme 5. Formation of ester with HOSu.

But in this case we need an extra step to activate the acid by DCC and react the ester formed with HOSu.



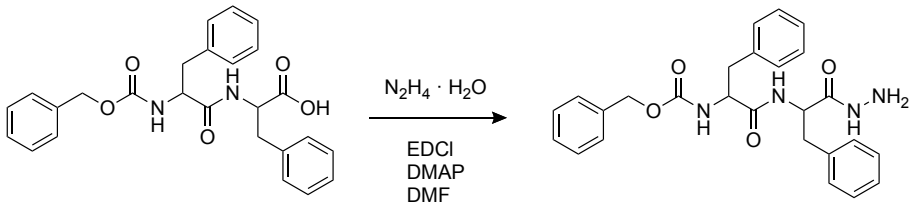
Scheme 6. Methodology reacting with HOSu and DCC.

The compound can not be purified due to the formation of the dicyclohexylurea sub-product (DCU), from the DCC, which binds to the AA being very difficult to remove it later. So we need to remove the DCU generated before to add the hydrazine.



Scheme 7. DCC reacting with carboxylic acid.

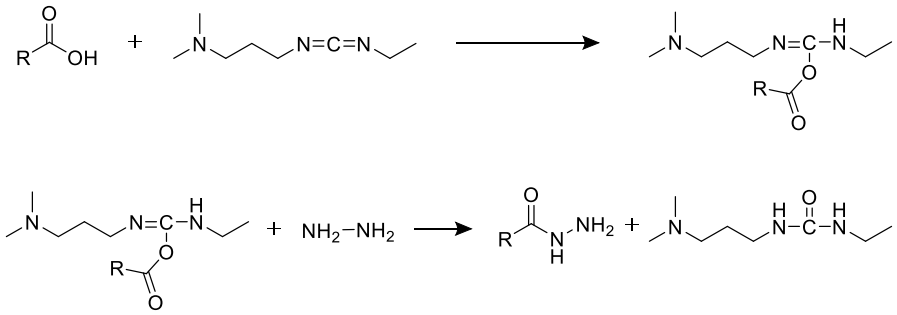
We thus choose another route of action based on the article by Liu and co-workers[16], in which EDCl is used as the acid activating group, and DMAP as a base.



Scheme 8. Mechanism using EDCl as activating group.

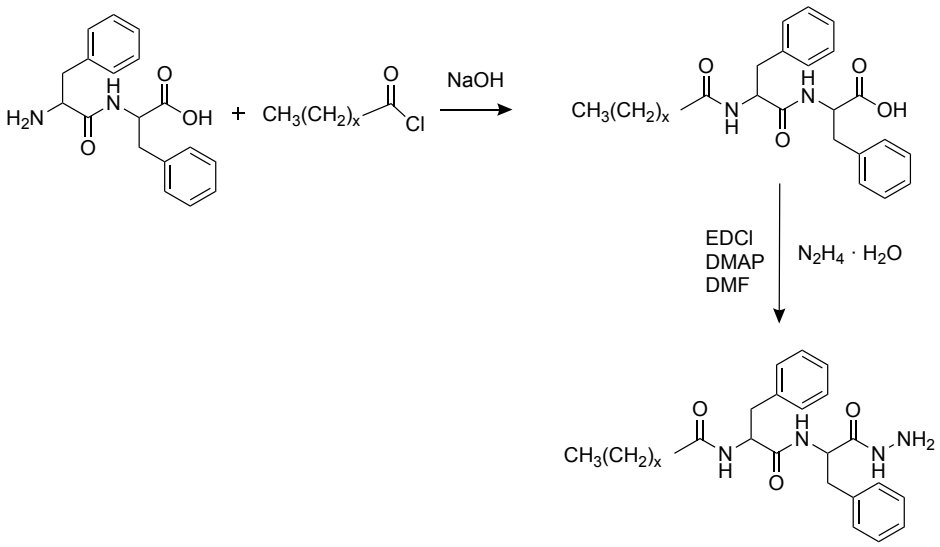
In the same line as DCC, EDCl reacts with the carboxyl group to increase its reactivity, favouring the attack of the amine.

This methodology has been tested in two different solvents, THF and DMF, being the latter one the chosen as the final solvent for its better solubility and product purification.
Therefore, it was decided to synthesize the 3 peptides that we pretend to carry out to study ZFFNHNH$_2$, C12FFNHNH$_2$ and C16FFNHNH$_2$ following this last methodology.



Scheme 9. EDCl reacting with carboxylic acid.

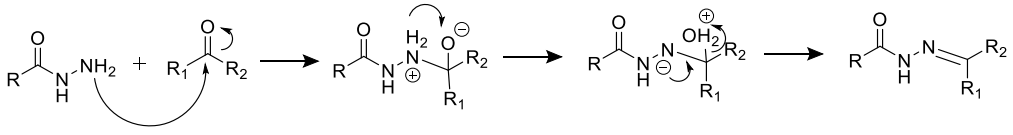
It has been necessary to synthesize compounds C$_{16}$FFOH and C$_{12}$FFOH[13] from which our desired compounds are obtained. NaOH is used as dissolvent, the AA is mixed with the respective chloride that provides the respective tail (reported on the experimental section).



Scheme 10. Synthesis of C$_{12}$FFNH$_2$ and C$_{16}$FFNHNH$_2$.

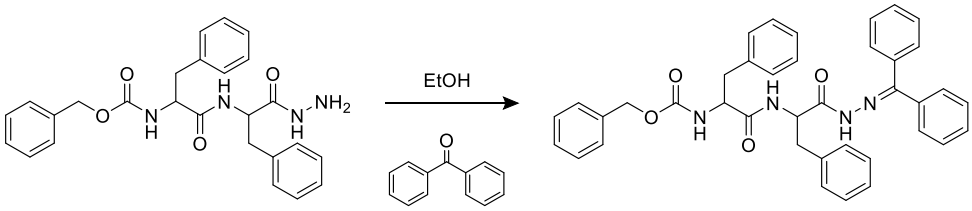
Synthesis of peptide-hydrazones.

Once obtained our desired peptide-hydrazides, we would try to assembly the dipeptide to a carboxyl group from a ketone or an aldehyde group with ZFFNHNH$_2$ as model product.



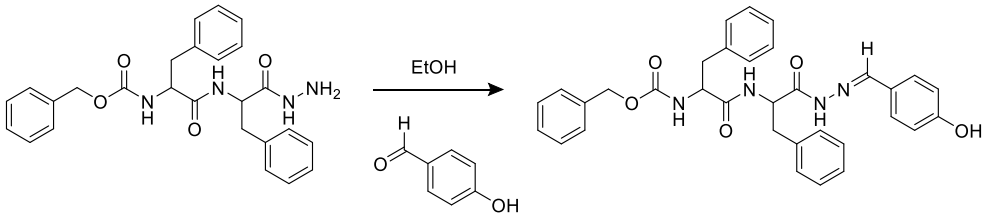
Scheme 11. Hydrazone bond mechanism.

Based on the article by Matson and Stupp[9] we studied the ketone assembly.



Scheme 12. Assembly of ketone to the peptide.

The assembly of the aldehyde group was based on the studies by Dyniewicz and co-workers[10] and Zhu and co-workers[11].



Scheme 13. Assembly of aldehyde to the peptide.

Both reported on the experimental section. The studies were not completely satisfactory because although we obtained several products, none of them was the indicated one and they presented many impurities. These reactions need to be improved in the future

Gelation studies

Once synthetized our peptides we have to prove their ability to form gels into different solvents. One of the most intuitive ways to know if a compound forms a gel is through the vial inversion test, which consists in turning the small vial where the gel has been formed and check if this resist the gravity effect, it means not flowing down and stay suspended. It consists in dissolving a known amount of sample inside a vial together with a quantity of volume also known, thus being able to know the exact concentration inside the vial. Therefore, to improve this dissolution, the sample is heated with a heat gun until complete dissolution of our product. At this moment, our particles will get a huge energy that when it is allowed to cool to room temperature, this loss of energy will cause the forming of a three-dimensional structure, because our compound try to hide the hydrophobic tails from the aqueous medium. If the gelation has turned out, it should not flow to the bottom and stay in suspension when the vial be turned. Thereby if the test is passed, a new quantity of known volume will be added, and the process will be repeated, until the concentration where the test will fail, proclaiming MGC the previous one.
Our study focus will be obtaining the minimum gel concentration (MGC) of the compounds that gelate, equal to the last sample concentration that passes the inversion test. At a lower MGC, better gelling product.

Four different solvents have been chosen to prove the gelation:

- ***H_2O***
- ***PBS pH= 7,4***
  It is a no toxic saline solution that contains NaCl, Na_3PO_4, KCl and K_3PO_4. Its ion concentration (Cl-, Na + and K +) are very similar to the mammals extracellular fluid. It is an isotonic solution, equal concentration inside and outside the cell, while the phosphates groups keep the pH 7,4 constant.

- ***Tris HCl pH= 7***
  It is an amino alcohol $(CH_2OH)_3CNH_2$, biologically inert with low toxicity, which supplements the buffering capacity of the hydrogen carbonate buffer system in the blood, accepting protons, which allows the regeneration of hydrogen carbonate and the reduction of the partial pressure of carbon dioxide to the blood arterial. Like the buffer range for Tris (pH 7-9) matches with the physiological pH of most living organisms, it is very useful for physiologists and pharmacologists who want to study the effects of alterations in the acid-base balance.

- ***Tris HCl pH= 5***
  The designation Tris-HCl, does not indicate that the buffer was prepared with Tris hydrochloride, but may have been prepared with Tris base and the precise amount of HCl to adjust the pH to the wanted value. Tris HCl allow us to simulate a desire pH.

ZFFNHNH$_2$

The gelation studies for ZFFNHNH$_2$, with molecular weight of 460,53 g/mol, verify self-assembly ability to form a gel easily and fast. The tests were done in four different solvents:

-5,05 mg of product were taken to dissolve it in H_2O as solvent, establishing the MGC as **10,97 mM**. It's the solvent in which it takes longer to dissolve the sample.

Figure 10. ZFFNHNH$_2$ gelation studies with H_2O.

-5,07 mg of product were taken to dissolve it in PBS pH= 7,4 as solvent, establishing the MGC as **22,02 mM**.

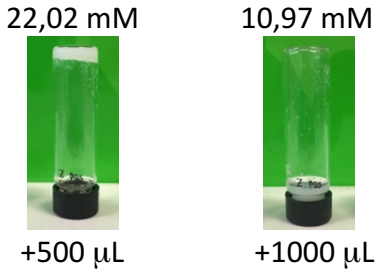


Figure 11. ZFFNHNH$_2$ gelation studies with PBS.

-5,04 mg of product were taken to dissolve it in TRIS HCl pH= 5 as solvent, establishing the MGC as **10,94 mM**.

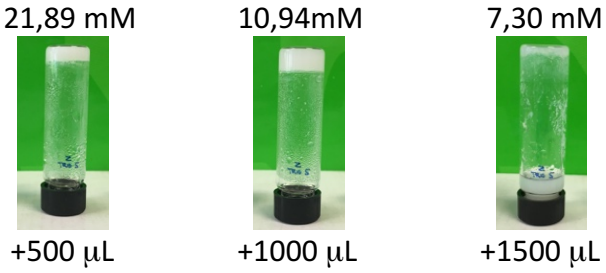


Figure 12. ZFFNHNH$_2$ gelation studies with TRIS HCl pH=5.

-5,25 mg of product were taken to dissolve it in TRIS HCl pH= 7 as solvent establishing the MGC as **7,60 mM**.

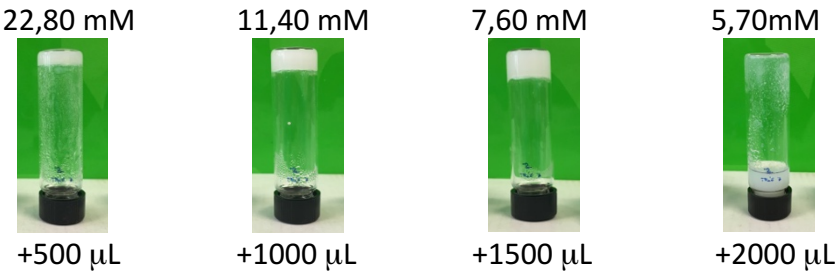


Figure 13. ZFFNHNH$_2$ gelation studies with TRIS HCl pH=7.

C_{12}FFNHNH$_2$

The gelation studies for C_{12}FFNHNH$_2$, with molecular weight of 508,70 g/mol, have not been satisfactory so we can say that this compound does not form gels in this means. We tried to change the concentration and cool it down in the ultrasound but it does not work. The tests were subjected to a four different solvents:

-5,19 mg of product were taken to dissolve it in H_2O as solvent. It is the solvent in which it takes longer to dissolve the sample.

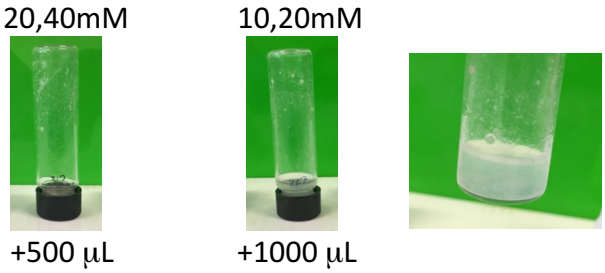


Figure 14. C_{12}FFNHNH$_2$ gelation studies with H_2O.

-5,05mg of product were taken to dissolve it in PBS pH= 7,4 as solvent. Appeared some brown spheres permanents like if the compound was molten at 500μL of solvent. However, we decide to continue, the spheres disappear but it does not form a complete gel.

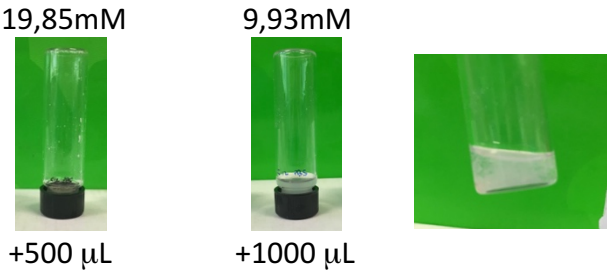


Figure 15. C_{12}FFNHNH$_2$ gelation studies with PBS.

-5,00 mg of product were taken to dissolve it in TRIS HCl pH= 5 as solvent.

19,66mM 9,83mM

+500 μL +1000 μL

Figure 16. C_{12}FFNHNH$_2$ gelation studies with TRIS HCl pH=5.

-5,14 mg of product were taken to dissolve it in TRIS HCl pH= 7 as solvent.

20,21 mM 10,10mM

+500 μL +1000 μL

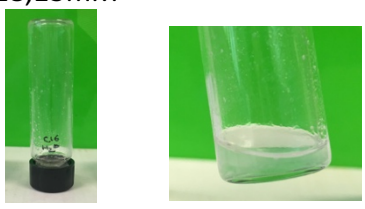
Figure 17. C_{12}FFNHNH$_2$ gelation studies with TRIS HCl pH=7.

C_{16}FFNHNH$_2$

The gelation studies for C_{16}FFNHNH$_2$, with molecular weight of 564,81 g/mol, have not been satisfactory so we can say that this compound is not gelling in these solvents. We tried change the concentration but it does not work. The tests were subjected to a four different solvents:

-5,12 mg of product were taken to dissolve it in H_2O as solvent. It is the solvent in which it takes longer to dissolve the sample.

18,13mM

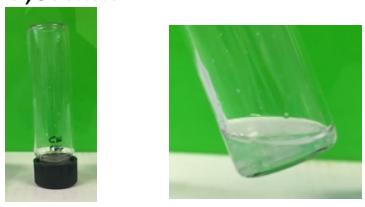


+500 μL

Figure 18. C_{16}FFNHNH$_2$ gelation studies with H_2O.

-4,99 mg of product were taken to dissolve it in PBS pH= 7,4 as solvent.

17,67mM

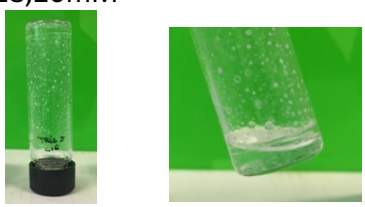


+500 μL

Figure 19. C_{16}FFNHNH$_2$ gelation studies with PBS.

-5,14 mg of product were taken to dissolve it in TRIS HCl pH= 5 as solvent.

18,20mM

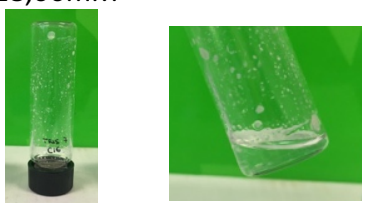


+500 μL

Figure 20. $C_{16}FFNHNH_2$ gelation studies with TRIS HCl pH=5.

-5,10 mg of product were taken to dissolve it in TRIS HCl pH= 7 as solvent. Appeared some permanent spheres like if the compound was molten at 500μL of solvent.

18,06mM



+500 μL

Figure 21. $C_{16}FFNHNH_2$ gelation studies with TRIS HCl pH=7.

After carrying out the gelation studies, we conclude that there is only gelation using compound Z, that is the reason because of we decided to characterize this by electron microscopy, TEM and SEM.
For this we prepare two gels of compound Z, one in H_2O medium and the other one in PBS pH=7,4 , the concentrations of which are 11,4 mM and 23 mM respectively.

Electron microscopy

In order to really observe the nanostructure of our gel, we will characterize it by means of electron microscopy techniques. Thanks to these we can obtain information about the self-assembly of our structures and the size of the fibers formed.

Both techniques used here (TEM and SEM) are similar when it comes to scientific fundamentals, but there are some differences. While SEM obtain the information through the electrons that are emitted by the sample when is irradiated with an electron beam, TEM takes the information of the electrons able to crossing the sample. This makes us give different important aspects of the sample, SEM focus on the surface of the sample, so it will give us morphological information in 3-D and TEM allows us to obtain more detailed information of the sample by providing an image 2-D with a higher resolution.

SEM samples must be lyophilized to remove the solvent and obtain a dry gel (xerogel). The drying process could affect the sample, leading the formation of holes in the surface of the sample. After this process, the samples are covered by a metallic layer in

order to obtain these secondary electrons emitted by the sample with the electron beam. While the TEM samples adhere to the special slide a thin layer of hydrogel.

Both techniques help us to know the morphology, the size of the fibers formed and also if the chirality of the molecules is transcribed to the nanostructure and it shows this effect adopting helical forms.[17]

Z H_2O

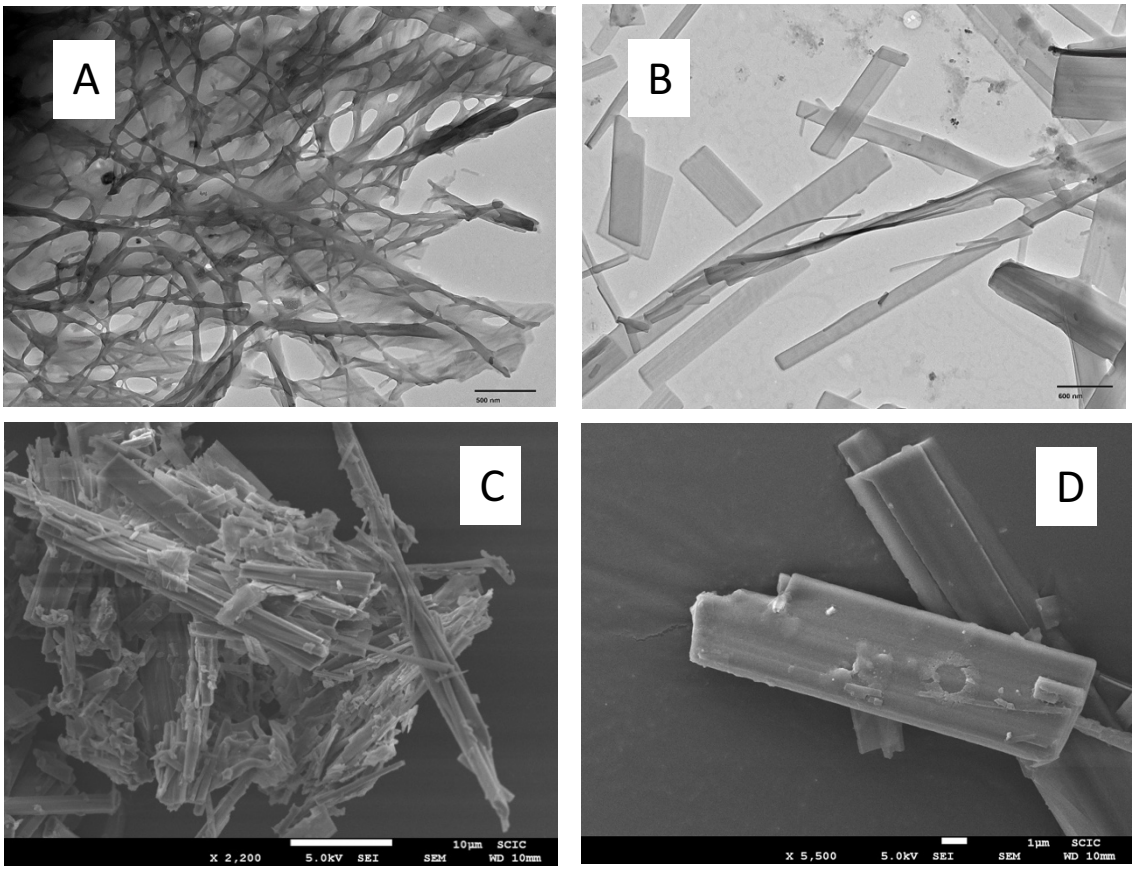


Figure 22: (A)(B) TEM; (C)(D) SEM

Figure 22A shows a fiber net, of an order of length bigger than 500 nm, and an approximate width of less than 100 nm. We can observe in figure 22B the coexistence of another type of structure in the form of tape or sheets that coexists with the structure that is formed in figure 22A which seems wrinkled. This could be due to the formation of aggregates, under kinetic and thermodynamic control, thus one creating a more stable structure than the other. We would have to play with the cooling speed to achieve the formation of a single structure, thus controlling the two parameters, both kinetics and thermodynamics. In the figure 22C a mass of aggregates is observed, which can be observed closer in figure 22D, with an order of length bigger than 12 µm, and an approximate width of more than 3 µm. A small gap of the order of 1µm is observed, probably caused in the drying process of the gel solvent.[18]
Thanks to the fact that the two techniques used are complementary, we can know the structure of the gel.

Z PBS

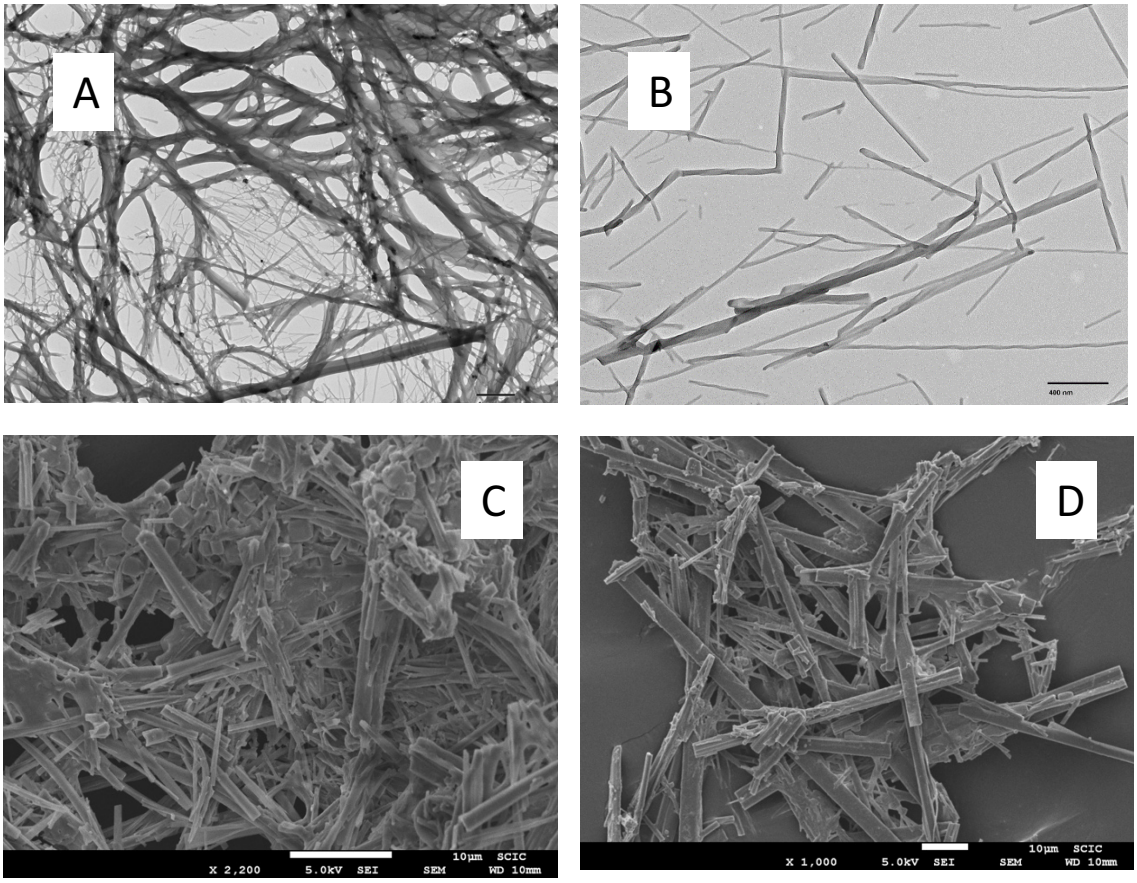


Figure 23: (A)(B) TEM; (C)(D) SEM

We can see a structure not as compact as the previous one in water, we observe large spaces between the fibers. Small points appear, which could be due to the formation of salts coming from the buffer attached to the structure. The fibers are more uniform, so they have the same structure, opposite to the previous compound. In the figure 23B we can observe some helical fibers, which indicates that there is chirality in the molecules, causing these folds in the structure. These fibers are of an order bigger than 400 nm in length and less than 100 nm of width. The structure 3-D of the fiber network mentioned above can be seen in figures 23C and 23D, of an order bigger than 10 μm in length and less than 5μm.

4. CONCLUSION

Having started from the compounds ZFFOH, C_{12}FFOH and C_{16}FFOH, the methodology based on J. Med. Chem. 2010,53, 1370-1382[16] is the one chosen to synthesize all compounds bounded to hydrazide.

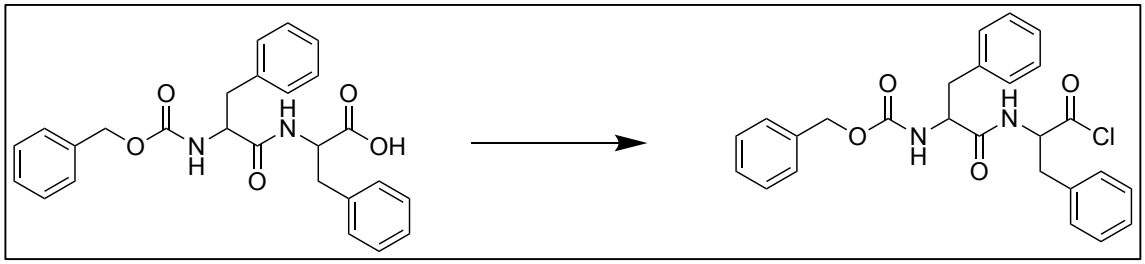
Once all the experimental work has been done, we can conclude that of the three gelators synthesized only the compound ZFFNHNH_2 is able to form a gel at different pH and in different media, being thus the most indicated to the union of ketone and aldehyde groups.

In the second step, no conclusive results have been obtained, so that the synthesis method will have to be optimized, then the gelation and drug release tests will be carried out, firstly performing tests analysing the amount of drug released in a medium added on the gel in the same vial after a certain time.

If this test is conclusive, the tests will be taken to a biological laboratory for the first tests ex-vivo and afterwards on animals.

5. EXPERIMENTAL SECTION

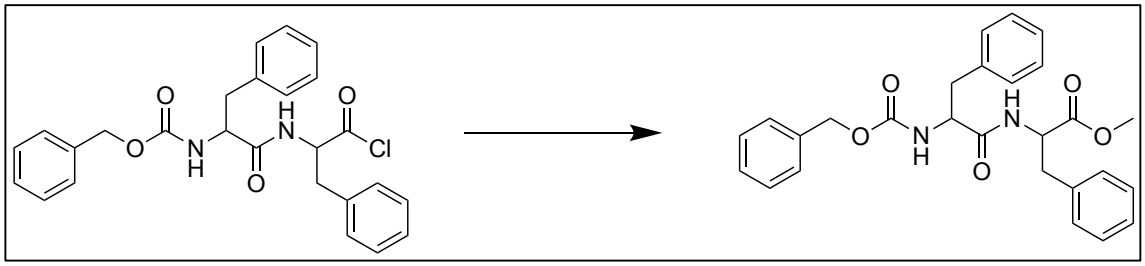
1- Formation of ZFFOCl



ZFFOH	446,5 g/mol	1,12 mmol	0,500 g
Thionyl chloride			20 mL

Inside a two-necked round bottom flask, ZFFOH was dissolved in thionyl chloride with reflux and nitrogen atmosphere, overnight. Afterwards, the system was connected to a vacuum microdistillator, to evaporate the thionyl chloride excess and the HCl produced, until dryness.

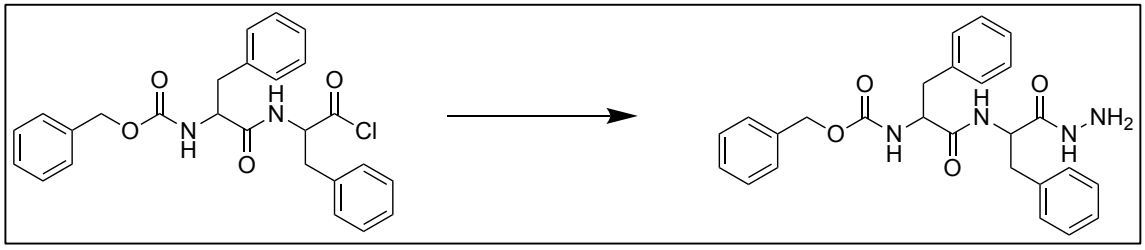
2- Formation of ZFFOMe from ZFFOCl



Et_3N	101,19 g/mol	1,12 mmol	1 eq	164 μl
MeOH				20 mL

ZFFOCl was dissolved in MeOH under nitrogen atmosphere and stirring, and after that the Et_3N was added by syringe, drop by drop (appeared a white fume and precipitate inside caused by the neutralization of the present acid).
After the sample was evaporated, and dissolved in CH_2Cl_2 and washed with H_2O and brine. The organic phase was dried by Na_2SO_4, filtered, washed with CH_2Cl_2 and evaporated until dryness. 0.475 g of impure product were obtained.
The sample was purified by column chromatography 9:1, CH_2Cl_2 : MeOH, obtaining 0.046 g of product.
ZFFOMe 0,046 g
Yield: 8,9 % no pure

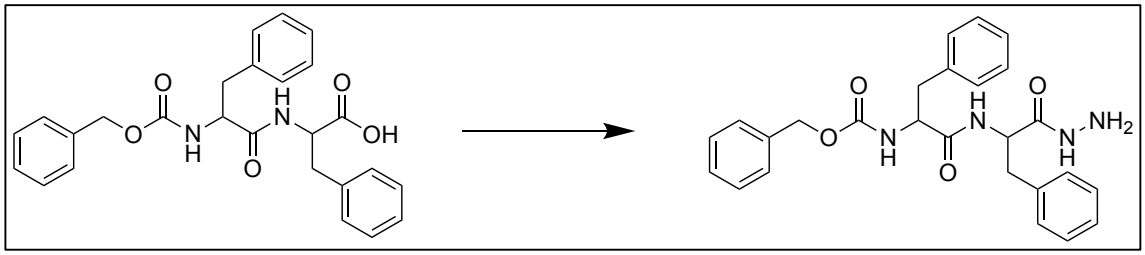
3- Formation of $ZFFNHNH_2$ from ZFFOCl



NH_2-NH_2·H_2O	32,05 g/mol	5,6 mmol	5 eq	186 μL
THF				20 mL

ZFFOCl was dissolved in 10 mL THF. After that the NH_2-NH_2 volume mixed with 10 mL of THF was added through an addition funnel inside an ice-bath and N_2 atmosphere. Leaving the reaction overnight and at room temperature.
After vacuum filtration, this filtered was washed with H_2O to eliminate side products with HCl. The organic phase was dried by anhydrous Na_2SO_4, filtered, washed with THF and evaporated until dryness. 0,660 g of impure product were obtained.
The sample was purified by column 95:5, CH_2Cl_2:MeOH. obtaining 0.115 g of product.
$ZFFNHNH_2$ 0,115 g
Yield: 22,08% no pure

4- Formation of ZFFNHNH$_2$ from ZFFOH



ZFFOH	446,5 g/mol	1,12 mmol	1eq	0,500 g
HOSu	155,09 g/mol	1,23 mmol	1,1 eq	0,191 g
DCC	206,33 g/mol	1,12 mmol	1 eq	0,231 g
NH_2-NH_2·H_2O	32,05 g/mol	5,6 mmol	5 eq	27 mL
THF				20 mL

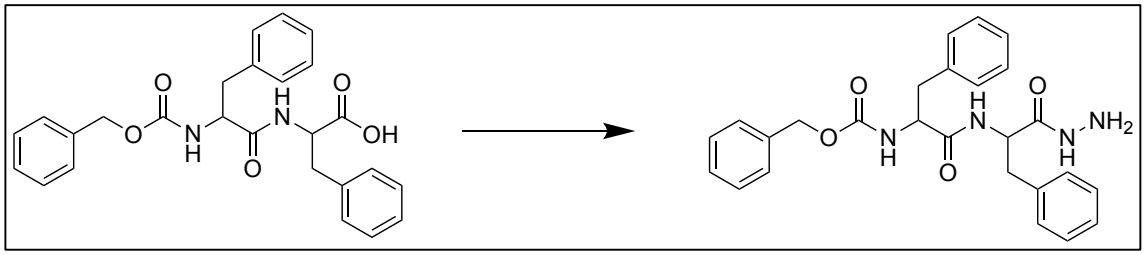
ZFFOH mixed with HOSu were dissolved with 10 mL of THF. After DCC mixed with 10 mL of THF was added, through an addition funnel inside an ice-bath and N_2 atmosphere. The reaction was leaved 3 h and NH_2-NH_2 mixed with 6 mL of THF was added trough an addition funnel inside an ice-bath and N_2 atmosphere. Leaving the reaction overnight and at room temperature.

The mixture was vacuum filtered and washed with THF to eliminate the DCU formed. A $NaHCO_3$:H_2O solution was added to precipitate the product, which was vacuum filtered and washed with $NaHCO_3$ and H_2O, and finally dried in 60 ºC oven overnight. 0.144g of impure product were obtained.

The sample was purified by column chromatography 98:2, CH_2Cl_2:MeOH, 0.109 g was obtained.

ZFFNHNH$_2$ 0,109 g
Yield: 21,14 % no pure

5- Formation of ZFFNHNH$_2$ from ZFFOH



ZFFOH	446,5 g/mol	1,12 mmol	1eq	0,500 g
NH_2-$NH_2 \cdot H_2O$	32,05 g/mol	1,12 mmol	1 eq	35 μl
EDCl	191,70g/mol	1,68 mmol	1'5 eq	0,322 g
DMAP	122,17 g/mol	1,68 mmol	1,5 eq	0,205 g
THF				10 mL

ZFFOH was weighed in a two-necked round bottom flask. After that, DMAP was added dissolved in 4 mL of THF and after 10 min EDC was added with 6 mL of THF. The NH_2-NH_2 volume was added by syringe inside an ice-bath and N_2 atmosphere. Leaving the reaction overnight and at room temperature.
To eliminate the sub-product of EDC, HCl 0,1M and brine were added until product precipitation, which was filtered, washed with THF and dried overnight.
The reaction was purified through two different methodologies.

Methodology A:

0,490 g of sample were suspended in $NaHCO_3$ stirring overnight. After vacuum filtered, the precipitate was dried in heater overnight, obtaining 0,368 g of impure product.
This product was suspended in HCl 0,1M stirring overnight. After vacuum filtered, the precipitate was dried in oven overnight, obtaining 0,317 g of impure product.
Finally the sample was purified by column chromatography 98:2, CH_2Cl_2:MeOH, obtaining 0,083 g of product.

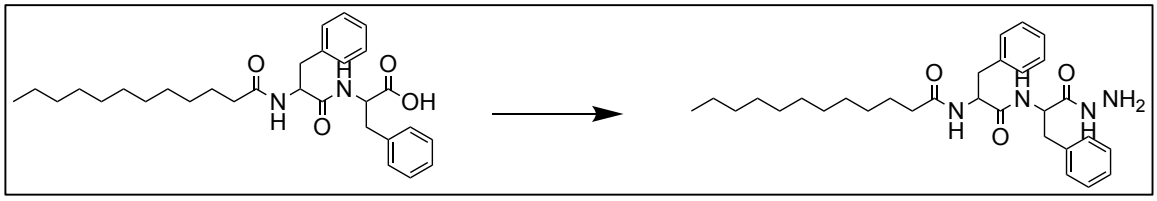
ZFFNHNH$_2$ A 0,083g
Yield: 16,09 % no pure

Methodology B:

0,287 g of sample were washed with HCl 0,1 M, $NaHCO_3$, KOH, H_2O and a bit of Et_2O. Then was dried overnight, obtaining 0,209g of impure product which was putted with 0,054g recovered from the first filtered.
The sample was washed with HCl 0,1 M, $NaHCO_3$, KOH, H_2O and a bit of Et_2O. Then was dried overnight, obtaining 0,208g of impure product.

ZFFNHNH$_2$ B 0,208g
Yield: 40,33 % no pure

6- Formation of C12FFNHNH$_2$ from C12FFOH



C12FFOH	494,31 g/mol	0,716 mmol	1eq	0,354 g
NH_2-NH_2·H_2O	32,05 g/mol	0,716 mmol	1 eq	22 μl
EDCl	191,70g/mol	1,074 mmol	1'5 eq	0,205 g
DMAP	122,17 g/mol	1,074 mmol	1,5 eq	0,131 g
THF				10 mL

C12FFOH was weighed in a two-necked round bottom flask. After that, DMAP was added dissolved in 4 mL of THF and after 10 min EDC was added with 6 mL of THF. The NH_2-NH_2 volume was added by syringe inside an ice-bath and N_2 atmosphere, leaving the reaction overnight and at room temperature.
To eliminate the side product of EDC, HCl 0,1M and brine were added until product precipitation, which was filtered, washed with THF and dried overnight.
The reaction was purified through two different methodologies.

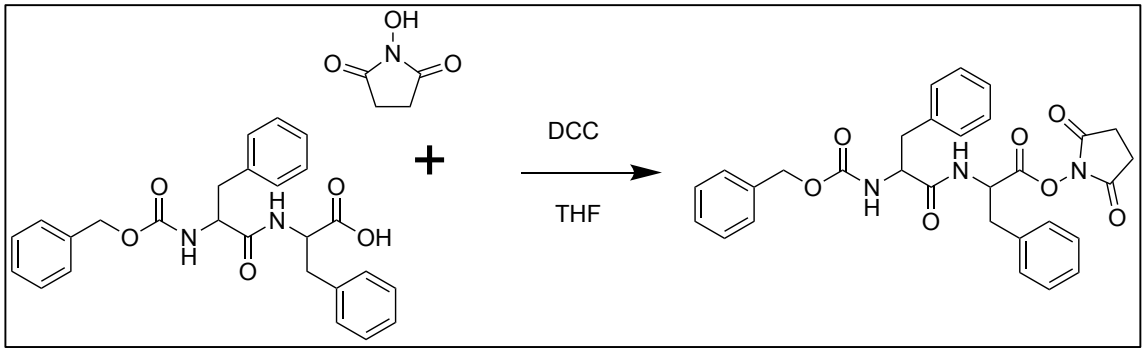
Methodology A:
0,184 g of sample were washed with THF stirring overnight. The precipitate was dried overnight after being vacuum filtered. The sample was suspended in H_2O heating and stirring 2 hours. Then the precipitate was dried overnight after being vacuum filtered, obtaining 0,035 g[1*] of impure product

Methodology B:
C12FFOH 0,501 g/ **NH_2-NH_2** 31μl
EDCl 0,292 g/ **DMAP** 0,185g/ **THF** 10mL
0,103 g[2*] of impure product were obtained.
The filtered after THF was decanted with ethyl acetate, and concentrated at vacuum evaporator, obtaining 0,848 g[3*] of impure product.

The three samples 1*,2*,3* were putted together because there was our product present but not purified, then the new sample was washed with HCl 0,1 M, H_2O and a bit of Et_2O. Then it was dried overnight. 0,267 g of product was obtained.

C12FFNHNH$_2$ 0,267 g
Yield: 30,34 % no pure

7- Formation of ZFFOSu from ZFFOH

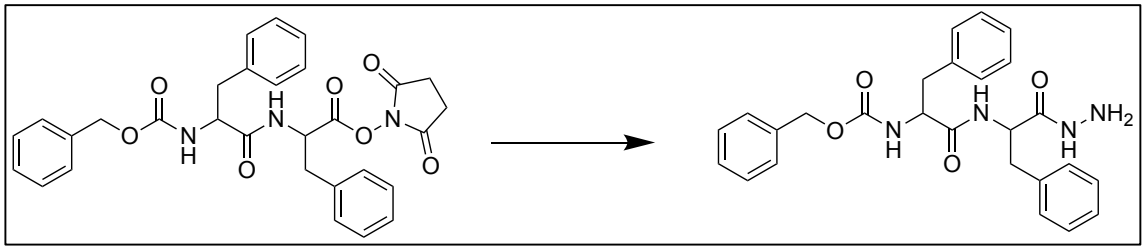


ZFFOH	446,5 g/mol	1,12 mmol	1eq	0,500 g
HOSu	155,09 g/mol	1,23 mmol	1,1 eq	0,191 g
DCC	206,33 g/mol	1,12 mmol	1 eq	0,231 g
IPA				20 mL

ZFFOH mixed with HOSu was dissolved with 10 mL of THF. After DCC mixed with 10 mL of THF was added, through an addition funnel inside an ice-bath and N_2 atmosphere. Leaving the reaction overnight at room temperature.
The sample was washed with THF after being vacuum filtered and the filtered was concentrated because it contained our product. 20 mL of IPA, at boiling point, were added to recrystallize. The impurities kept dissolved and our product precipitated, inside an ice-bath. The sample was filtered and washed with IPA. 0,438 g of product were obtained.

ZFFOSu 0,438 g
Yield: 71,95 %

8- Formation of $ZFFNHNH_2$ from ZFFOSu



HOSu	543,58 g/mol	0,806 mmol	1 eq	0,438 g
NH_2-NH_2 · H_2O	32,05 g/mol	0,806 mmol	1 eq	25 μL
DME				15 mL

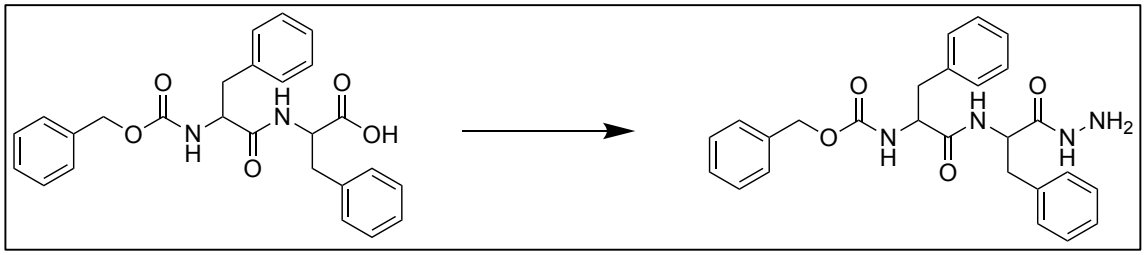
ZFFOSu was dissolved with 10 ml of DME. The NH_2-NH_2 was mixed with 5 ml of DME and was added through an additon funnel inside an ice-bath and N_2 atmosphere. Leaving the reaction overnight and at room temperature.
The sample was washed with DME after vacuum filtered. The precipitate was suspended in CH_2Cl_2, filtered and washed with CH_2Cl_2, KOH, H_2O and Et_2O.Finally was dried overnight. 0,337g of impure product were obtained.
The precipitate was suspended and stirred in 5 ml of HCl 0,1M, after was filtered and dried overnight, obtaining 0,170 g

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.15 (s, 1H), 8.08 (d, J = 8.2 Hz, 1H), 7.40 (d, J = 8.6 Hz, 1H), 7.35 – 7.13 (m, 15H), 4.93 (s, 2H), 4.47 (s, 1H), 4.22 (s, 3H), 2.97 – 2.75 (m, 4H)

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 171.53, 170.44, 137.97, 137.46, 129.69, 129.61, 128.76, 128.54, 128.48, 128.13, 127.86, 126.76, 126.66, 65.68, 56.59, 53.06

$ZFFNHNH_2$ 0,170 g
Yield: 32,88%

9- Formation of ZFFNHNH$_2$ from ZFFOH



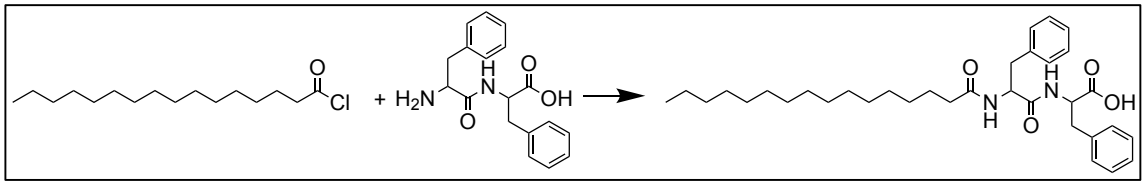
ZFFOH	446,5 g/mol	0,607 mmol	1eq	1 g
NH_2-NH_2 · H_2O	32,05 g/mol	0,692 mmol	1,1 eq	79 μL
EDCl	191,70g/mol	0,692 mmol	1,14 eq	0,490 g
DMAP	122,17 g/mol	1,384 mmol	2,28 eq	0,622 g
DMF				50 mL

ZFFOH was dissolved with 15 mL of DMF. After that, DMAP was added and then the NH_2-NH_2 by syringe inside an ice-bath and N_2 atmosphere. After 10 min EDC was added, leaving the reaction 2 h in an ice-bath and then overnight at room temperature.

NH_4Cl saturated was added to help the precipitation of our product in an ice-bath, but it did not work, NaOH was added to change the pH rounding 8 to precipitation, and finally H_2O was added until complete precipitation, the sample was filtered and washed with HCl 0,1M, H_2O and dried overnight. 0,421 g of product were obtained.

ZFFNHNH$_2$ 0,421 g
Yield: 40,8 %

10- Formation of C_{16}FFOH from HFFOH



HFFOH	312,37 g/mol	1,60 mmol	1eq	0,5 g
Palmitoyl Chloride	274,88 g/mol	3,20 mmol	2 eq	970μL
NaOH 0,1M				35 mL

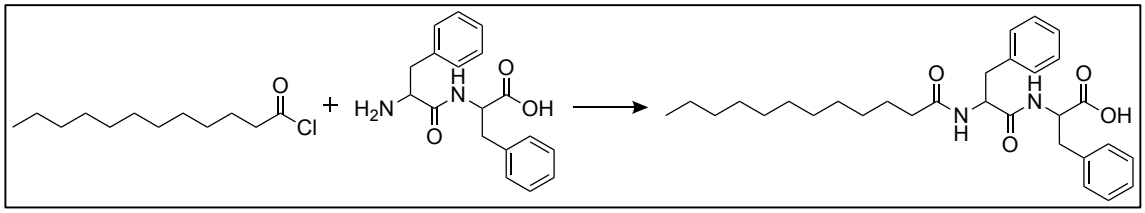
HFFOH was dissolved in 30 mL of 1 M NaOH in a two-necked round-bottomed flask. The solution was stirred in an ice bath. After, palmitoyl chloride and 5 mL of 1 M NaOH were added dropwise at the same rate inside an ice-bath. The mixture was stirred 48 h at room temperature. The suspension was acidified with 7 mL of 5 M HCl. The residue obtained after filtration was dried and washed with H_2O. The precipitate was suspended in Hexane and stirred 1 hour. Finally it was filtered and dried overnight. 0,549 g of product were obtained.

^{1}H NMR (400 MHz, 30 ºC, DMSO-[d_6]) δ ppm 8.08 (d, *J* = 8.1 Hz, 1H), 7.44 (s, 1H), 7.33 – 7.03 (m, 10H), 4.31 (s, 2H), 2.17 (t, *J* = 7.3 Hz, 2H), 1.48 (s, 2H), 1.25 (s, 28H), 0.86 (t, *J* = 6.7 Hz, 3H).

^{13}C NMR (100 MHz, 30 ºC, DMSO-[d_6]) δ ppm 172.85, 172.44, 171.18, 138.77 (2C), 129.94 (2C), 129.56 (2C), 128.36 (2C), 128.26 (2C), 126.49, 126.38, 54.85, 54.49, 37.81, 37.34, 35.69, 34.20, 31.75, 29.50, 29.48, 29.36, 29.25, 29.20, 29.15, 29.02, 28.92, 25.60, 24.99, 22.55, 14.40.

C_{16}FFOH 0,549 g
Yield: 60,7 %

11- Formation of C_{12}FFOH from HFFOH



HFFOH	312,37 g/mol	1,60 mmol	1eq	0,5 g
Lauroyl Chloride	218,76 g/mol	3,20 mmol	2 eq	700μL
NaOH 0,1M				35 mL

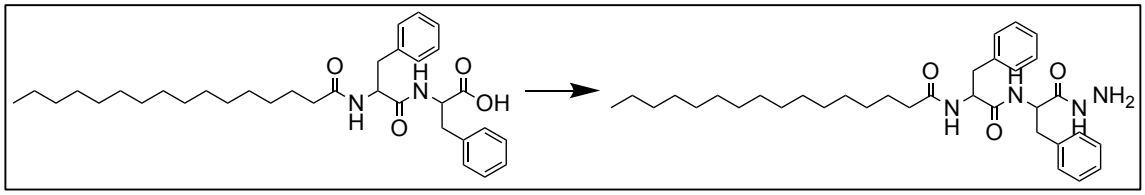
HFFOH was dissolved in 30 mL of 1 M NaOH in a two-necked round-bottomed flask. The solution was stirred in an ice bath. After, 3.20 mmol of lauroyl chloride and 5 mL of 1 M NaOH were added dropwise at the same rate. The mixture was stirred 48 h. The suspension was acidified with 7 mL of 5 M HCl. The residue obtained after filtration was dried and washed with H_2O and Et_2O.

^{1}H NMR (400 MHz, 30 ºC, DMSO-[d_6]) δ 8.14 (d, *J* = 6.4 Hz, 1H), 7.89 (d, *J* = 8.7 Hz, 1H), 7.2 (m, 10H), 4.54 (m, 1H), 4.43 (m, 1H), 3.13 – 2.85 (m, 2H), 2.18 (t, *J* = 7.4 Hz, 2H), 1.50-1.45 (m, 2H), 1.24 (s, 18H), 0.86 (t, *J* = 6.8 Hz, 3H)

^{13}C NMR (100 MHz, 30 ºC, DMSO-[d_6]) δ 174.95 (3C), 129.62 (6C), 128.64 (4C), 128.35 (2C), 53.86 (2C), 34.13 (2C), 31.75 (2C), 29.44 (2C), 29.35, 29.18, 29.15, 29.00, 24.95, 22.55, 14.41.

C_{12}FFOH 0,502g
Yield: 63,45 %

12- Formation of C16FFNHNH$_2$ from C16FFOH



C_{16}FFOH	550,38 g/mol	0,550 mmol	1eq	0,303 g
NH_2-NH_2·H_2O	32,05 g/mol	0,605 mmol	1,1 eq	22 μL
EDC	191,70g/mol	0,627 mmol	1,14 eq	0,122 g
DMAP	122,17 g/mol	1,254 mmol	2,28 eq	0,153 g
DMF				15 mL

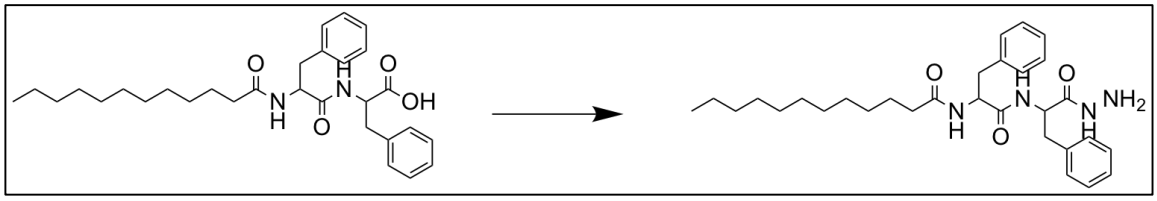
C_{16}FFOH was dissolved with 15 mL of DMF. After that, DMAP was added and then the NH_2-NH_2 by syringe inside an ice-bath and N_2 atmosphere. After 10 min EDC was added, leaving the reaction 2 h in an ice-bath and then overnight at room temperature.
NH_4Cl saturated was added to help the precipitation of our product in an ice-bath, then was filtered and dried overnight. 0,174g of product were obtained.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.12 (s, 1H), 7.97 (s, 1H), 7.89 (s, 1H), 7.21 (s, 10H), 4.49 (s, 3H), 2.94 (d, *J*=23,2 Hz, 2H), 2.80 (s, 2H), 1.49 (d, *J*=74,6 Hz, 2H), 1.25 (s, 24H), 0.86 (d, J=6.8 Hz, 3H).

^{13}C NMR (100 MHz, DMSO-d_6**)** δ ppm 129.64, 129.58, 128.53, 128.37, 34.14, 31.73, 29.47, 29.33, 29.24, 29.16, 29.14, 28.99, 24.92, 22.53, 14.40.

C16FFNHNH$_2$ 0,174 g
Yield: 55,96%

13- Formation of C12FFNHNH$_2$ from C12FFOH



C12FFOH	494,31 g/mol	0,607 mmol	1eq	0,300 g
NH_2-NH_2 · H_2O	32,05 g/mol	0,692 mmol	1,1 eq	22 μL
EDC	191,70g/mol	0,692 mmol	1,14 eq	0,133 g
DMAP	122,17 g/mol	1,384 mmol	2,28 eq	0,169 g
DMF				15 mL

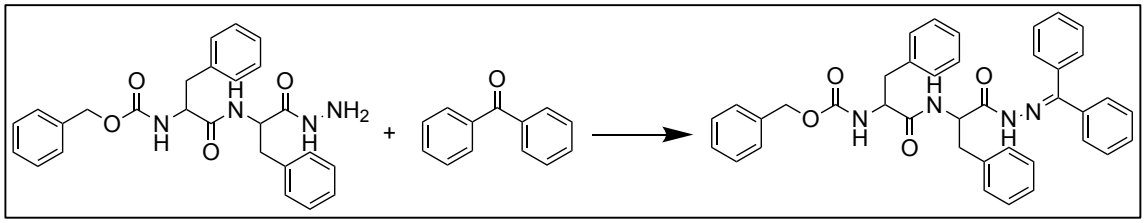
C_{12}FFOH was dissolved with 15 mL of DMF. After that, DMAP was added and then the NH_2-NH_2 by syringe inside an ice-bath and N_2 atmosphere. After 10 min EDC was added, leaving the reaction 2 h in an ice-bath and then overnight at room temperature.
NH_4Cl saturated was added to help the precipitation of our product in an ice-bath, then it was filtered and dried overnight. 0,257g of impure product were obtained which was washed with HCl 0,1M and H_2O, and dried overnight, obtaining 0,159 g of product.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.12 (s, 1H), 7.97 (s, 1H), 7.89 (s, 1H), 7.21 (s, 10H), 4.49 (s, 3H), 2.94 (s, 2H), 2.80 (s, 2H), 1.98 (s, 2H), 1.49 (s, 2H), 1.25 (s, 16H), 0.86 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 174.97, 129.64, 129.58, 128.54, 128.37, 34.14, 31.75, 29.44, 29.35, 29.15, 28.99, 28.88, 25.61, 24.95, 22.54, 14.41.

C12FFNHNH$_2$ 0,159 g
Yield: 51,50 %

14- Addition of diphenylketone to a ZFFNHNH$_2$



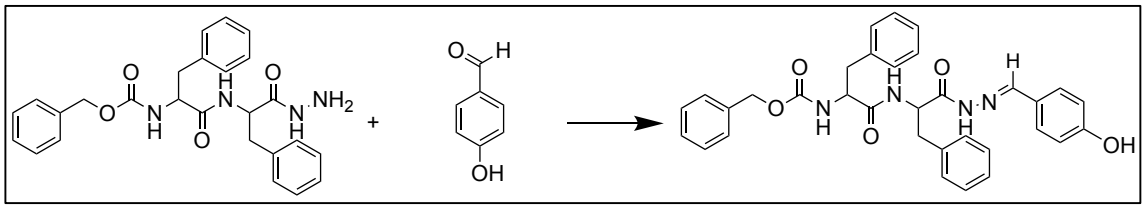
ZFFNHNH$_2$	460,53 g/mol	0,178 mmol	1eq	0,082 g
Diphenylketone	182,22 g/mol	0,214 mmol	1,2 eq	0,039 g
EtOH			50mg/mL	3 mL

ZFFOH was mixed with diphenylketone in a 25mL round-bottom flask with 3 mL of EtOH. To improve the dissolution, we took it to the ultrasounds. The reaction was leaved stirring overnight at reflux and N_2 atmosphere. Then 1 mL of DMF was added and during the afternoon we added 5-10% of acetic acid stirring overnight. H_2O was added until precipitation, then was filtered and dried overnight. 0,073g of impure product was obtained.

The sample was purified by column chromatography using CH_2Cl_2 as eluent. We obtained three fractions but any of them were the product, so the reaction was not working well.

F1: 0,014 g **F2:** 0,004 g **F3:** 0,003 g

15- Addition of 4-hydroxybenzaldehyde to ZFFNHNH$_2$



ZFFNHNH$_2$	460,53 g/mol	0,145 mmol	1eq	0,067 g
4-hydroxylbenzaldehyde	122,12 g/mol	0,174 mmol	1,2 eq	0,021 g
THF			50mg/mL	2 mL

ZFFOH was mixed with 4-hydroxybenzaldehyde in a 25mL round-bottom flask with 3 mL of EtOH. To improve the dissolution, we took it to the ultrasounds. The reaction was leaved stirring overnight at reflux and N_2 atmosphere. Then the sample was concentrated and dried at vacuum. The sample was suspended in H_2O, then was filtered and dried overnight. 0,046g of impure product were obtained.

The sample was purified by column chromatography CH_2Cl_2 , and 95:5 CH_2Cl_2: MeOH to obtain the second product. We obtained two fractions but any of them were the product, so the reaction was not working well.

F1: 0,004 g **F2:** 0,041 g

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8. ANNEXES

ZFFNHNH$_2$

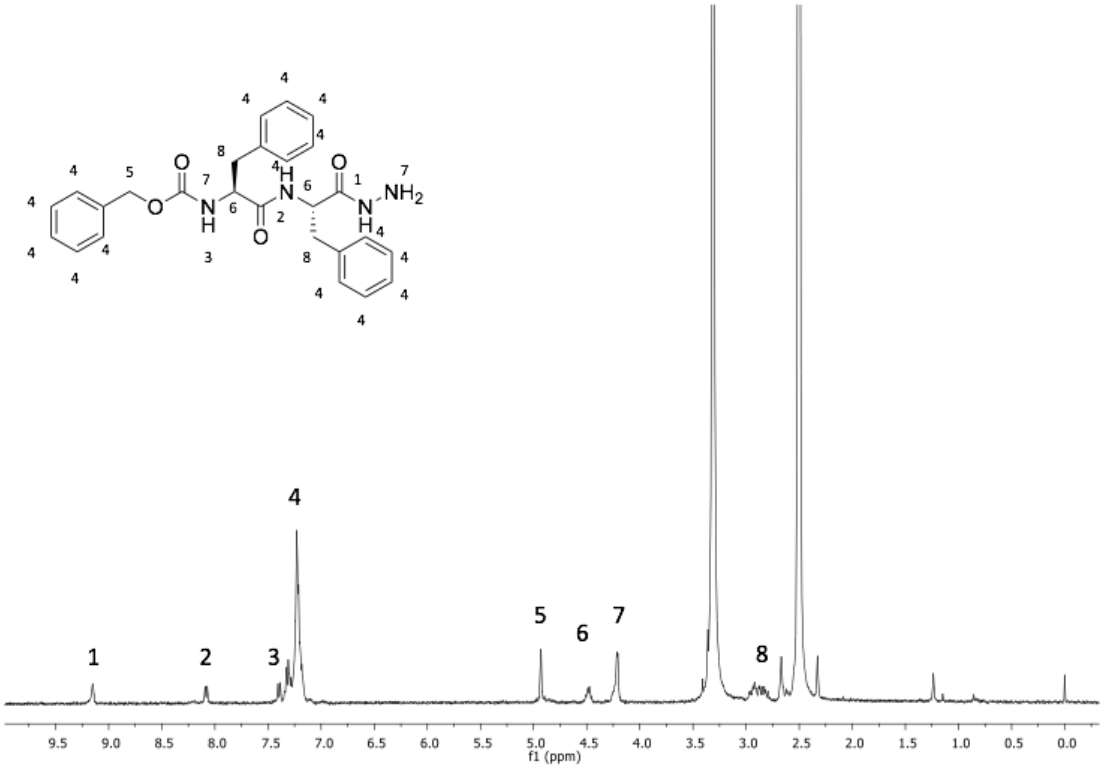


Figure A1. ZFFNHNH$_2$ ^{1}H NMR (400 MHz, DMSO-d$_6$)

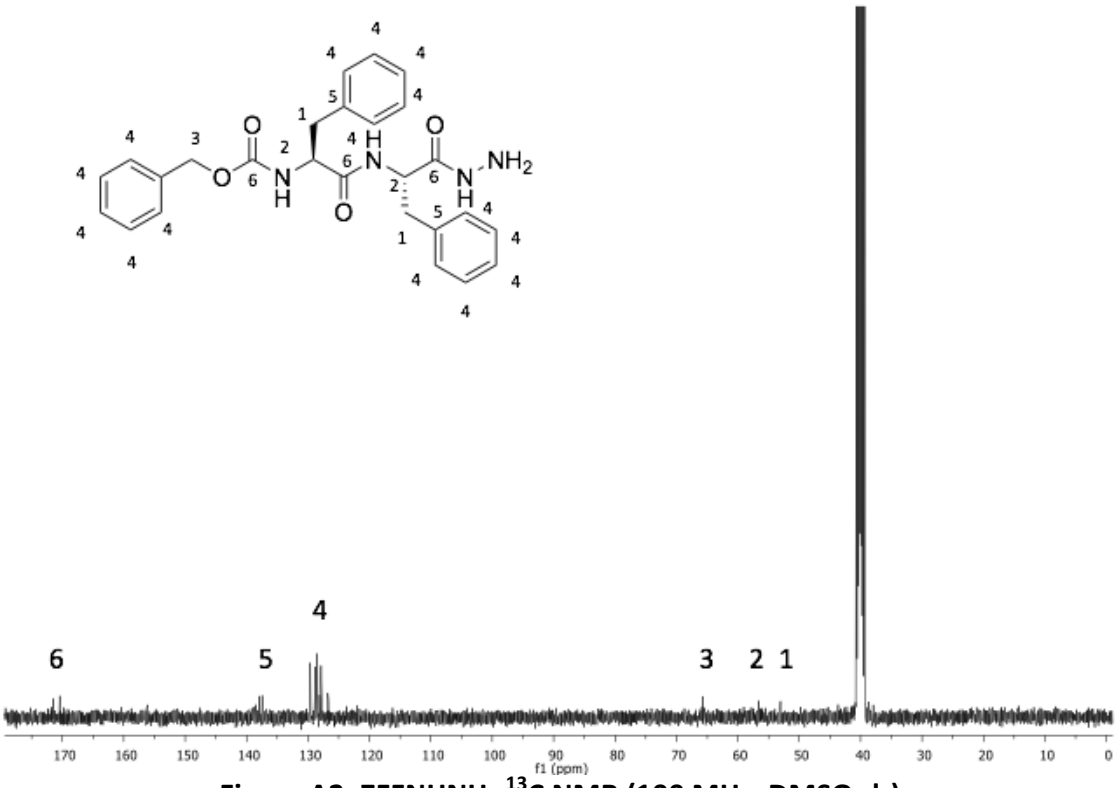


Figure A2. ZFFNHNH$_2$ ^{13}C NMR (100 MHz, DMSO-d$_6$)

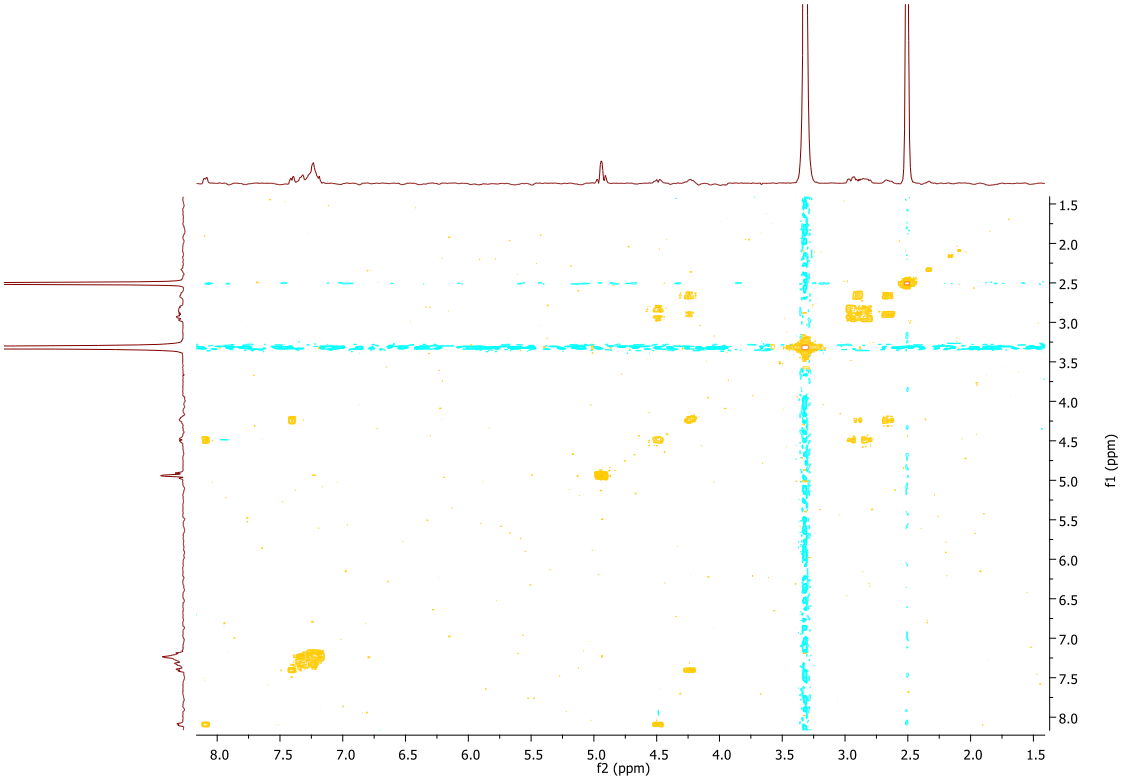
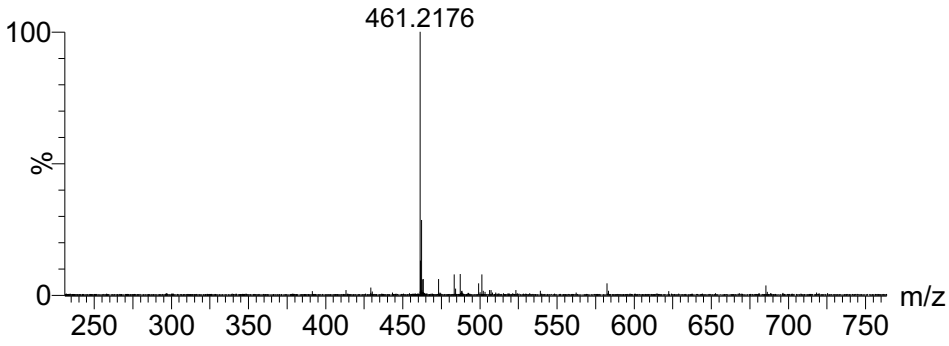


Figure A3. ZFFNHNH$_2$ COSY NMR



$[M+H]^+$=461.2189
$[M+H]^+$=461.2176 (2.8 ppm)

Figure A4. ZFFNHNH$_2$ MS (ES-QTOF)

C_{12}FFNHNH$_2$

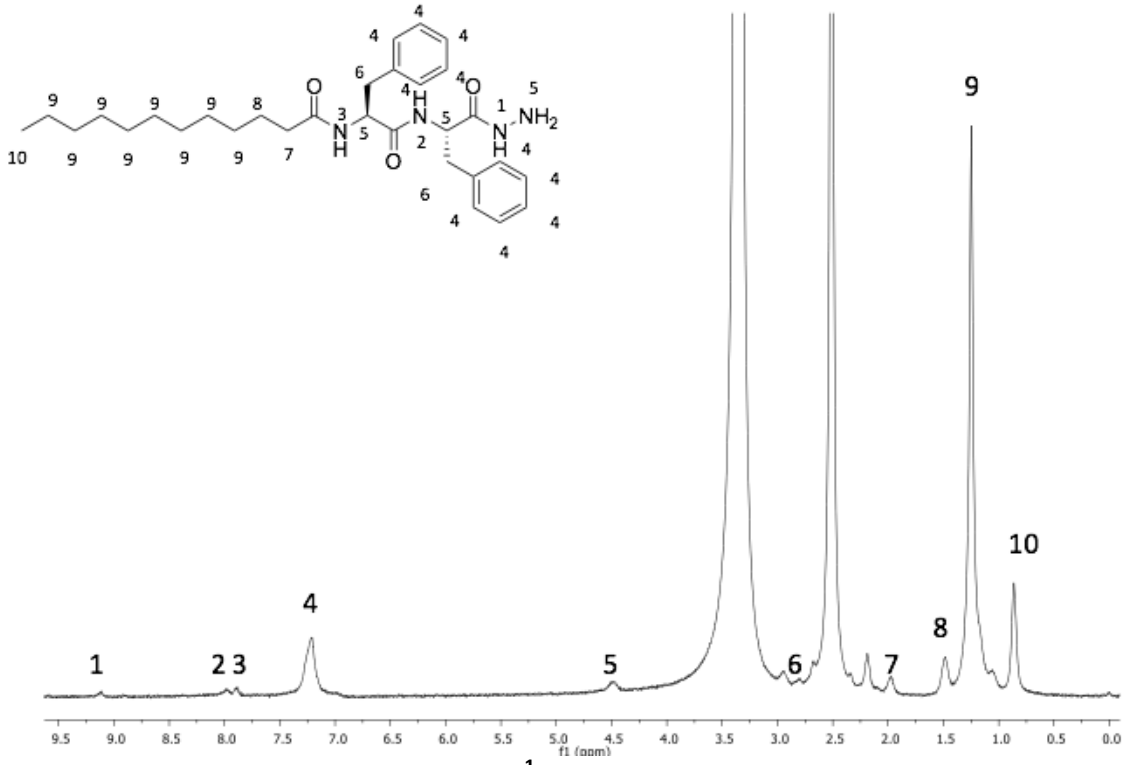


Figure B1. C_{12}FFNHNH$_2$ ^{1}H NMR (400 MHz, DMSO-d$_6$)

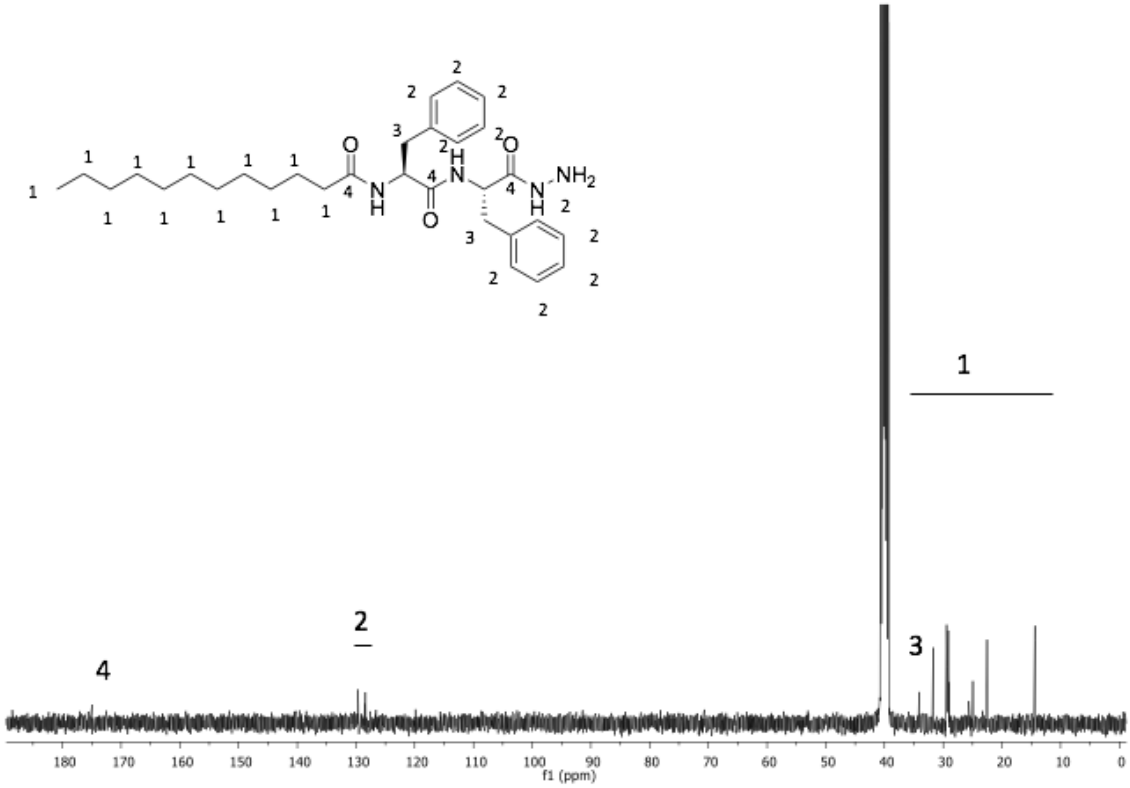


Figure B2. C_{12}FFNHNH$_2$ ^{13}C NMR (100 MHz, DMSO-d$_6$)

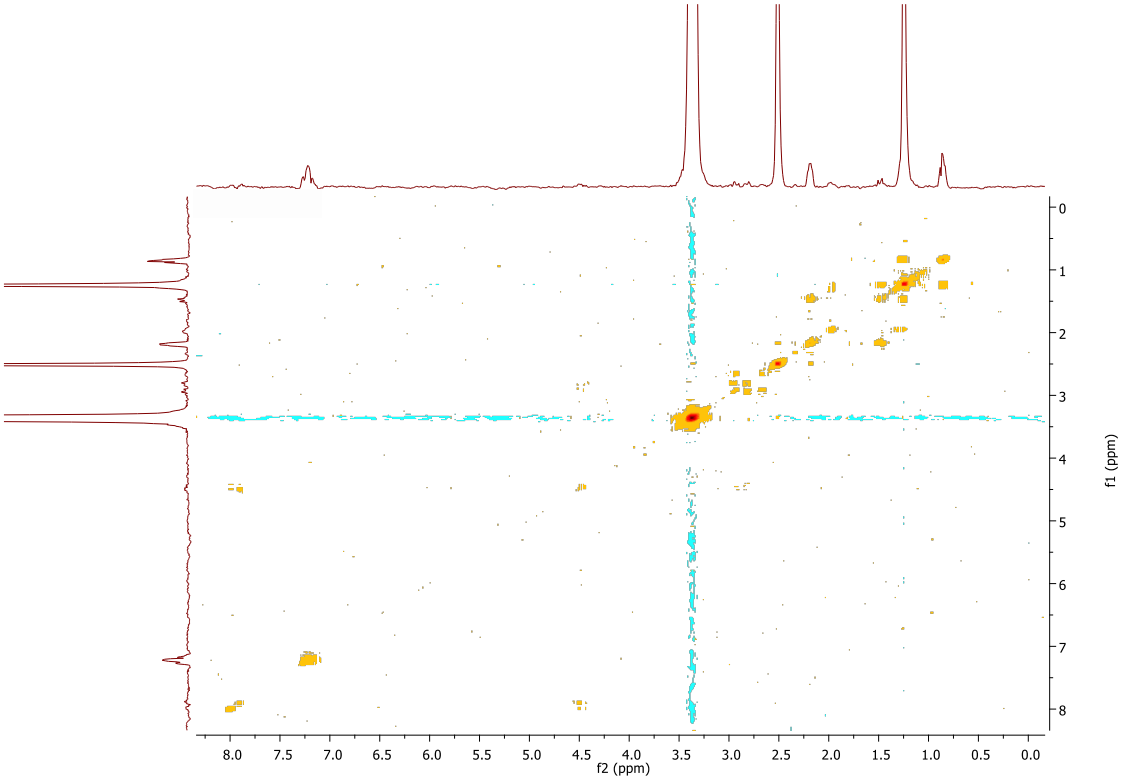
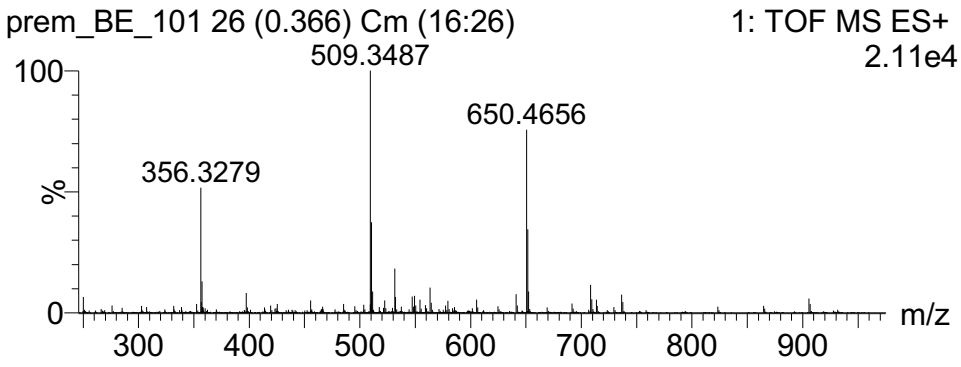


Figure B3. C_{12}FFNHNH$_2$ COSY NMR



$[M+H]^+$ =509.3492

$[M+H]^+$=509.3487 (1.0 ppm)

Figure B4. C_{12}FFNHNH$_2$ MS (ES-QTOF)

C_{16}FFNHNH$_2$

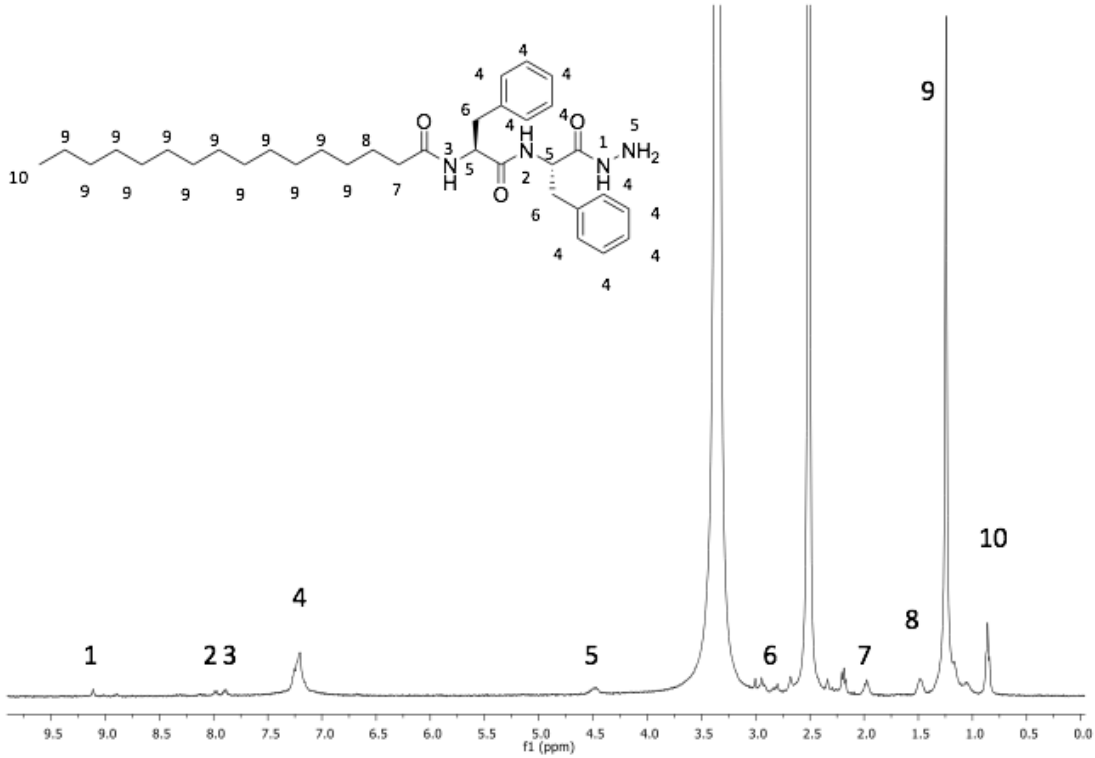


Figure C1. C_{16}FFNHNH$_2$ ^{1}H NMR (400 MHz, DMSO-d$_6$)

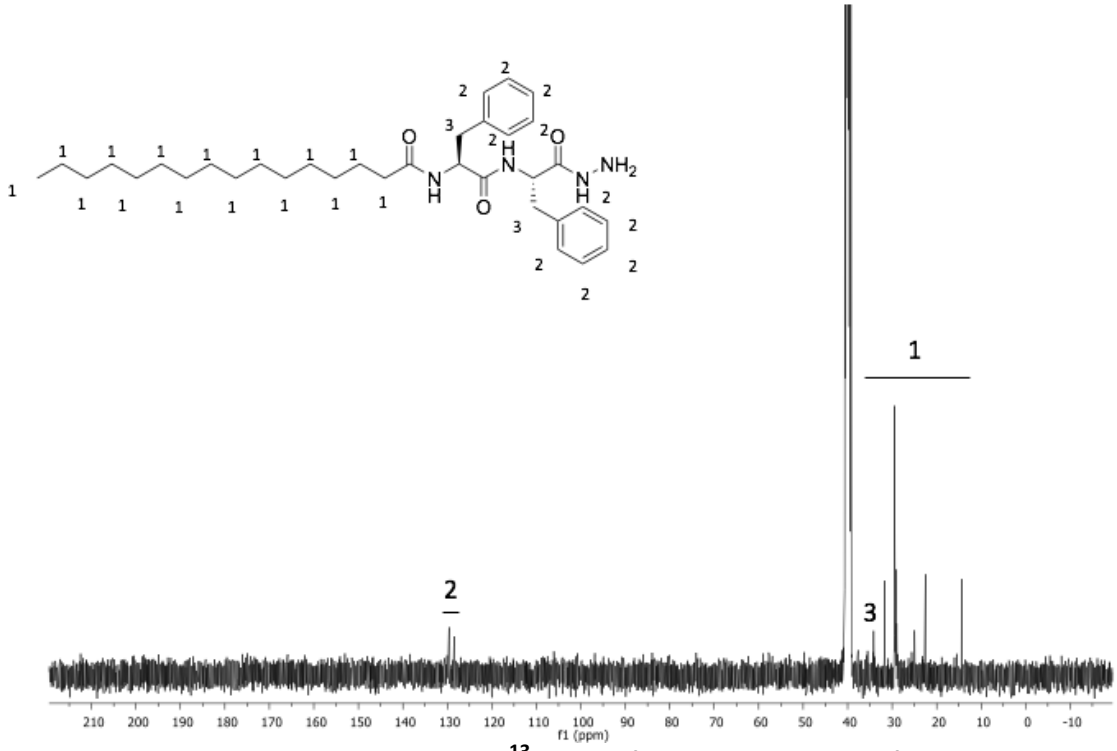


Figure C2. C_{16}FFNHNH$_2$ ^{13}C NMR (100 MHz, DMSO-d$_6$)

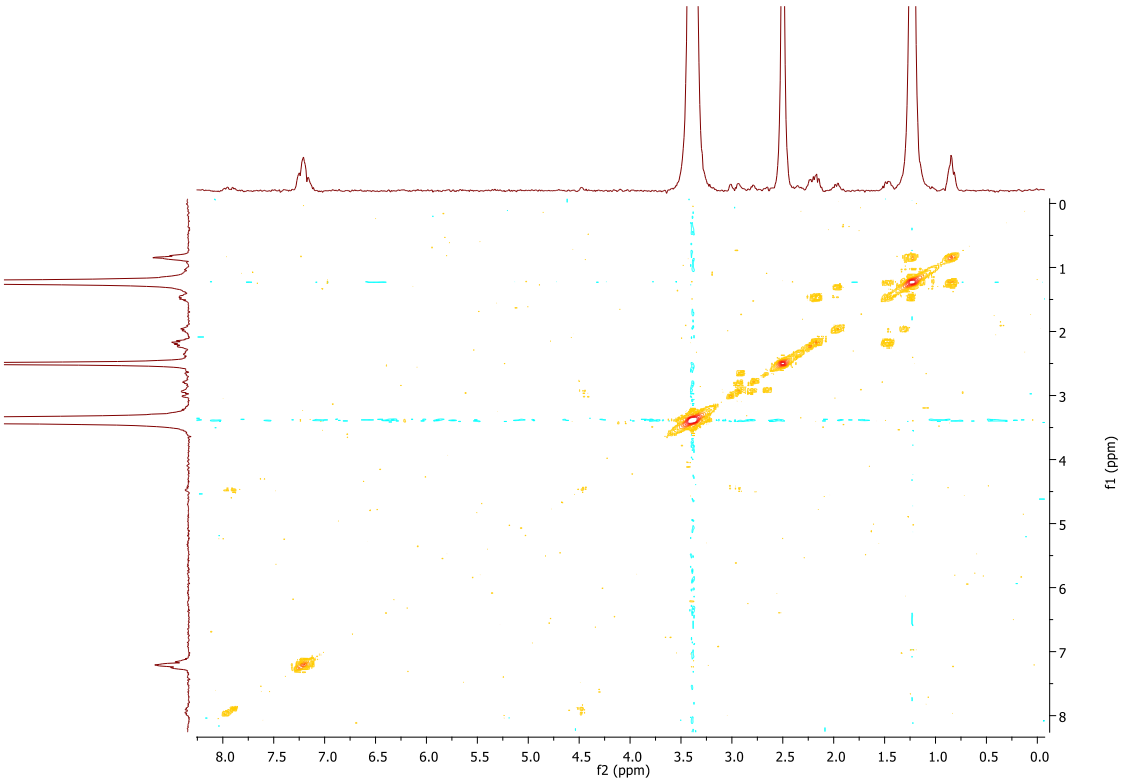
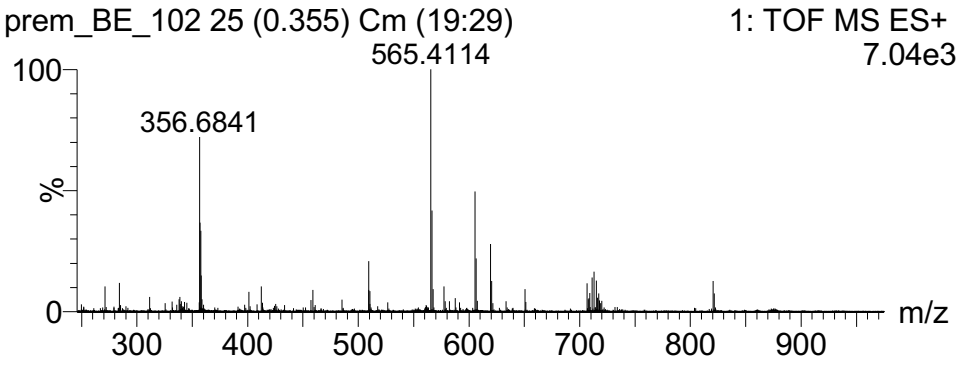


Figure C3. $C_{16}FFNHNH_2$ COSY NMR



$[M+H]^+$=565.4118

$[M+H]^+$=565.4114 (0.7 ppm)

Figure C4. $C_{16}FFNHNH_2$ MS (ES-QTOF)