

**Escuela superior de Tecnología y Ciencias Experimentales
Departamento de Química Inorgánica y Orgánica
Grupo de Nanomateriales Moleculares Orgánicos con Aplicaciones
Biomédicas**



**STUDIES OF BIOMIMETIC DEAMIDATION IN
MODEL COMPOUNDS DERIVED FROM
SUCCINIC AND GLUTARIC ACIDS**

**CELIA M. RUEDA NAVARRO
CHEMISTRY DEGREE RESEARCH PROJECT
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El Dr. Juan Felipe Miravet Celades, Profesor Titular, y el Dr. César Augusto Angulo Pachón, Investigador, pertenecientes al Departamento de Química Inorgánica y Orgánica de la Universitat Jaume I de Castellón de la Plana,

CERTIFICAN

Que el trabajo fin de grado con el título **STUDIES OF BIOMIMETIC DEAMIDATION IN MODEL COMPOUNDS DERIVED FROM SUCCINIC AND GLUTARIC ACIDS** ha sido realizado por Celia María Rueda Navarro bajo su dirección, en el grupo de Nanomateriales Moleculares Orgánicos con Aplicaciones Biomédicas del Departamento de Química Inorgánica y Orgánica de la Universitat Jaume I de Castellón de la Plana.

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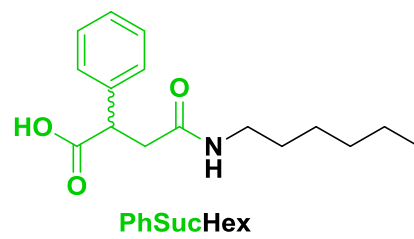
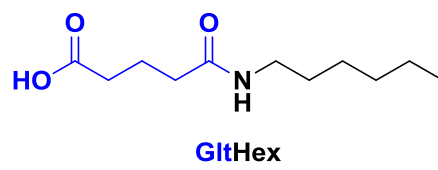
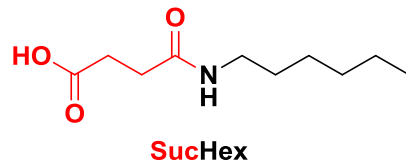
Fdo. Dr. Juan F. Miravet Celades

Fdo. Dr. César A. Angulo Pachón

Abbreviations

Suc	Succinic acid radical
Glt	Glutaric acid radical
Ph	Phenyl radical
Hex	Hexyl radical
NMR	Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
D ₂ O	Deuterated water
DMSO	Dimethyl sulfoxide
THF	Tetrahydrofuran
AcOEt	Ethyl acetate
HPLC	High-Performance Liquid Chromatography

Synthesized and studied compounds



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Every experience in life is different and every of them has to be exhilarating. This one was gratifying, also giving me a better understanding of the research field.

Everything will be alright in the end.

If it is not alright, it is not yet the end.

Unknown

Index

1. Chapter: Introduction	1
1.1. The amide bond	1
1.2. Amides synthesis	2
1.3. Reactivity of amides	4
1.3.1. Deamidation	5
1.3.2. Amides hydrolysis	6
2. Chapter: Objectives	9
3. Chapter: Results and Discussion	11
3.1 Synthesis of SucHex and GltHex	11
3.2 Synthesis of PhSucHex	11
3.3 Hydrolytic reaction studies by NMR	18
3.4 Study of the possible formation of succinimide intermediates by NMR	21
3.5 Hydrolytic analysis by mass spectrometry	22
4. Chapter: Conclusions	27
5. Chapter: Experimental Section	29
5.1. General methods	29
5.1.1. Synthesis of SucHex, GltHex and PhSucHex	30
5.1.2. General procedure	30
5.2. General procedure of hydrolytic reactions	31
6. Chapter: NMR spectra	33
7. Chapter: Mass spectra	37
7.1. Molecular Mass	37
7.2. Exact Mass	39

Introduction

1 Chapter: Introduction

1.1 The amide bond.

Nature is a very wide concept, as such are proteins that are on it. Proteins are constituted by juxtaposition of peptides forming peptide bonds between them. A peptide bond is a covalent bond formed by condensation of a carboxylic group and an amine group. This mentioned bond is an amide (CO-NH). Amide moiety is ubiquitous and plays an important role in life.

In nature, protein synthesis are carried out in the ribosome where there are enzymes that catalyse the complex process, probably to safeguard the unique and precisely defined amino acid sequence of every protein¹.

The amide functionality is also a common feature in small or complex synthetic molecules. Synthetic amides are in very divers environments, they can go from the textile industry with the polyamides, passing through cosmetics and personal care products, to medicine.

Amide bonds are very common in active pharmaceutical ingredients (API).² An in-depth analysis of the Comprehensive Medicinal Chemistry database revealed that carboxamide group appears in more than 25% of known drugs³, including paracetamol and penicillin (Figure 1-1). This last, heralding the dawn of the antibiotic age.⁴

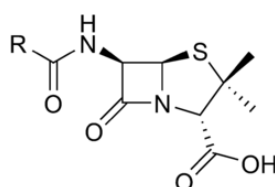


Figure 1-1. Penicillin structure.

¹ Montalbetti, C. A. G. N.; Falque, V. *Tetrahedron* **2005**, 61, 10827–10852.

² Carey, J. S.; Laffan, D.; Thomson, C.; Williams, M. T. *Org. Biomol. Chem.* **2006**, 4, 2337-2347

³ Ghose, A. K.; Viswanadhan, V. N.; Wendoloski, J. J. *J. Comb. Chem.* **1999**, 1, 55–68.

⁴ Aldridge, S.; Parascandola, J.; Sturchio, J. L. Commemorative booklet: "The discovery and development of penicillin 1928-1945". *Am. Chem. Soc.* **1999**.

Peptide bond is very noticeable for its stability capacity, reason why there are lots of amide bonds in the manufacture of rigid structures, like the polyamides in nylon (Figure 1-2) for such a range of textile applications; and Kevlar, very used for body armor.⁵

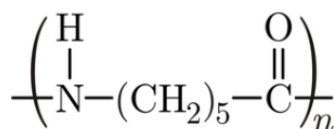


Figure 1-2. Nylon 6 structure.

As mentioned, peptide bond is involved in proteins. Caseins are the major fraction of milk proteins. Thus, they are widely studied. Apart from cheese farming, caseins have been mainly used as a food ingredient for enhancing physical properties such as foaming, whipping, thickening, for water binding, texture, and also for improving the nutritional values.⁶ Furthermore, caseins are exploited for nanoencapsulating the medicinal drugs. In particular, b-casein with some complexes were used as nanovehicles for the delivery of a platinum anticancer drug.⁷

1.2 Amides synthesis

There are lots of methods to synthesize amides.¹ The simplest method is by coupling a carboxylic acid with an amine, but this is only straightforward on paper. Even though this reaction is thermodynamically favorable, high activation energy is needed. This high energy is largely due to the first step in the reaction is the amine deprotonating the carboxylic acid, which reduces its reactivity, increasing this way the energy required. Consequently, the direct reaction often requires high temperatures.

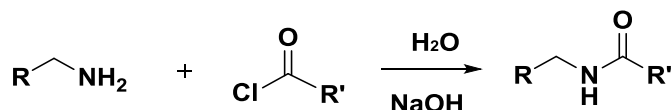
To catalyze the synthesis, many methods are being studied. Most of them involving converting the carboxylic acid to a better electrophile. Two reaction methods are common: *Schotten-Baumann reaction*⁸ and *Lumière-Barbier method*. The first one, uses chlorid acid and a base to drive the equilibrium to the right (Scheme 1-1). And the second one uses anhydrides.

⁵ Majzoubi, G. H.; Zaheri, F. M. *Int. Jour. of Eng.* **2017**, *30*, 791-799.

⁶ Sinha, S; Mishra, S.; Sen, G. *Int. J. Biol. Macromol.* **2013**, *60*, 141.

⁷ Razmi, M.; Divsalar, A.; Saboury, A. A.; Izadi, Z.; Haertle, T.; Mansuri-Torshizi, H. *Colloids Surf. B.* **2013**, *112*, 362.

⁸ Schotten, C. *Ber. Dtsch. Chem. Ges.* **1884**, *17*, 2544-2547.



Scheme 1-1. Schotten-Baumann reaction

The *Lumière–Barbier method* is employed for acetylating aromatic amines in aqueous solutions.⁹ It can be explained by the acetylation of aniline. First, aniline is dissolved in water using one equivalent of the strong hydrochloric acid. Then, an anhydride (acetate anhydride) and a base solution (aqueous sodium acetate) are used as it is seen in scheme. At the end, acetate acts as a leaving group and the amide (acetanilide) is formed.

Both examples of reaction use strong conditions to be carry out, concerning the stability of the amide bond. Moreover, exist more methods not mentioned, most of them using metals as catalysts.^{10,11}

Process chemists have a wide array of methods at their disposal to generate amides, but the limitations imposed by large-scale operations have focused the selection to a narrowerfield of reagents. For plant production, an amide coupling reagent has to be selected. The ideal considerations for the reagent is to be inexpensive, widely available, nontoxic, safe, simple to handle, easy to purge from reaction mixtures, and contribute only minimally to waste streams.¹² However, this conditions are not easy to handle.

Amide bond in some contexts is called peptide bond. Peptides form part of many things as polymers, that human use in everyday life. Moreover, proteins are of huge relevance for the body. Thus, peptide synthesis is a very important issue in society. The first peptide synthesised was the oxytocin (Figure 1-3), a hormone that among other things plays a role in reproduction between people and mammals, and in facilitating trust and attachment between individuals. So that, it

⁹ Clayden, J.; Greeves, N.; Warren, S. **2001**. "Organic chemistry". *Oxford university press*. 188 ISBN: 978-0-19-850346-0.

¹⁰ L. Zhang; Shunpeng, S.; Hongping, W.; Shaowu, W.; *Tetrahedron* **2009**, 65, 10022–10024.

¹¹ Gunanathan, C.; Milstein, D. **2014**. "Catalysis by Pincer Complexes: Synthesis of Esters, Amides, and Peptides". *Szabó, K. J.; Wendt, O. F.* ISBN: 9783527334421.

¹² Dunetz, J. R.; Magano, J.; Weisenburger, G. A. *Org. Process Res. Dev.* **2016**, 20, 140–177.

is often dubbed as "the love hormone"¹³ and it was obtained by Vincent du Vigneaud in 1953, who got the Nobel Prize for along others, this discovering.¹⁴

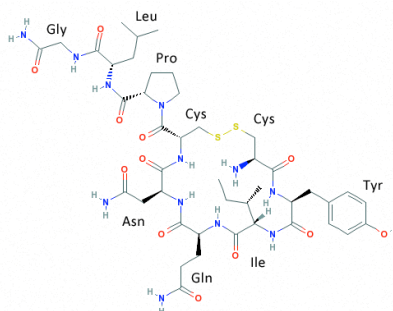


Figure 1-3. Oxytocin hormone structure.

1.3 Reactivity of amides

Amides are the least reactive of the neutral carboxylic acid derivatives, even less reactive than esters. Nevertheless, they undergo many chemical reactions such as hydrolysis, but in heavy conditions: hot alkali or in strong acidic conditions. Moreover, amides are versatile precursors to many functional groups. Some reactions amides can experiment, are going to be briefly described. Reduction, to produce an amine or an aldehyde, depending on the quantity of substitution amide poses. Amides can be reduced using reducing agents as LiAlH_4 and by catalytic hydrogenation together with high pressure.¹⁵

Most amides are unreactive because of the electron donation character of nitrogen atom, donating a pair of electrons to the carbonyl group, and because if there are functional groups coupled with the nitrogen atom, they may block the approach of attacking reagents.

Amide reactivity is of huge relevant in proteins field. When proteins are ingested in an organism, they must be broken into smaller peptides for absorption of the amino acids in the intestine. The breakdown of proteins is called proteolysis, and the enzyme that develops this function, by a hydrolysis reaction, are the

¹³ Grillon, C.; Krinsky, M.; Charney, DR.; Vytal, K.; Ernst, M.; Cornwell, B. *Mol. Psychiatry*. **2013**, *18*, 958–60.

¹⁴ "Vincent du Vigneaud - Facts". *Nobelprize.org*. Nobel Media AB **2014**.

¹⁵ Everett, W.; Lane, J. *Organic Reactions*. **1946**, *3*, 267–306

proteases. Every enzyme, is selective to cleave in different parts of the peptide sequence. This shows the strength of the amide bond. For instance, trypsin is able to break down the protein by the carboxylic side of arginine and lysine.

The design of artificial enzymes capable of cleaving peptide bonds of proteins has been the focus of intense effort for a number of years as a model for the study of hydrolysis of amides.¹⁶

1.3.1 Deamidation

A chemical reaction in which an amide in the side chain of amino acids is removed or converted to another functional group is named deamidation. These reactions in a protein or peptide are of huge importance because they may alter its structure, stability or function, and may lead to protein degradation. Deamidation is a very slow process in mild conditions, being considerate as a factor that limit the useful lifetime of proteins. However, peptides have a extend range of half-lives. Significant factors affect peptide bond half-life, such as pH, temperature, sequence, amount of peptide, modifications, etc. It proceeding much more quickly at elevated pH (>10) and temperature. Enzymatically, have short half-lives¹⁷, but non-enzimatically have a much longer half-life. At neutral pH and 25°C, the hydrolysis of a deactivated peptide bond has an approximate half-life of 500 years.¹⁸ This reaction proceeds much more quickly if the susceptible amino acid is followed by a small, flexible residue such as glycine who has a very low steric hindrance.¹⁹

It is reported that spontaneous degradation of proteins can take place in peptides and proteins containing asparaginyl and aspartyl residues. This process takes place via succinimide intermediates in an intramolecularly catalyzed nonenzymatic deamidation, resulting in modified proteins.^{20,21} Scheme 1 shows the intramolecular attack of the peptidic nitrogen atom to the amide group in the

¹⁶ Kahne, D.; Still, W. C. *J. Am. Chem. Soc.*, **1988**, *110*, 7529–7534.

¹⁷ Mathur, D.; Prakash, S.; Anand, P.; Kaur, H.; Agrawal, P. *Sci. Rep.* **2016**, *6*, 36617.

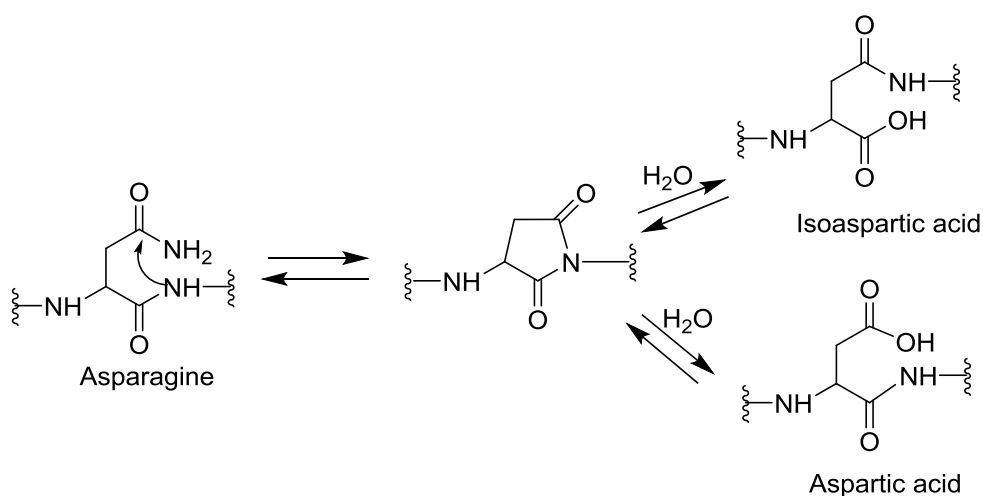
¹⁸ Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.*, **1996**, *118*, 6105–6109.

¹⁹ Stephenson, RC.; Clarke, S. *J. Biol. Chem.* **1989**, *264*, 6164-6170.

²⁰ N. H. Shah and T. W. Muir, *Chem. Sci.*, **2014**, *5*, 446-461.

²¹ C. A. Angulo-Pachon and J. F. Miravet, *Chem. Commun.*, **2016**, *52*, 5398-5401.

side chain of asparagine affording a succinimide intermediate, which upon hydrolysis can produce aspartic or isoaspartic acid.



Scheme 1. Simplified mechanism of protein deamidation.

A related chemical process is that found in inteins. Inteins are polypeptide sequences that are excised from the protein that contains them by a self-catalyzed protein-splicing reaction. It is a multi-step biochemical reaction comprised of both the cleavage and formation of peptide bonds.²² In the final step of the splicing reaction, an intramolecular attack takes place, leading to a succinimide unit and protein breakage.²³

1.3.2 Amides hydrolysis.

Due to amide poor reactivity, kinetic and mechanistic studies have been done almost exclusively with amides that are in extreme conditions, by activation with substituents, by ring strain,²⁴ by proximate functional groups or by metal complexes.²⁵ For example, in 1998, Menger and Ladika reported that the

²² Shah, N. H.; Muir, T. W. *Chem. Sci.*, **2014**, *5*, 446-461.

²³ Angulo-Pachón, C. A.; Navarro-Barreda, D.; Rueda, C. M.; Galindo, F.; Miravet, J. F. *J. Colloid Interface Sci.* **2017**, *505*, 1111-1117.

²⁴ Lopez, X.; Mujika, J. I.; Blackburn, G. M.; Karplus, M. *J. Phys. Chem. A*, **2003**, *107*, 2304-2315.

²⁵ Zhu, L.; Kostic, N. M. *Inorg. Chem.*, **1992**, *31*, 3994-4001.

pyrrolidyl amide of Kemp's triacid undergoes intramolecular hydrolysis. This compound has ring strain and two carboxylic acid groups and an amide group in axial of the conformation seen in Figure 3-1. This hydrolysis reaction lies on the geometric disposition of the functional groups, which forces the compounds to react. The cleavage can be explained on the basis of a pathway involving intramolecular nucleophilic displacement by the terminal carboxylic acid group on the amide function, in other words, due to the proximity between functional groups.²⁶

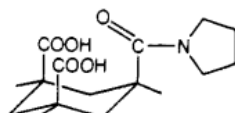


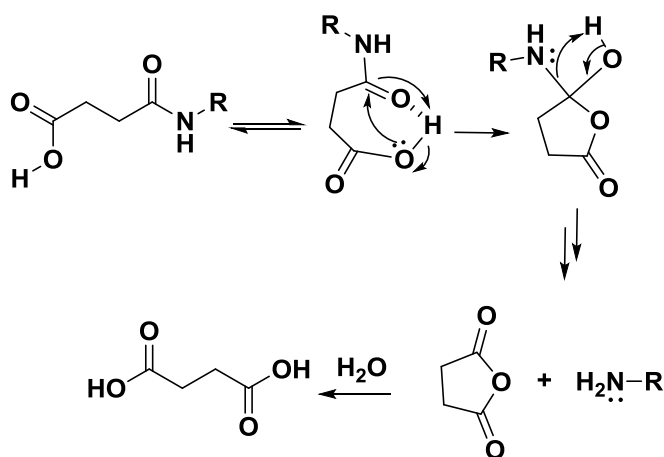
Figure 3-1. Pyrrolidyl amide of Kemp's triacid.

²⁶ Menger, F. M.; Ladika, M. *J. Am. Chem. Soc.* **1988**, *110*, 6794-6796.

Objectives

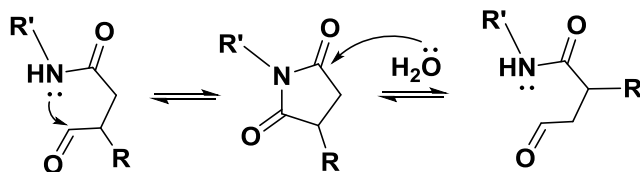
2 Chapter: Objectives

Previously, the research group synthesized a family of hydrogelators derived from different aminoacids with a succinic acid moiety. Surprisingly, the compounds showed hydrolysis in mild conditions.²⁷ They are simple chain compounds without any strain tension. The hydrolytic mechanism can be rationalized considering an intramolecular nucleophilic attack, as it is described in Scheme 2-1.



Scheme 2-1. Simplified hydrolytic deamidation mechanism.

As shown in the introduction, in proteins, deamidation occurs by forming a succinimide intermediate. Such process could also be operating in the succinic acid derivatives studied in the group, as shown in Scheme 2-2.



Scheme 2-2. Reaction mechanism through succinimide formation, similar to protein deamidation.

²⁷ Navarro-Barreda, D. 2016 "Synthesis and hydrolytic stability of succinic acid derived gelators" *Research Project, University Jaume I.*

Scheme 2-2 shows an isomerisation process that only will be apparent if an asymmetric succinic acid derivative is present.

The main goal of this work is to determine if these two proposed mechanisms, amide hydrolysis and isomerization via succinimide, can coexist.

The specific objectives are:

- ♣ Synthesize and characterize the following compounds to be studied by the research group (Figure 2-1).
 - ♣ PhSucHex: to study deamidation reaction through imide formation.
 - ♣ SucHex: to study hydrolytic intramolecular reaction.
 - ♣ GltHex: as a control for both reactions.

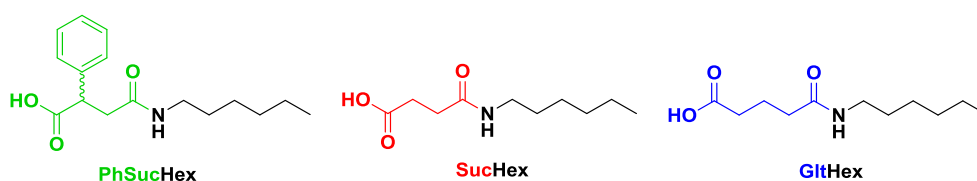


Figure 2-1. Compounds synthesized in the present project.

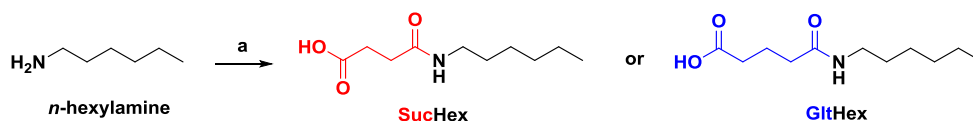
- ♣ Familiarize with 2D NMR techniques to elucidate the structure of the compounds.

Results and Discussion

3 Chapter: Results and Discussion

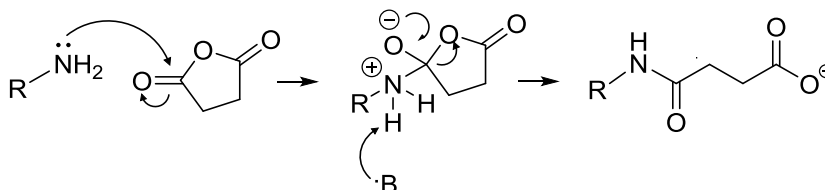
3.1 Synthesis of SucHex and GltHex:

Firstly, synthesis of SucHex and GltHex was performed in a one-step reaction, involving the coupling of an *n*-hexylamine and the correspondent acid activated in anhydride form (succinic anhydride for SucHex, and glutaric anhydride for GltHex), as it can be seen in the following Scheme 3-1:



Scheme 3-1. Reagents and conditions: a) Succinic or Glutaric anhydride, Na₂CO₃, THF, 16 h, 90 – 95%.

The mechanism of the reaction consists in the nucleophile attack of the amine to the carbonyl of the anhydride in soft basic conditions, creating the amide, as demonstrated in Scheme 3-2.

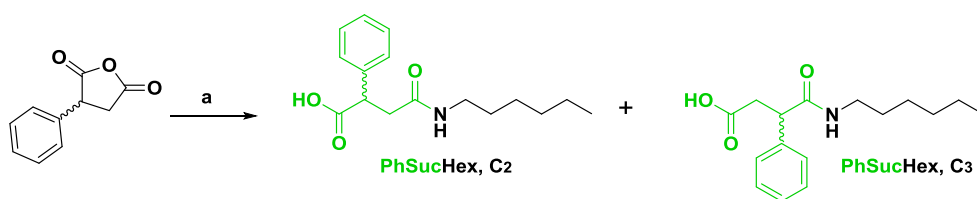


Scheme 3-2. Reaction mechanism to synthesize the amide bond.

SucHex and GltHex were synthesised successfully and the reaction was performed obtaining a good yield, confirming the synthesis by characterization using NMR. (NMR in annex).

3.2 Synthesis of PhSucHex

Compounds PhSucHex (see Scheme 3-3), were obtained following the same procedure as above, except that racemic phenylsuccinic anhydride was used. A white solid was obtained with good yield.



Scheme 3-3. Reagents and conditions: a) *n*-hexylamine, Na₂CO₃, THF, 73%.

The products of the reaction obtained have a quiral centre, so that, four enantiomers (PhSucHex,C₂ and PhSucHex,C₃; see scheme 3-3) are being formed.

The NMR of the crude of reaction showed just two compounds (Figure 3-1), (due to NMR equipment cannot distinguish between enantiomers).

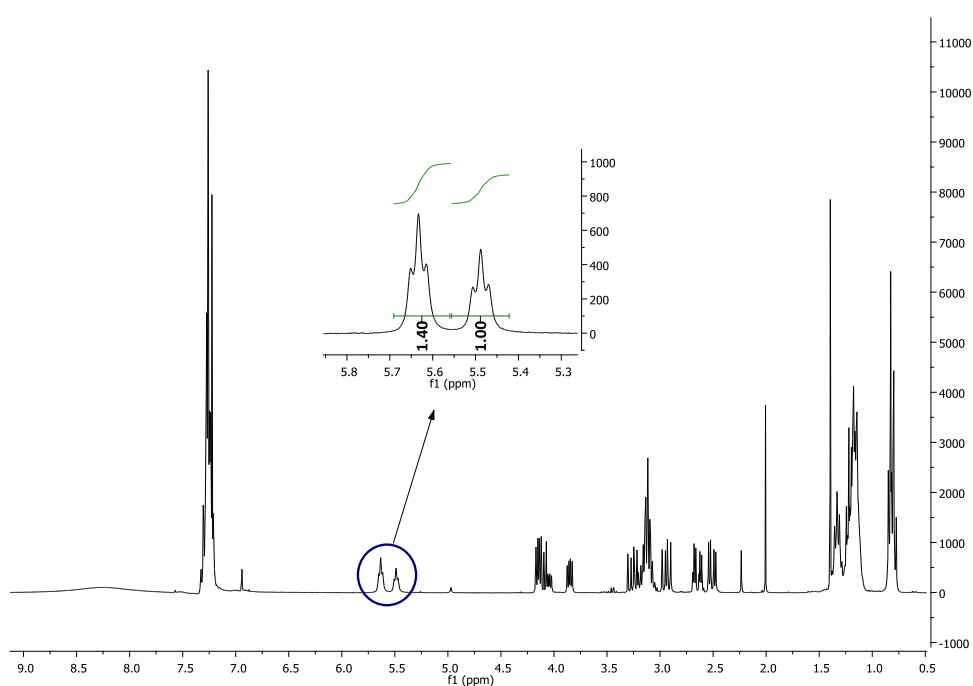


Figure 3-1. ¹H-NMR spectrum of products PhSucHex,C₂ and PhSucHex,C₃ in ratio 1.4:1.

As it can be appreciated with the signals corresponding to the protons belonging to the amides (signals at 5.4-5.7 ppm in figure 3-1), the proportion of the two isomers (Scheme 3-3) is 1.4:1.

Studies with more than one compound as a reactant are more complex to carry out due to the increased quantity of products formed. Therefore, more complicated also for their characterization. Hence, it was decided to optimize the reaction using a slower reaction rate to favor the formation of the kinetically favored isomer. For this, all the reaction was performed the same way but all developing in conditions of 0 °C of temperature. Moreover, a syringe pump was used to introduce very slowly (0.8 mL/min.) the solution of the phenylsuccinic anhydride. However, the reaction was unsuccessfully due to it did not improve the difference in the ratio of the isomers.

Finally, it was decided to separate the isomers by means of a chromatographic column.

TLCs in different ratios of dissolvent were carried out to analyze which solvent should be used for the separation. For instance, some of the mobile phases used were using solvents as THF, toluene, methanol, CH₂Cl₂ and AcOEt; spots were observed by treatment with ninhydrin revelator followed by heating. Finally, it was concluded that the best solvent to achieve the separation was ethyl acetate. The chromatography column used was 5cm diameter and 7cm of silica flash. The compounds were not possible to separate completely even though, by TLC compounds seemed to be segregate with this solvent.

As it was not possible to separate the isomers, fractions of the column elute were collected. Elute was separate in three different fractions, and each of this fraction was analyzed by NMR. ¹H-NMR of the third fraction, showed that the sample had been enriched with an isomer, present in a 81% as it can be observed in the following Figure 3-2.

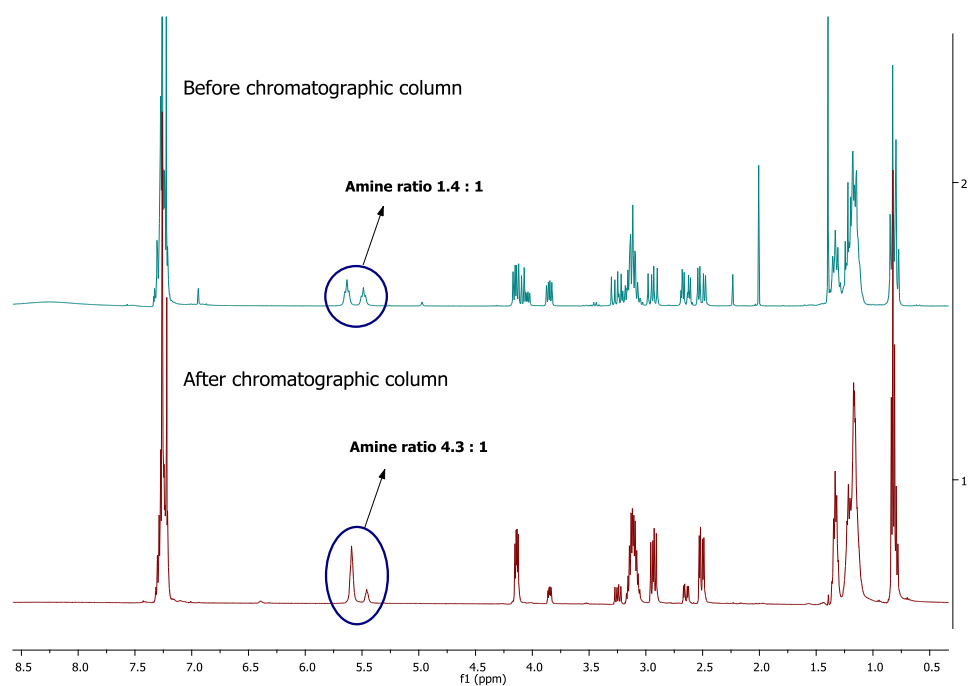


Figure 3-2. Comparative $^1\text{H-NMR}$ spectra before separation (top) and after the separation (bottom). Ratios of isomers can be observed in the figure, referring with the signal of the proton in the amides.

Another possible technique to separate the isomers would be HPLC, but there was no time to design an HPLC method for the separation due to many complications could take place hampering the project.

Figure 3-3 shows the two PhSucHex isomers wanted to be identified.

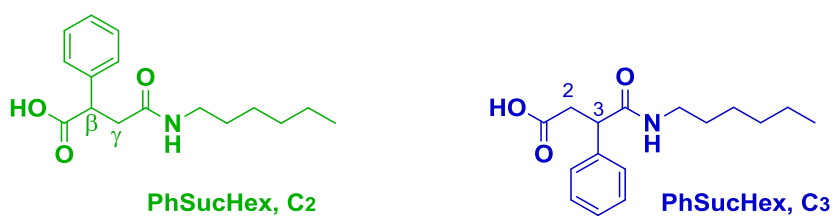


Figure 3-3. PhSucHex, C_2 and PhSucHex, C_3 .

Finally, it was decided to use NMR spectra for the identification of the major and minor isomer produced in the reaction. Therefore, this third fraction was used to carry out 2D NMR studies: ^1H - ^1H correlation (COSY), ^1H - ^{13}C correlation at one bond (HSQC) and ^1H - ^{13}C correlation at long distance, 2 or 3 bonds commonly (HMBC).

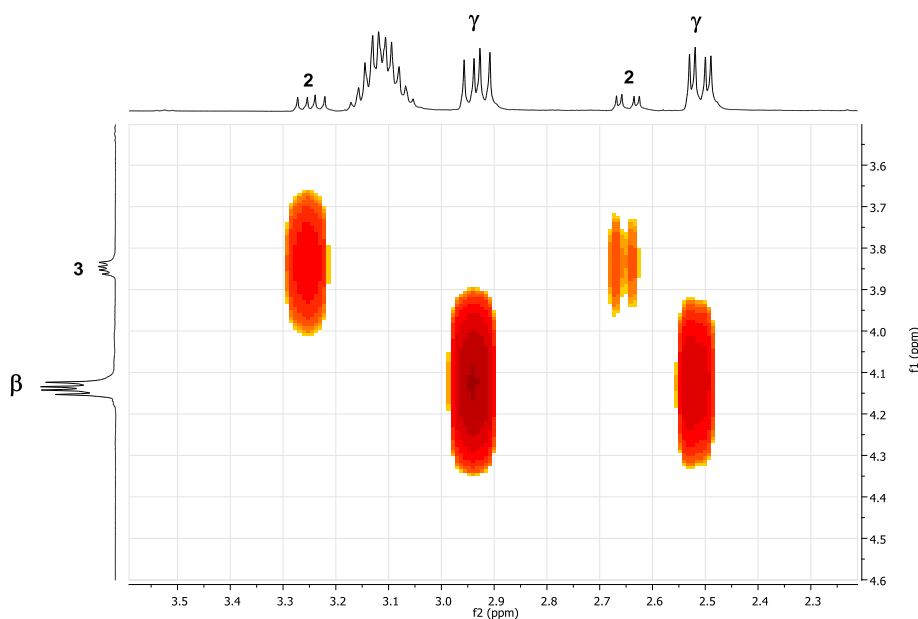


Figure 3-4. COSY of PhSucHex, C₂ and PhSucHex, C₃.

Firstly, by COSY, some proton signals were identified. Protons next to an aromatic group appear around 4 ppm due to the shielding, being the case of H₃ and H_β (seen in the vertical axis of Figure 3-4). Protons next to a carbonyl, appear around 2 ppm and 3 ppm, being H₂ and H_γ.

In figure 3-4, coupling between H₃ and H₂, and between H_β and H_γ can be observed.

Figure 3-5 shows the relevant atoms participating in the correlations observed in HSQC and HMBC.

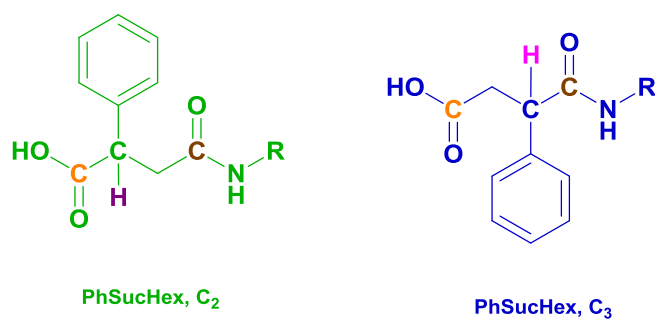


Figure 3-5. Relevant atoms participating in the interactions HSQC and HMBC for PhSucHex, C₂ and PhSucHex, C₃.

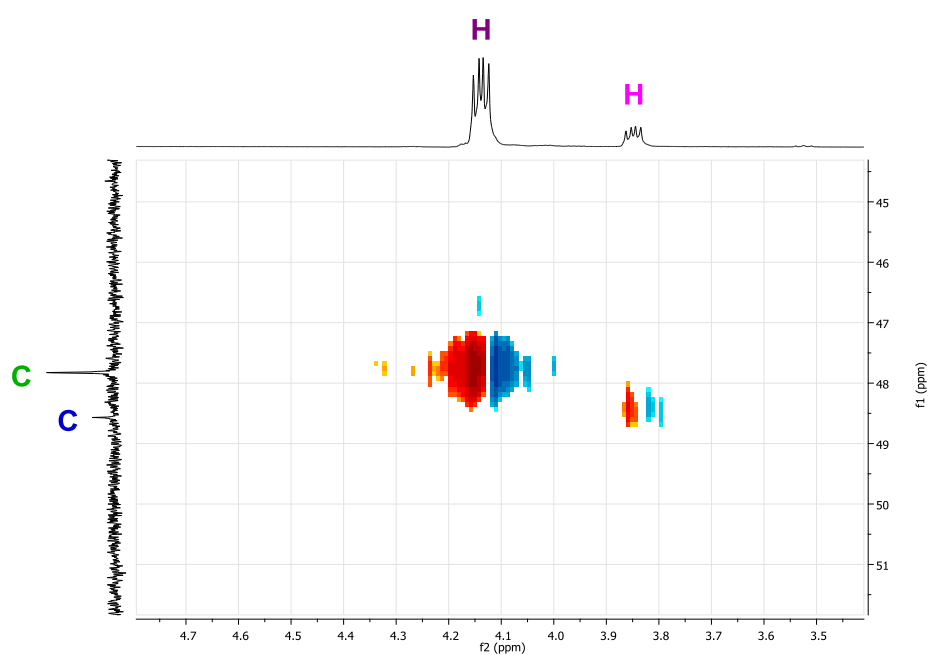


Figure 3-5 . HSQC enlargement of PhSucHex C₂, and PhSucHex C₃

From HSQC, it could be interpreted that purple H is coupling with green C, and pink H to blue C. Indicating which carbon is belonging with which signal.

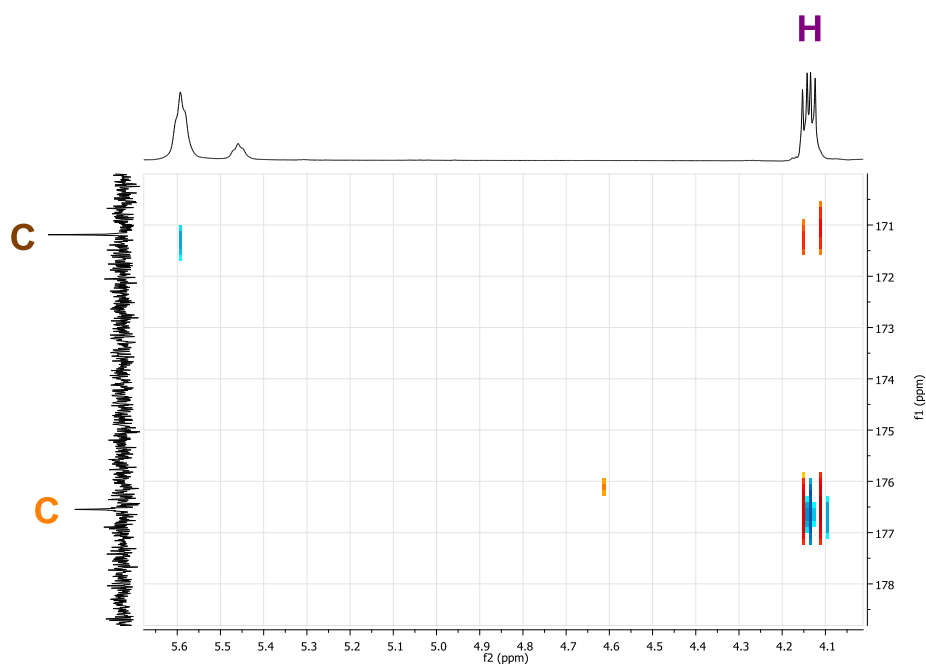


Figure 3-6. HMBC of PhSucHex, C₂, and PhSucHex, C₃.

This HMBC section shows the carbonyls of the ¹³C-NMR. Brown C is the carbon of the amide due to it correlates with the proton of -CONH-. The proton next to the phenyl group correlates with orange C better than with brown C, this last corresponding with the amide carbonyl. Therefore, proton signal at 4.15 ppm corresponding with the major isomer, is purple H. Thus, the major isomer proportion is the one with phenyl group in C₂: PhSucHex, C₂.

In short, by means of NMR spectra structure of compounds was elucidated (Figure 3-7).

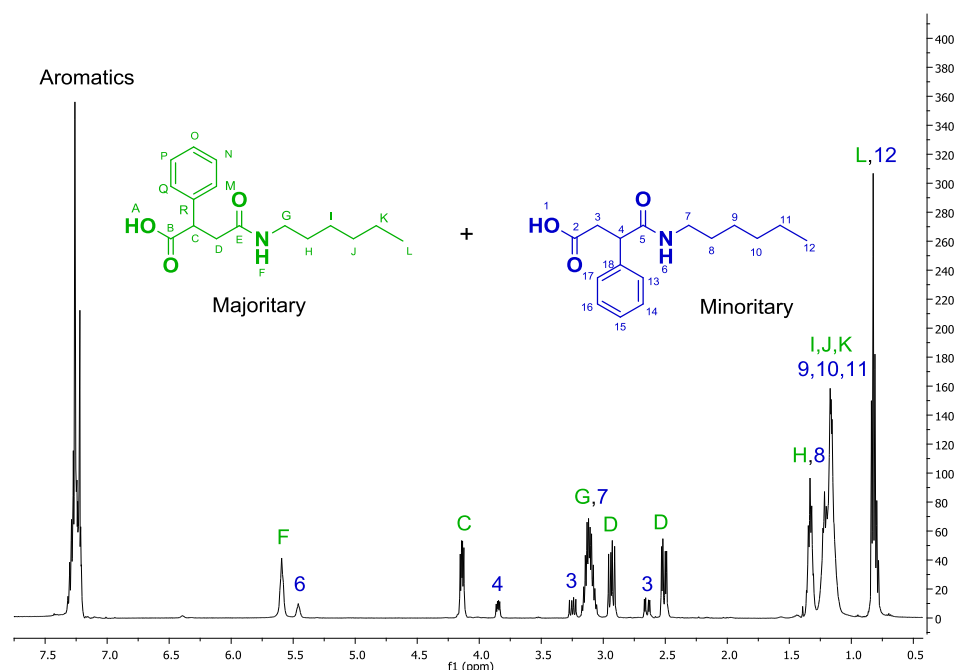
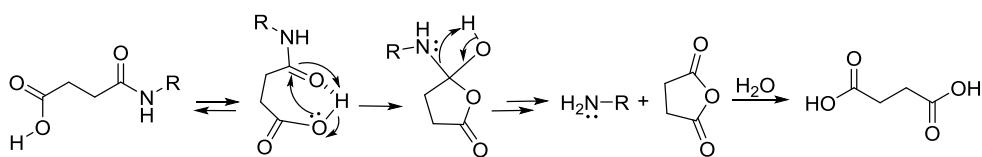


Figure 3-7. $^1\text{H-NMR}$ spectrum of PhSucHex identifying signals of both isomers.

3.3 Hydrolytic reaction studies by NMR.

It was studied by the research group that a hydrolytic process, related to protein deamidation or self-excision, could be taking place. To evaluate this possibility, simple models compounds were studied.

Hydrolysis reaction takes place through an intramolecular reaction. It could be explained on the basis of a pathway involving intramolecular nucleophilic attack by the terminal carboxyl group to the amide carbonyl group stabilised by intramolecular H-bonding with the vicinal amide group, yielding a tetrahedral intermediate that evolves to the excision of the molecule into the corresponding amine and succinic anhydride, which would be subsequently hydrolyzed into succinic acid (see proposed mechanism at Scheme 3-4).



Scheme 3-4. Simplified mechanism proposed for the deamidation reaction.

For the study of the hydrolytic stability of the compounds, several batches containing 5 mg of each compound were suspended in 1 mL of D₂O and sonicated for 10 minutes. After 18 h at 80 °C, SucHex and PhSucHex were almost completely hydrolyzed affording hexylamine and respective succinic acid (see Figure 3-8). Analysis of the samples at different time intervals was performed by ¹H-NMR. To achieve full solubility of the samples after the stipulated time, solid powdered NaOH was added until pH basic.

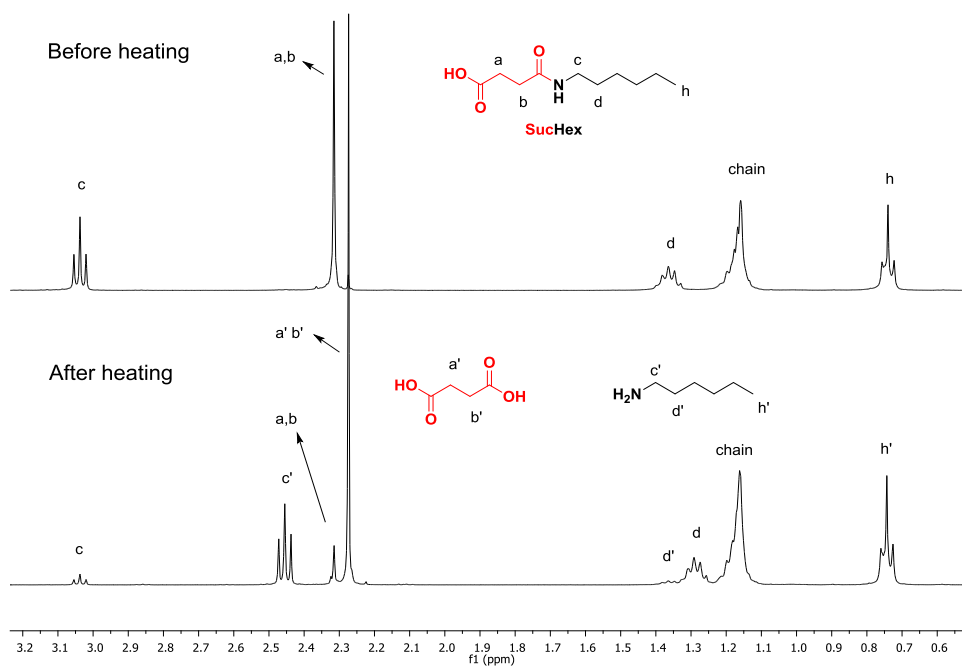


Figure 3-8. ¹H-NMR spectra of SucHex before (top) and after (bottom) heating at 80 °C in D₂O for 18 hours.

In this last Figure 3-8, the extreme lability in the studied succinic derivatives can be observed under the mild conditions used, and it is a remarkable fact, considering the robustness of amide bonds.

Conversely, glutaric derivative (GltHex) was also analysed, and it can be seen in Figure 3-9 that amide bond of GltHex does not break in such conditions.

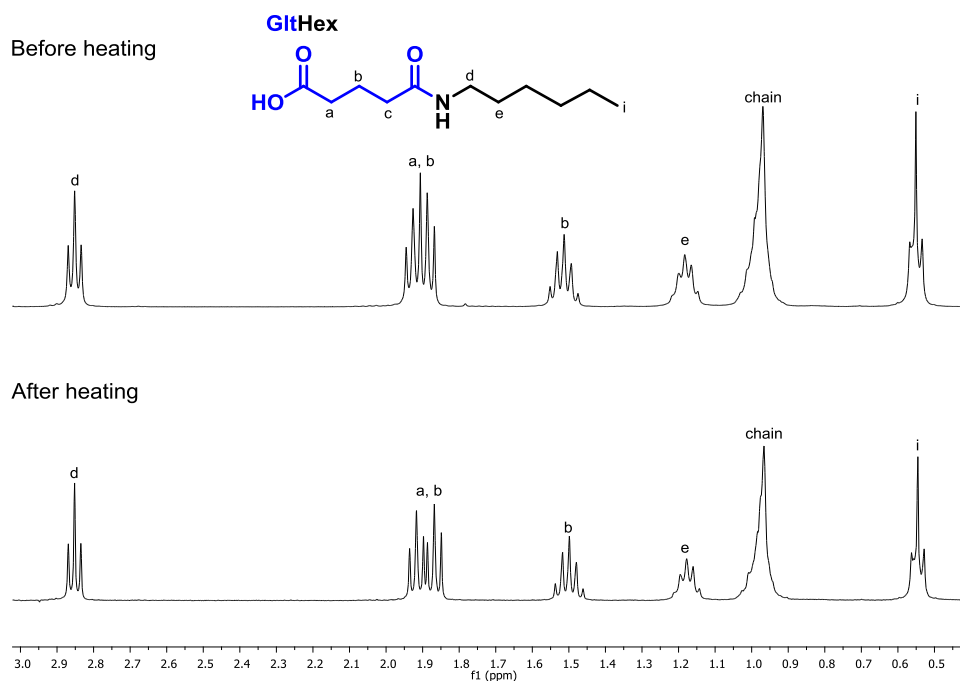


Figure 3-9. ¹H NMR spectra of GltHex before (top) and after (bottom) heating at 80 °C in D₂O for 18 hours.

Hydrolytic experiments confirm that the amide bond in SucHex breaks down. Analogue molecule GltHex was studied, being useful for supporting the intramolecular hypothesis reaction to be taken place. This molecule was found to be completely stable to hydrolysis in the same conditions for several hours, being such a dramatic difference when compared to succinic derivatives. Despite this, it seems reasonable that the presence of an additional methylene unit in GltHex would afford a rather less thermodynamically stable cyclic intermediate than in the case of SucHex for entropic reasons.

3.4 Study of the possible formation of succinimide intermediates by NMR.

Thermal treatment of the mixture of isomers of PhSucHex was carried out (Figure 3-10). It was observed that partial hydrolysis, as in the case of SucHex was taking place. However, even though hydrolysis of PhSucHex occurs, if an equilibrium exist forming succinimide intermediates, PhSucHex isomers proportion should vary after thermal treatment. In Figure 3-11, $^1\text{H-NMR}$ signals of the amide proton of a mixture of both isomers PhSucHex before and after heating, are shown. The proportion changed in relation major : minor, from 60% : 40% to 55% : 45%. This variation seems to indicate that the succinimide mediated isomerization could be taking place. However, the low change in the isomers ratio indicates that this result should be considered preliminary and further studies in different conditions should be done in the future.

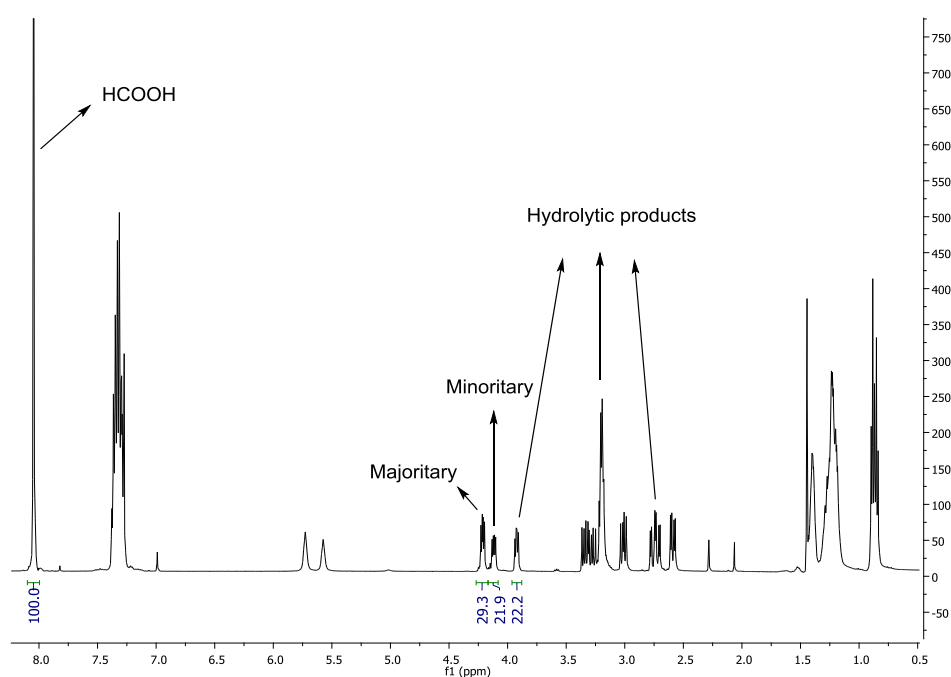


Figure 3-10. PhSucHex $^1\text{H-NMR}$ spectrum after hydrolysis in CDCl_3 and 0.5% HCOOH.

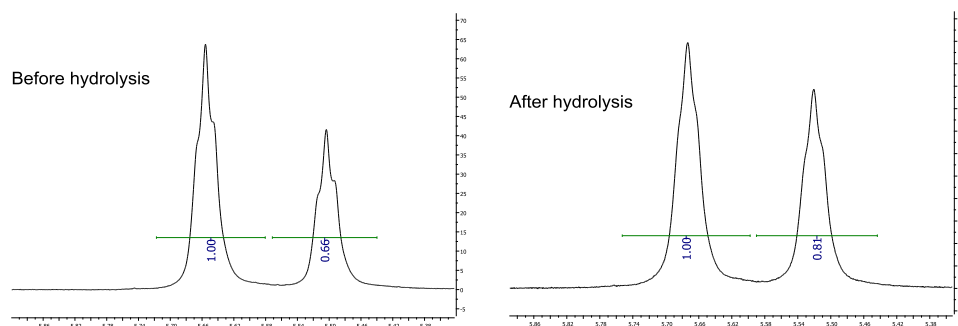


Figure 3-11. $^1\text{H-NMR}$ (CDCl_3) spectra of PhSucHex major and minor isomer, before (top) and after thermal treatment (bottom).

3.5 Mass spectrometry analysis of the reaction

To evaluate the possible presence of succinimide intermediates in minor quantities not observable by NMR, mass spectra were recorded for the samples studied (SucHex and PhSucHex), and they are showed in Figures 3-12 to 3-14 hereunder. Tables 3-1 and 3-2 describe information of the possible compounds formed in each case. Entry 1 corresponds to the starting compound, and 2-4 to the possible products of the hydrolysis, being entry 4, the succinimide intermediate.

Entry	Structure	Chemical Formula	Molecular Weight
1		$\text{C}_{10}\text{H}_{19}\text{NO}_3$	277,36
2		$\text{C}_{10}\text{H}_9\text{O}_4^-$	193,18
3		$\text{C}_6\text{H}_{16}\text{N}^+$	102,20
4		$\text{C}_{10}\text{H}_{17}\text{NO}_2$	183,25

Table 3-1. Masses of the possible products of SucHex hydrolysis.

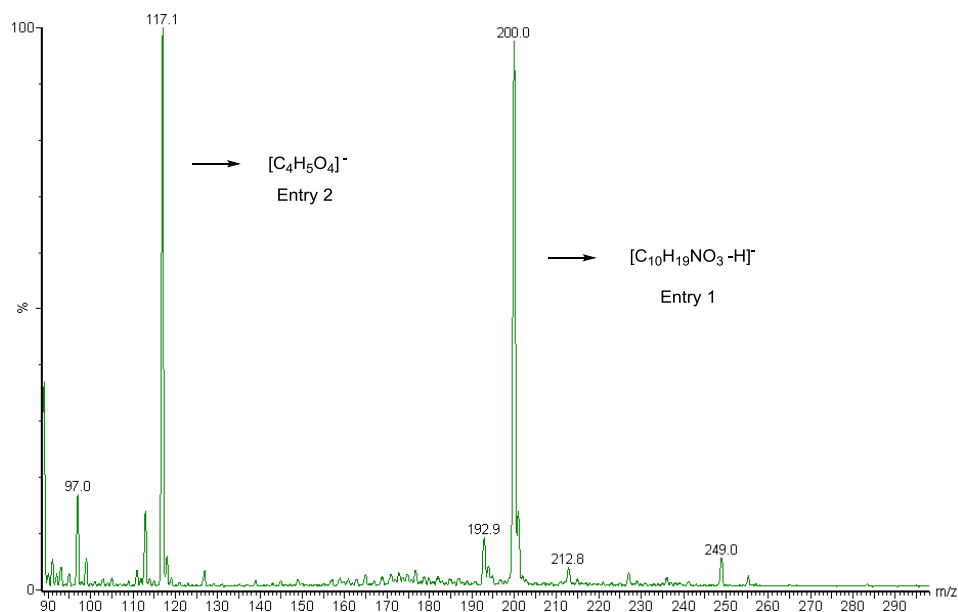


Figure 3-11. Hydrolysed SucHex mass spectrometry spectrum. Negative scan.

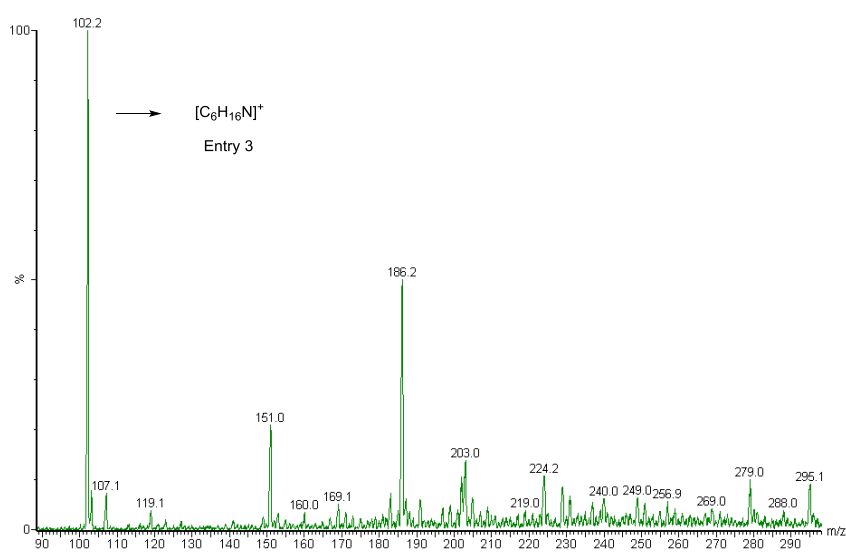


Figure 3-12. Hydrolysed SucHex mass spectrometry spectrum. Positive scan.

It can be seen that mass analysis of SucHex shows compounds in entries: 1, corresponding with the starting compound; 2, with the acid; 3, with the amine. But compound in entry 4, which is the intermediate, is not found.

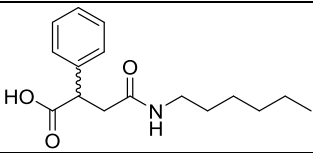
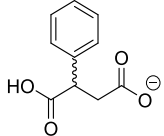
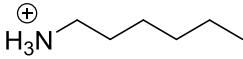
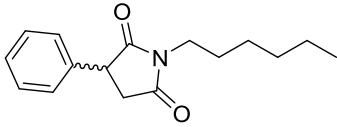
Entry	Structure	Chemical Formula	Molecular Weight
1		$C_{16}H_{23}NO_3$	277,36
2		$C_{10}H_9O_4^-$	193,18
3		$C_6H_{16}N^+$	102,20
4		$C_{16}H_{21}NO_2$	259,34

Table 3-2. Masses of the possible products of PhSucHex hydrolysis.

It can be seen that mass spectrometry analysis of PhSucHex shows compounds in entries 1,2,3 but not compound in entry 4, which is the intermediate.

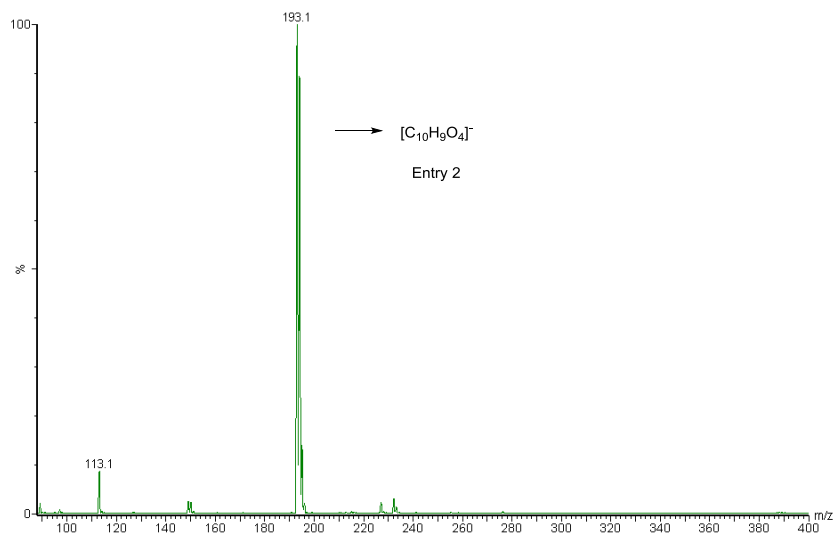


Figure 3-13. Hydrolysed PhSucHex mass spectrometry spectrum. Negative scan.

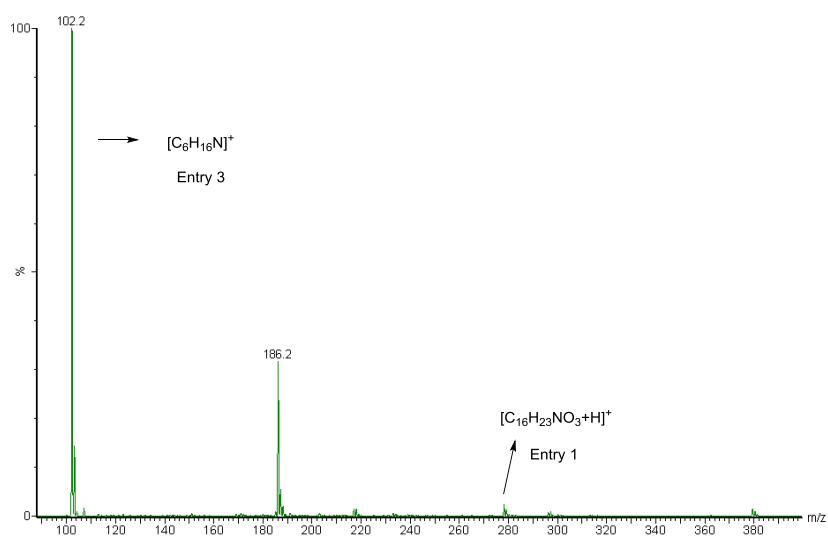


Figure 3-14. Hydrolysed PhSucHex mass spectrometry spectrum. Positive scan.

Conclusions

4 Chapter: Conclusions

- ♣ Compounds SucHex, PhSucHex and GltHex were successfully synthesised and characterized.
- ♣ NMR and mass studies were performed to analyse the hydrolytic reaction and possible formation of succinimide intermediates.
- ♣ The observed hydrolysis is attributed to an intramolecular effect, which favors the nucleophilic attack of the carboxylic acid moiety to the neighbor amide group.
- ♣ Succinimide intermediate affording isomerization of PheSucHex cannot be discarded completely because a slight difference in the ratio of isomers was observed after thermal treatment. However, no succimide type compound was detected by NMR. To clarify this issue more experiments with different temperatures and reaction times should be performed.

Experimental Section

5 Chapter: Experimental Section

5.1 General methods.

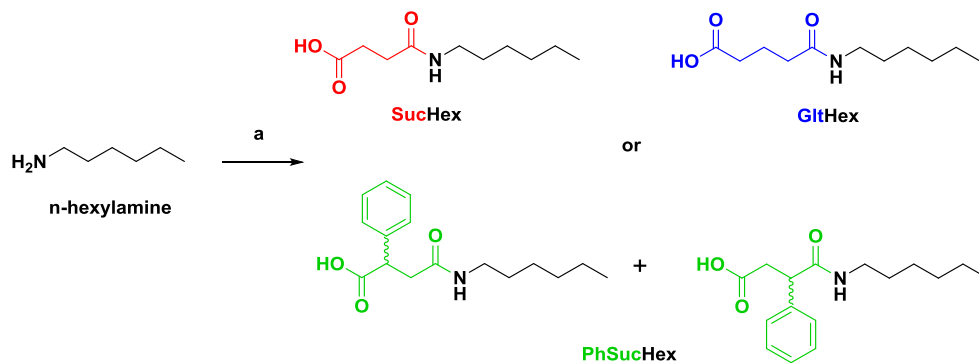
^1H -NMR and ^{13}C -NMR spectra were recorded on Agilent VNMR System spectrometer (500 MHz for ^1H -NMR, 125 MHz ^{13}C -NMR) or Bruker Avance III HD spectrometers (400 MHz and 300 MHz for ^1H -NMR, 101 MHz and 75 MHz for ^{13}C -NMR) in the indicated solvent at 30 °C. Signals of the deuterated solvent (DMSO- d_6 in all the cases unless otherwise indicated) were taken as the reference. ^1H -NMR chemical shifts (δH) and ^{13}C -NMR chemical shifts (δC) are quoted in parts per million (ppm) downfield from trimethylsilane (TMS) and coupling constants (J) are quoted in Hertz (Hz). Abbreviations for NMR data are s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet) and m (multiplet).

^1H and ^{13}C signals were assigned with the aid of 2D methods (COSY, HSQC and HMBC). Reactions which required an inert atmosphere were carried out under N_2 . Commercially available reagents and HPLC grade solvents were used as received. All compound synthesised were dried in a vacuum oven at 60 °C to constant weight (except for the free amines which are dried under vacuum pump at room temperature).

Exact mass spectra were run by the electro-spray mode (ESMS). Masses spectra were recorded at Mass Spectrometry Triple Quadrupole Q-TOF Premier (Waters) with simultaneous Electrospray and APCI Probe.

Mass spectra studies were obtained using a Triple Quadrupole Mass Spectrometer (QuattroLC, Micromass). The cone voltage was set at 20 V unless otherwise stated using Methanol as the mobile phase solvent. Sample solutions have been infused via syringe pump directly connected to the ESI source at a flow rate of 12 $\mu\text{L}/\text{min}$ and a capillary voltage of 3.5 kV was used in the positive and negative scan modes.

5.1.1 Synthesis of SucHex, GltHex and PhSucHex.



Scheme 5-1. Reagents and conditions: a) Succinic, Glutaric or Phenylsuccinic anhydride, Na₂CO₃, THF, 16 h; 90, 95, 73%.

5.1.2 General procedure.

A solution of commercially available *n*-hexylamine (10 mmol) in THF (100 mL) was treated at 0 °C under air with solid Na₂CO₃ (38 mmol, 3.8 eq.). The mixture was stirred for 15 minutes at 0 °C, after it was added with a dropping funnel to a solution of commercial available correspondent anhydride (20 mmol, 2.0 eq.) in THF (25 mL). The mixture was further stirred vigorously for 16 h at room temperature. After this time, the solution was concentrated under reduced pressure and the crude residue was dissolved in water (50 mL); then concentrated hydrochloric acid (37%) was added dropwise at 0 °C until pH = 3. The aqueous mixture was extracted three times with chloroform. The combined organic layers were then dried over anhydrous Na₂SO₄, filtered and concentrated to dryness under reduced pressure. The solid obtained was washed with water and dissolved again with chloroform. Finally, removal of all volatiles under reduced pressure to obtain the pure compound.

- Compound *4-(hexylamino)-4-oxobutanoic acid (SucHex)*: Compound obtained using succinic anhydride. A yellow solid was obtained (yield 95%).

^1H NMR (300 MHz, DMSO- d_6): δ 12.03 (s, 1H), 7.77 (t, J = 5.3 Hz, 1H), 3.00 (dd, J = 12.6, 5.4 Hz, 2H), 2.45 – 2.23 (m, 4H), 1.45 – 1.31 (m, 2H), 1.31 – 1.15 (m, 8H), 0.85 (t, J = 6.7 Hz, 3H).

^{13}C NMR (101 MHz, DMSO- d_6): δ 173.8, 170.7 (C=O), 38.5, 31.0, 30.0, 29.2, 29.1, 26.0, 22.0 (x 2) (CH_2), 13.9 (CH_3).

HR ESMS: m/z calcd. for $\text{C}_{11}\text{H}_{21}\text{NO}_3$ [$\text{M} + \text{H}$] $^+$ = 202.1443; found = 202.1447

- Compound *5-(hexylamino)-5-oxopentanoic acid (GltHex)* Compound was obtained following the same procedure as above except that glutaric anhydride was used. A white solid was obtained (yield 90%).

^1H NMR (300 MHz, DMSO- d_6): δ 12.00 (s, 1H), 7.74 (t, J = 5.2 Hz, 1H), 3.01 (dd, J = 12.6, 6.8, 5.8 Hz, 2H), 2.18 (t, J = 7.4 Hz, 2H), 2.07 (t, J = 7.4 Hz, 2H), 1.69 (p, J = 7.5 Hz, 2H), 1.49 – 1.30 (m, 2H), 1.30 – 1.15 (m, 6H), 0.85 (t, J = 6.7 Hz, 3H).

^{13}C NMR (101 MHz, DMSO- d_6): δ 174.1, 171.3 (C=O), 38.4, 34.5, 33.0, 31.0, 29.1, 26.0, 22.0, 20.7 (CH_2), 13.9 (CH_3).

HR ESMS: m/z calcd. for $\text{C}_{11}\text{H}_{21}\text{NO}_3$ [$\text{M} + \text{H}$] $^+$ = 216.1600; found = 216.1598.

- Compound *4-(hexylamino)-4-oxo-2(3)-phenylbutanoic acid (PhSucHex)*: Compound was obtained following the same procedure as above except that phenylsuccinic anhydride was used. The residue was purified by means of column chromatography on silica flash (AcOEt) obtaining a white solid (yield 73%).

Major ^1H NMR isomer signals (500 MHz, CDCl_3): δ 7.37 – 7.14 (m, 5H), 5.59 (s, 1H), 4.20 – 4.17 (m, 1H), 3.18 – 3.01 (m, 2H), 2.93 (dd, J = 15.0, 9.3 Hz, 1H), 2.51 (dd, J = 15.0, 5.3 Hz, 1H), 1.41 – 1.28 (m, 2H), 1.26 – 1.09 (m, 6H), 0.88 – 0.74 (m, 3H).

Minor ^1H NMR isomer signals (500 MHz, CDCl_3): δ 7.37 – 7.14 (m, 5H), 5.46 (s, 1H), 3.85 (dd, J = 9.1, 5.0 Hz, 1H), 3.23 (dt, J = 28.3, 14.2 Hz, 1H), 3.18 – 3.01 (m, 2H), 2.65 (dd, J = 16.6, 5.0 Hz, 1H), 1.41 – 1.28 (m, 2H), 1.26 – 1.09 (m, 6H), 0.88 – 0.74 (m, 3H).

HR ESMS: m/z calcd. for $\text{C}_{16}\text{H}_{23}\text{NO}_3$ [$\text{M} + \text{H}$] $^+$ = 278.1756; found = 278.1753.

5.2 General procedure of hydrolytic reactions.

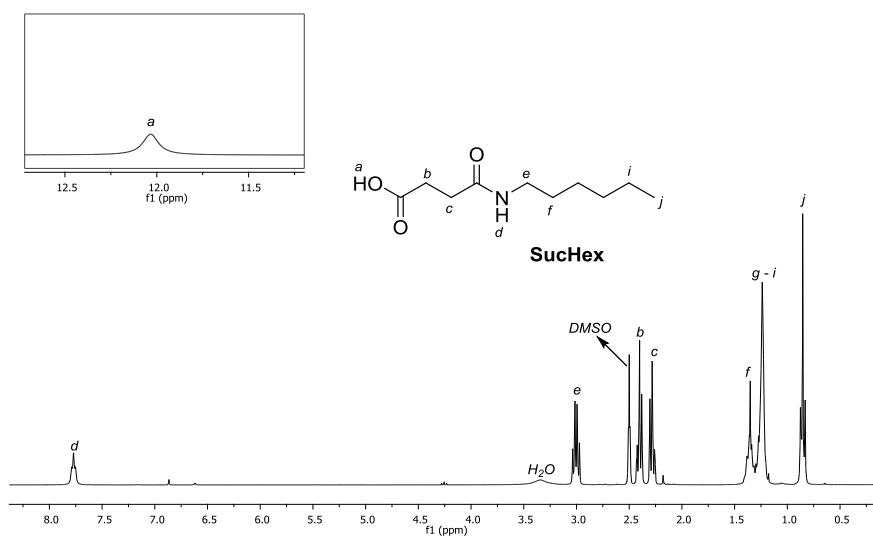
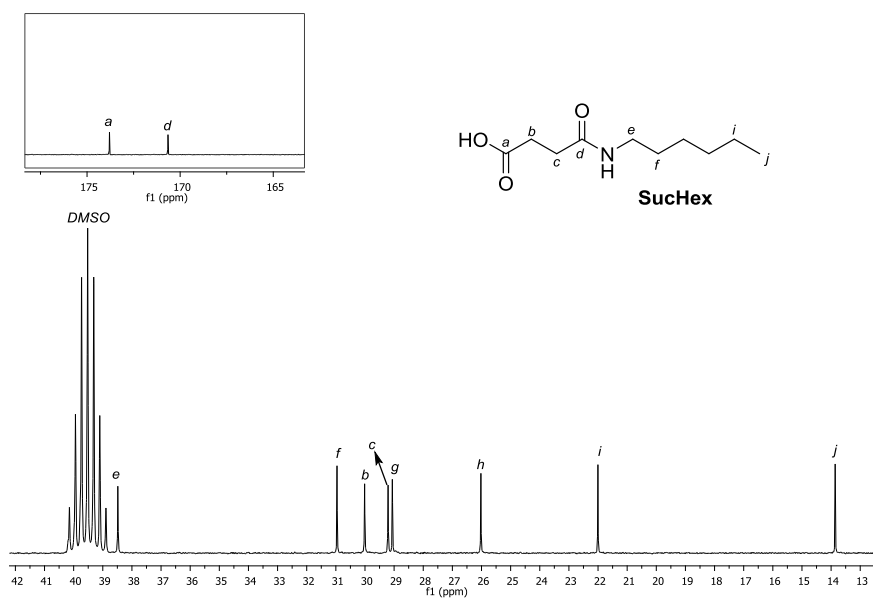
In a screw vial, 10mg of each succinic or glutaric derivative were suspended in 2mL D_2O by sonication for 10 minutes, obtaining a concentration of 5 mg/mL. The

solution was acidic in all cases. Vials were placed into a thermo-regulated bath at 80 °C overnight.

After that, solvent was evaporated in a vacuum oven at 60 °C, and DMSO or CDCl_3 was used to record more NMR spectra in case signals overlapped with deuterated water.

NMR Spectra

6 Chapter: NMR spectra

Figure 6-1. ^1H NMR spectrum of SucHex in DMSO.Figure 6-2. ^{13}C NMR spectrum of SucHex in DMSO.

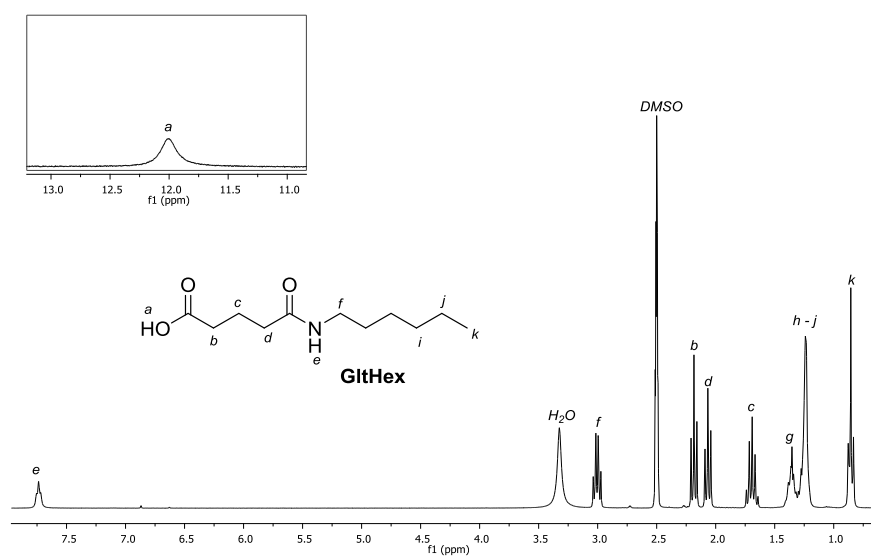


Figure 6-3. ^1H NMR spectrum of GltHex in DMSO.

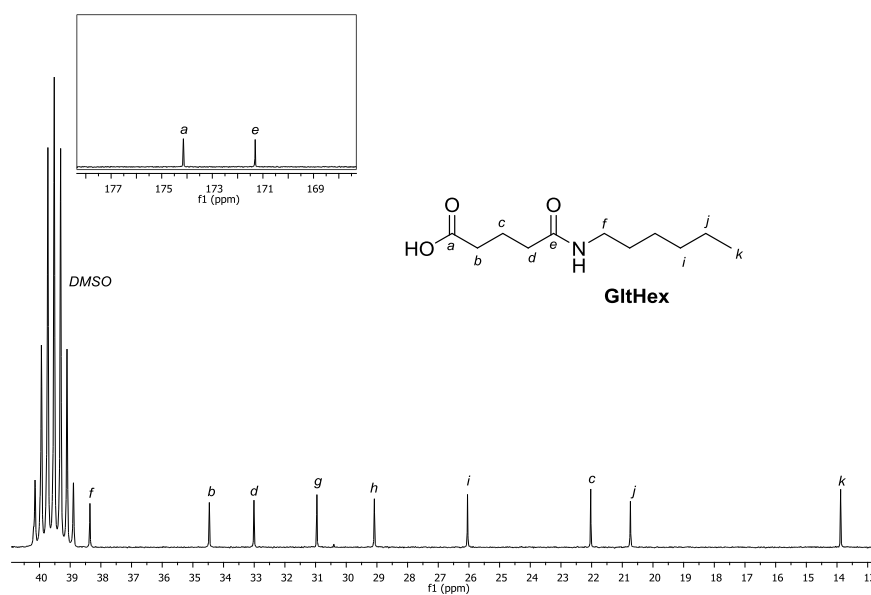


Figure 6-4. ^{13}C NMR spectrum of carboxylic acid GltHex in DMSO.

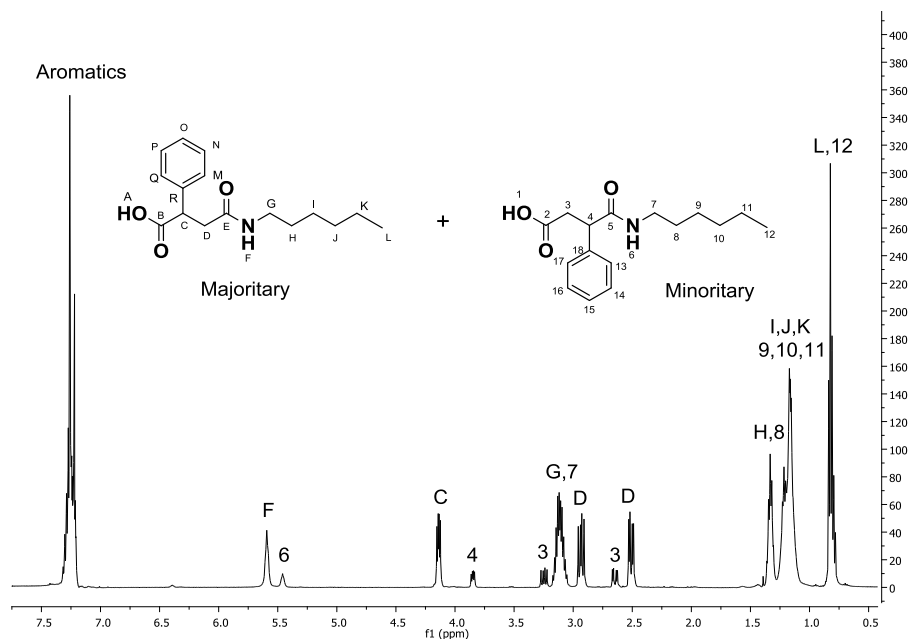


Figure 6-5. ^1H NMR spectrum of PhSucHex in CDCl_3 .

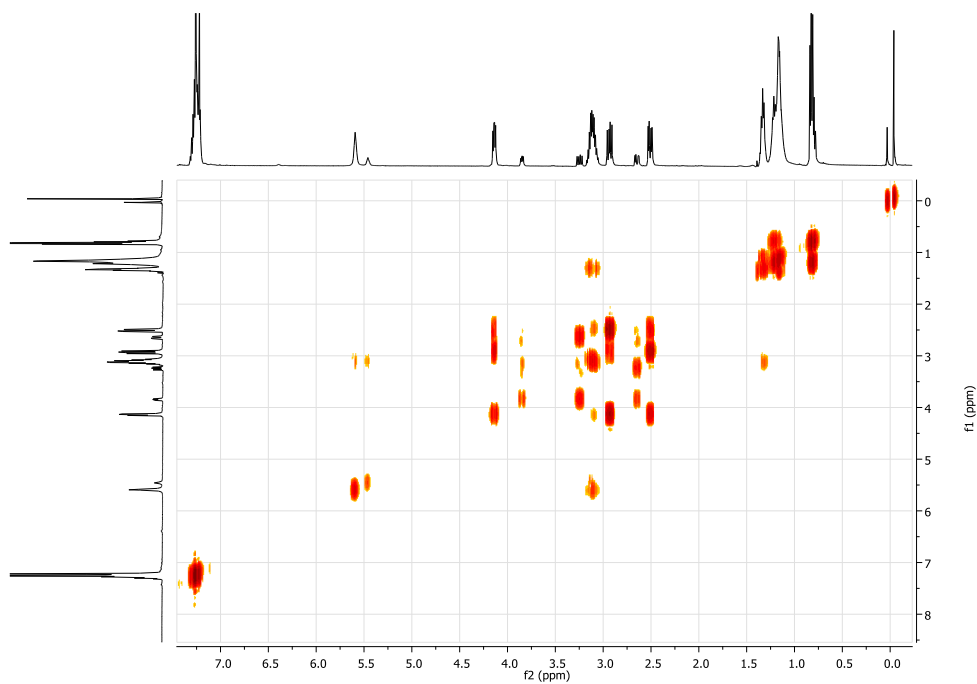


Figure 6-6. COSY of PhSucHex, CDCl_3 .

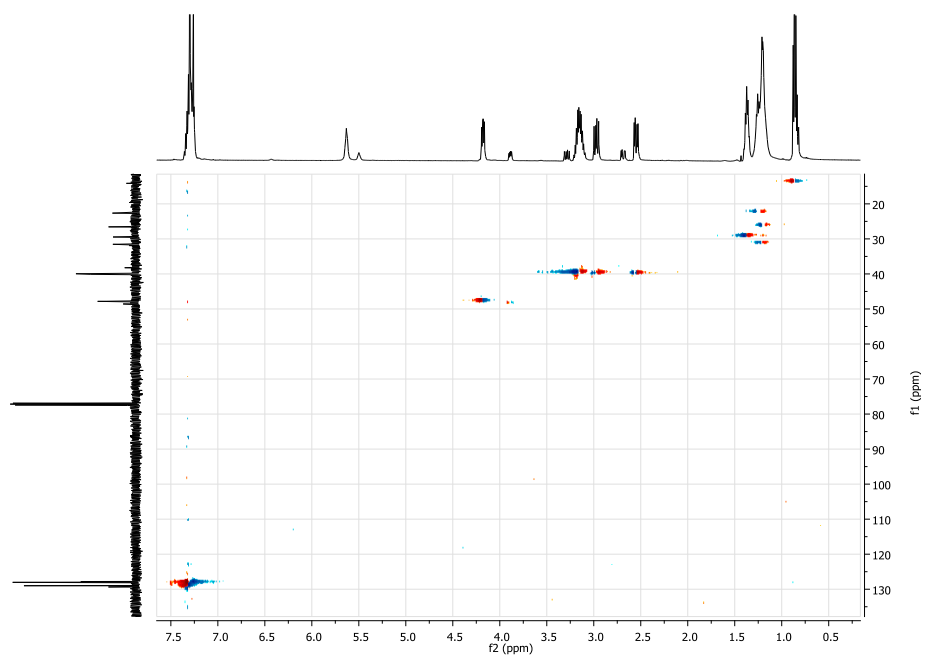


Figure 6-7. HSQC of PhSucHex, CDCl_3

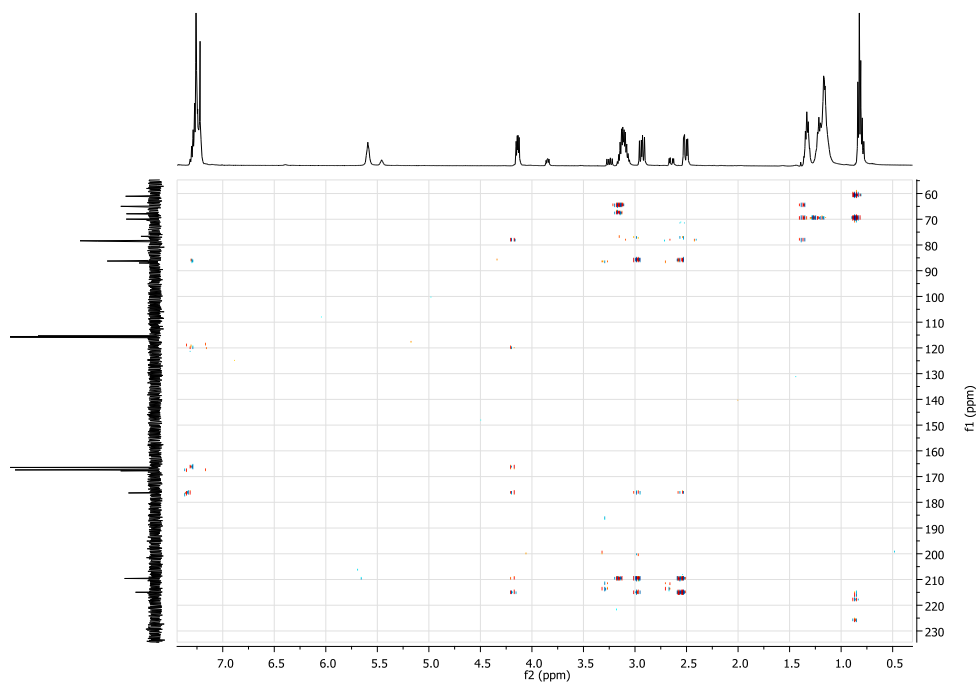


Figure 6-8. HMBC PhSucHex, CDCl_3

Mass spectra

7 Chapter: Mass spectra

7.1 Molecular Mass.

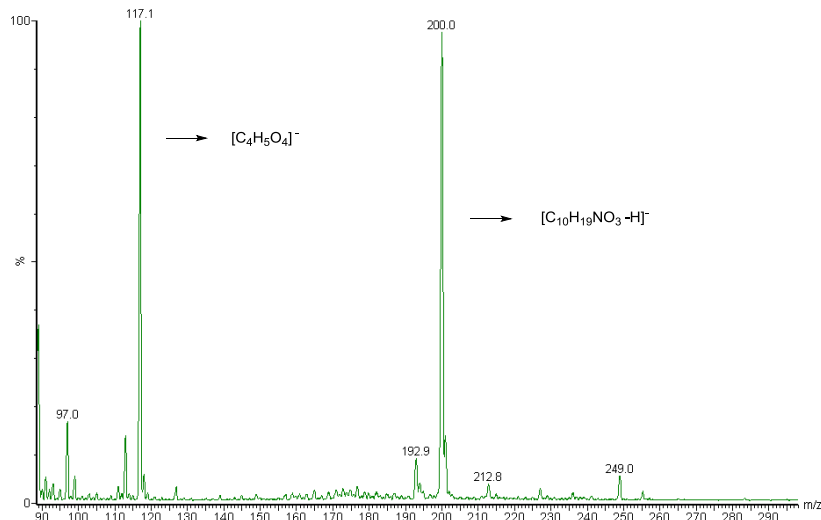


Figure 7-1. Hydrolysed SucHex mass spectrometry spectrum. Negative scan.

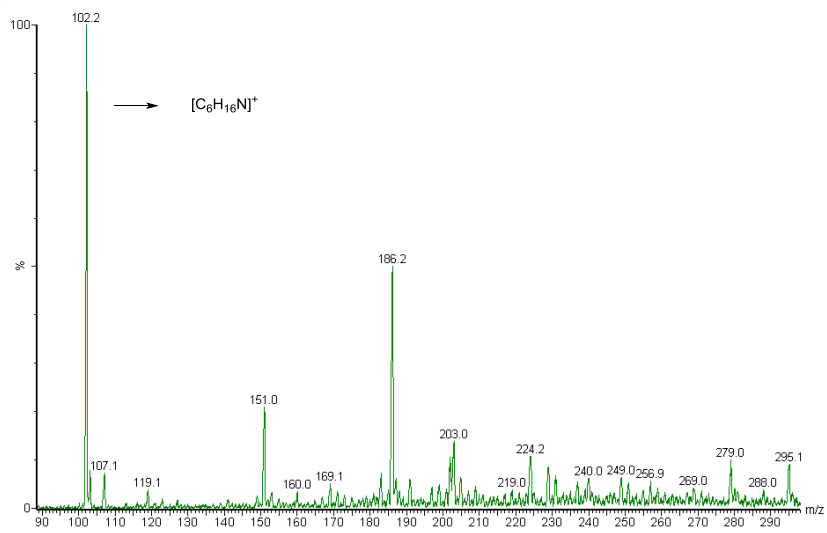


Figure 7-2. Hydrolysed SucHex mass spectrometry spectrum. Positive scan.

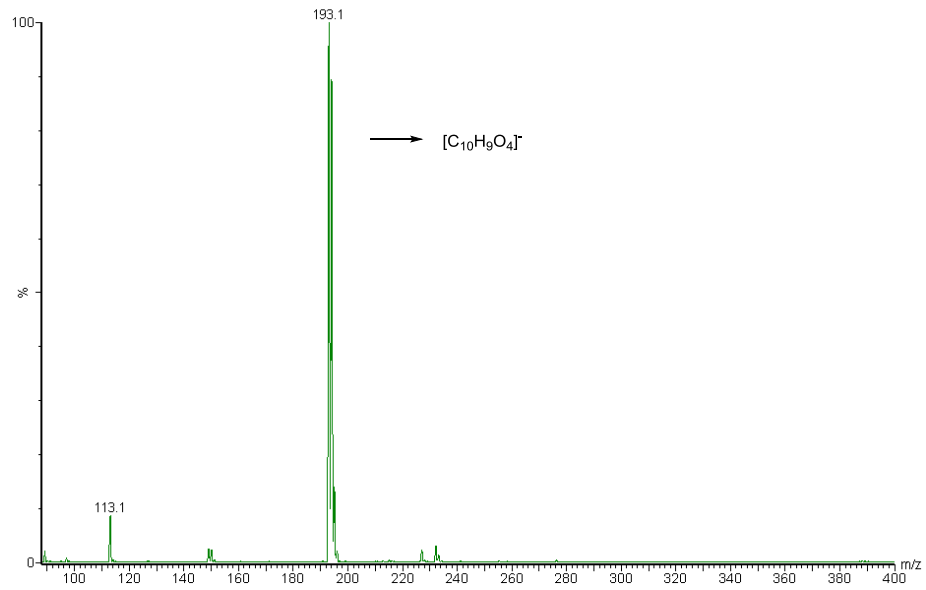


Figure 7-3. Hydrolysed PhSucHex mass spectrometry spectrum. Negative scan.

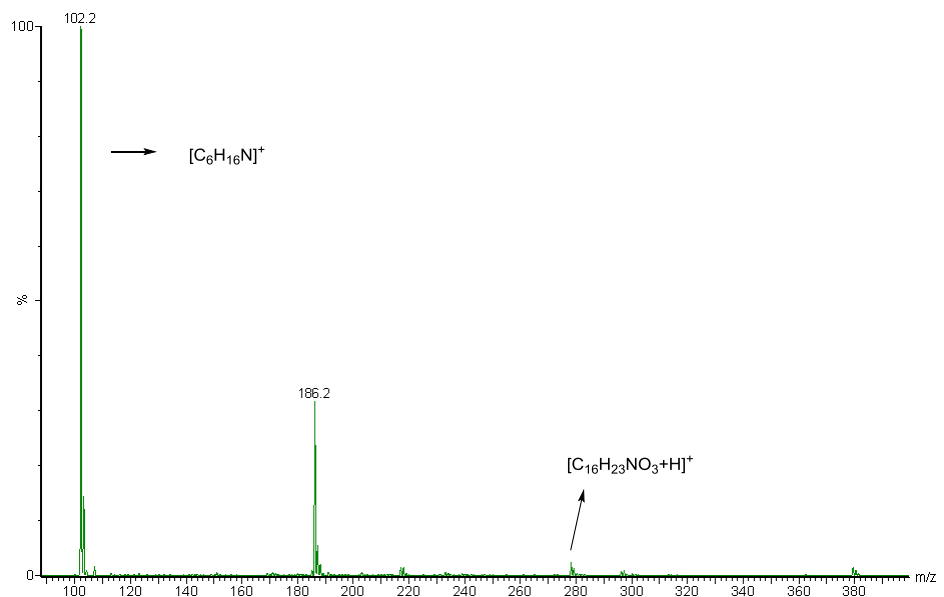
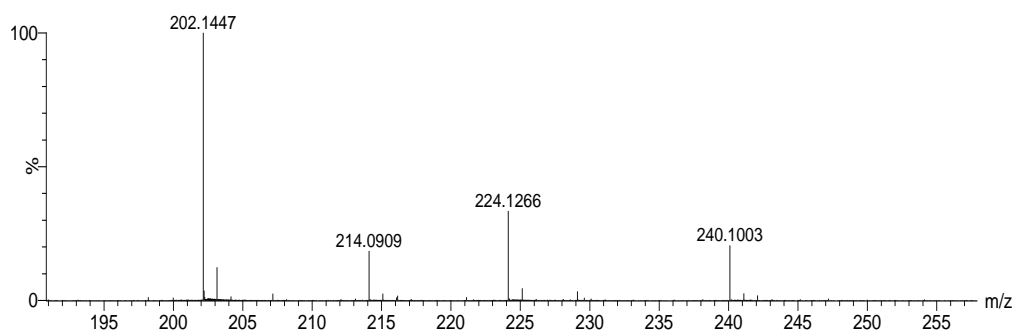
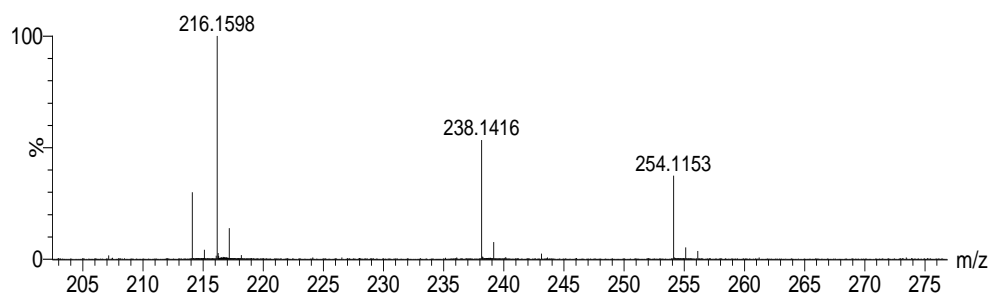
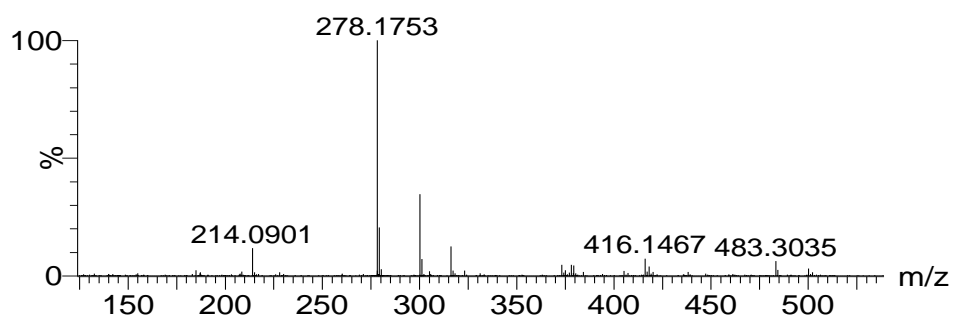


Figure 7-4. Hydrolysed PhSucHex mass spectrometry spectrum. Positive scan.

7.2 Exact Mass.**Figure 7-5.** Exact mass spectrometry spectrum SucHex.**Figure 7-6.** Exact mass spectrometry spectrum GlitHex.**Figure 7-7.** Exact mass spectrometry spectrum PhSucHex.

