Universidade de Lisboa

Faculdade de Farmácia

Design of bioorthogonal tools for bioconjugation and drug delivery

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Mestrado Integrado em Ciências Farmacêuticas

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Abstract

Bioconjugation applied to protein modification is a promising technology to address several existing biotherapeutic gaps. With the development of two generations of Antibody-Drug conjugates (ADCs), it became evident that bioselectivity and biostability is still far from intended, giving rise to a tremendous challenge.

Maleimides, commonly used to establish covalent bonds between the residue's thiol and the drug by Michael addition , are part of this problem, since it has been shown that thiosuccinimide linkage is less robust than previously thought. Thus, in a biological environment, thiosuccinimides can undergo two competitive reactions: breaking the linkage of thiosuccinimide through the retro-Michael elimination, which results in a premature release of the drug and exchange with biological thiols, and hydrolysis of the ring, the product of which has been demonstrate resistance to the previous reaction. To circumvent the problem of stability, the scientific community focused on the development of designs aimed at making the thiosuccinimide bond more robust. An example of this development is the introduction of electrowithdrawing groups as N-α substituents that aims to accelerate the hydrolysis of thiosuccinimide.

The purpose of this fieldwork is to synthesize and carry out comparative studies between 3 maleimides (ethyl as N-α substituent; Oxygen as N-α substituent and Nitrogen as N-α substituent), in order to understand the influence of the N-α substituent on susceptibility to hydrolysis, Michael addition and Michael elimination, and to define if these maleimides would be suitable for bioconjugation.

Resumo

A bioconjugação aplicada a modificação de proteínas é uma tecnologia promissora para resolver várias lacunas bioterapeuticas existentes. Com o desenvolvimento de duas gerações de Antibody-Drug conjugates (ADCs) emergiram problemas com a obtenção de bioseletividade e bioestabilidade, dando origem a um desafio tremendo.

As maleimidas, comumente usadas para estabelecer ligações covalentes entre o tiol do resíduo e o fármaco (reação de Michael), fazem parte deste problema, já que se veio a demonstrar que a ligação da tiosuccinimida é menos robusta do que outrora se pensava. Desta forma, em meio biologico as tiosuccinimidas podem ser sujeitas a duas reações competitivas: quebra da ligação atravez da eleminação de retro-Michael, que resulta numa libertação prematura do fármaco e troca com tióis biologicos, e hidrolise do anel, cujo produto se veio a demonstrar resistente a reação anterior. Ao fim de contornar o problema da estabilidade, a comunidade cientifica centrou-se no desenvolvimento de metodologias destinadas a tornar a ligação tiosuccinimida mais robusta, sendo um exemplo deste desenvolvimento a introdução na meleimida de grupos eletroatratores como substituintes N-α que visam a acelarar a hidrolise da tiosuccinimida.

O objetivo deste trabalho de campo é síntese e realização de estudos comparativos entre 3 (etilo com substituinte N-α; Oxigénio com substituinte e Azoto com substituinte) maleimidas, ao fim de compreender a influencia do substituinte N-α na suscetibilidade a tanto a ocorrencia de adição e eleminação de Michael como da hidrólise, e definir se estas maleimidas seriam adequandas para bioncojugação.

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Abbreviation

ACN – Acetonitrile

ADC – Antibody-Drug conjugate

Boc - Tert-Butyloxycarbonyl protecting group

Boc_2O - Di-*tert*-butyl dicarbonate

DCM - Dicloromethane

DMF - Dimethylformamide

Et_2O - Diethyl Ether

KOtBu - Potassium tert-butoxide

h - Hours

m - Multiplet

M - Molarity

MS – Molecular sieves

NMR - Nuclear magnetic resonance

PBS - Phosphate-Buffered Saline

Rf – Retention factor

s - Singlet

t - Triplet

TEA - Triethylamine

THF – Tetrahydrofuran

TFA - Trifluoroacetic acid

TLC - Thin layer chromatography

δ - Chemical shift

UV-Vis- Ultraviolet-Visible

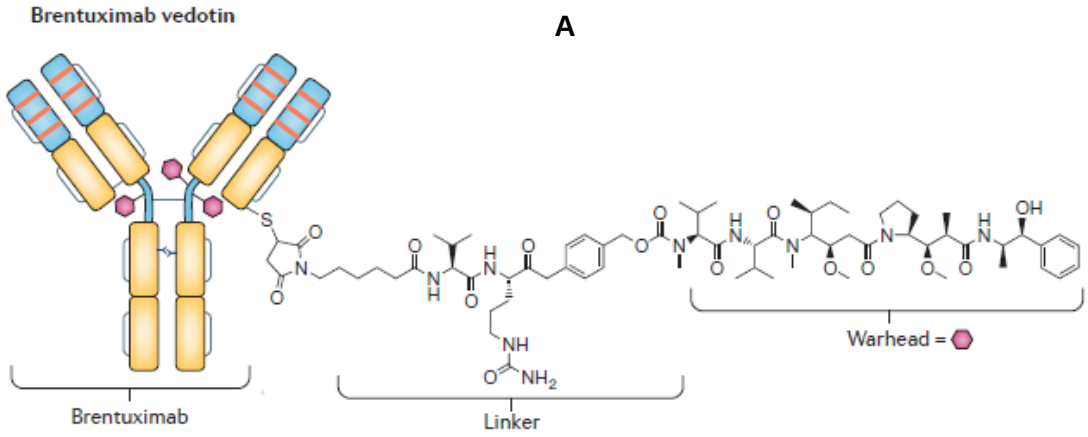
ESI-MS - Electrospray ionization mass spectrometry

1. Introduction

Peptide bioconjugation chemistry

Bioconjugation is a set of techniques that allow the creation of site-specific covalent link between a biomolecule and an exogenous moiety, endowing it with desirable properties (1). It has come a long way since its inception in 1962 as a selective reaction between the p-arsonylbenzenediazonium ion and the antigen-combining site of a rabbit anti-p-azobenzenearsonate antibody by Wofsey and co-workers In 2003 the term bioorthogonal chemistry was coined by Carolyn R. Bertozzi, opening a new sea of endless possibilities regarding *in vivo* reactions.(2,3) Bioorthogonal reactions must obey several precepts like high selectivity, to avoid side reactions; biological inertness, as the resulting reactive products should not disrupt the native functionality of the organism under study, and fast and efficient kinetics. These reactions should occur within minutes with full conversion and should be biocompatible, nontoxic and functioning at physiological pH, taking in account the aqueous environment and temperature.(3,4)

Protein modification has been important in the biotechnology industry -examples of popular applications of this method include PEGylation of therapeutic proteins to increase serum half-life (5) and the conjugation of cytotoxins to cancer-targeting elements, resulting in ADCs.(4) ADCs are recombinant monoclonal antibodies that are covalently linked to a cytotoxic drug via a linker, combining the advantages of therapeutic immunoselectivity and cytotoxicity. A key characteristic for linker optimization on ADC development is its stability in blood circulation in order to limit off-target toxicity, but allow the drug to be released when it reaches its target. (6)



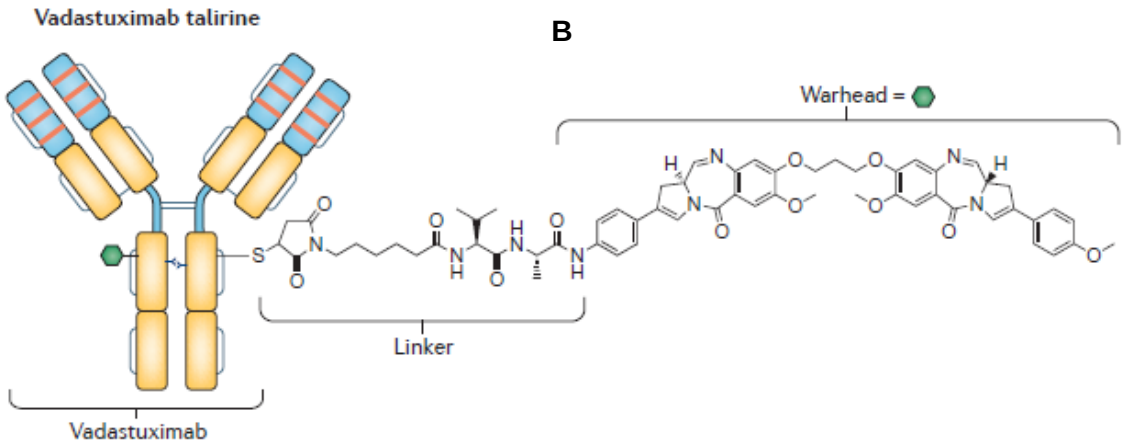


Figure 1: A - The structure of Bretuximab Vedotin; B – The structure of Vadastuximab talirine; Adapted from (6)

The preparation of stable, well-defined and homogeneous protein constructs is a major challenge. Classical methods of bioconjugation regularly presentlow control over the site of modification, which may result in the loss of biological function of the target biomolecule. Thus, the new methods that have emerged aim to circumvent this problem, since they are highly specific to the site and cause minimal disturbance to the active form of the biomolecule (3). In the case of ADCs, these have to be designed taking into account the balance that has to exist between good stability in circulation for up to several days and efficient cleavage and drug release when it reaches the target, since premature release of cytotoxic drugs can lead to systemic toxicity and lowering of the therapeutic window. (6)

One of the strategies to ensure bioconjugate stability and native protein functionality is the use of genetic engineering tools to introduce non-canonical amino acids into the protein sequence, which can posteriorly undergo selective chemical modification via biorthogonal reactions.(7) Another strategy for bioconjugate production consists in direct chemical modification of native proteins which offers practicality as it exploits the innate reactivity profiles of different side chain functionalities. Initially, protein modification was performed by modifying lysine residues (e.g with N-hidroxysuccinamide esters). However, with the development of chemical tools it is now possible to modify natural residues with high specificity (e.g, lysine via α/β - unsaturated sulfonamides, tyrosine via ene-type reaction, etc.). Notwithstanding, one of the most chosen residue for site-selective modifications is cysteine due to its relatively low abundance and the high nucleophilicity of the sulfhydryl group (8).

To ensure that the bioconjugation reaction is complete an excess of reagents is used. However, this may lead to over-modification and/or erosion of chemospecificity, as in the case of maleimides, which, in addition to reacting with cysteine, may also react with lysines. Nevertheless, maleimides remain the most popular choice for site-selective cysteine modifications used as a linker in ADCs (9). In general, using maleimides as linkers brings many

advantages, like fast kinetics, quantitative conversion and high specificity (10). Examples of commercially available ADCs whose construction relies on maleimide chemistry include Trastuzumab emtansine, Brentuximab vedotin (Fig.1- **A**), and Vadastuximab talirine (Fig.1 - **B**). (6).

Maleimide chemistry for protein modification

Maleimides **a** display unique reactivity in thio-Michael additions as a result of synergistic LUMO lowering effects, which render them more reactive than other typical Michael acceptors.(11)The ring strain imposed by the alkene moiety further enhances the electrophilic nature of the conjugated imide functionality,(12) whereas the solvent, a basic environment, and the type of thiol are known to play an important role in both reaction kinetics and selectivity in the thiol-maleimide chemistry.(13) As this class of bioorthogonal reagents become more popular, the instability of the resulting thiosuccimide adducts **b** (Fig. 2) has come into light. In biological *milieu*, thiol release and subsequent exchange with competitive biological thiols is possible via the intermediacy of the parent maleimide originated by retro-Michael addition (Fig. 2). If cleavage of the thiosuccinimide adduct is premature, this translates into increased off-target toxicity and decreased drug efficacy. On the other hand, hydrolytic degradation of maleimides **a** (Fig. 2) and thiosuccimide adducts **b** (Fig. 2) impacts the success of bioconjugations in different ways. Whilst maleimide hydrolysis yields maleic amides **c** (Fig. 2)**,** which do not undergo thio-Michael additions under biological conditions,(14) thiosuccinimide hydrolysis leads to stable succinamic acid thioether bioconjugates **d I, II** (Fig. 2).

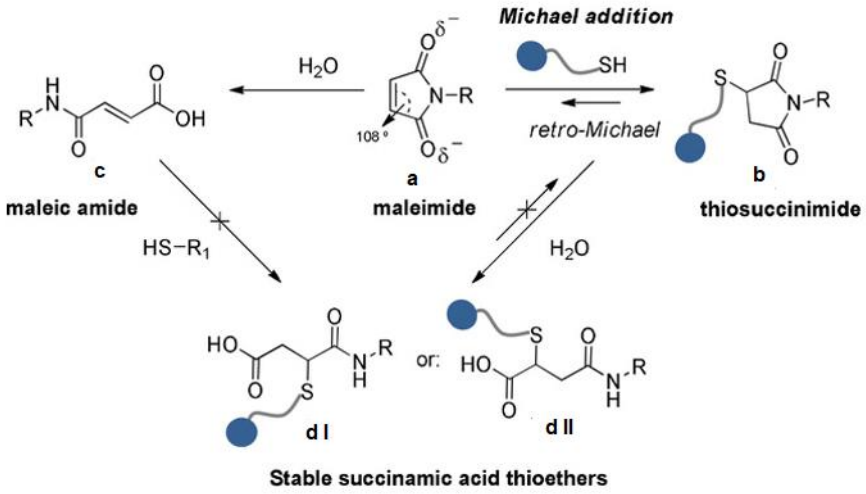


Figure 2 General Michael addition and hydrolytic pathways of maleimides and thiosuccinimides; Adapted from (8)

Studies have shown that hydrolyzed N-alkyl thiosuccinimide ADCs increase both efficacy and safety comparing to their non-hydrolyzed analogues, raising the low therapeutic window nowadays inherent to ADC therapy (14). These factors boosted the development of new maleimide reagents with higher hydrolysis rate for the thiosuccinimide, that is, reagents that aimed to reduce the occurrence of Retro-Michael elimination.

One of the result of this kind of development are ‘‘self-hydrolyzing maleimide’’ reagents (15,16). An example of this strategy, which aims to accelerate the hydrolysis of thiosuccinimide, relies on the presence of an adjacent basic amino group to promote intramolecular base catalyzed hydrolysis (16). (Fig. 3)

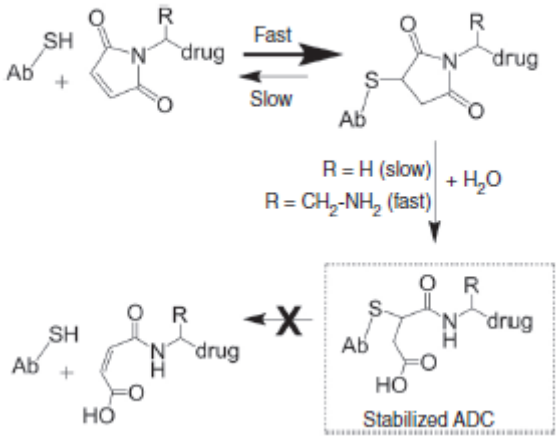


Figure 3:Influence of adjacent basic amino group on thiosuccinimide hydrolysis; Adapted from (16)

Another strategy focuses on preventing the occurrence of retro-Michael through altering the structure of the maleimides in order to decrease their stabilization by resonance (Fig. 3). This way, the exocyclic olefinic maleimides arose, whose resistance to thiol exchange is not directly dependent on the speed of hydrolysis of the ring, but rather on the stabilization of the first thiosuccinimide bond established (17).

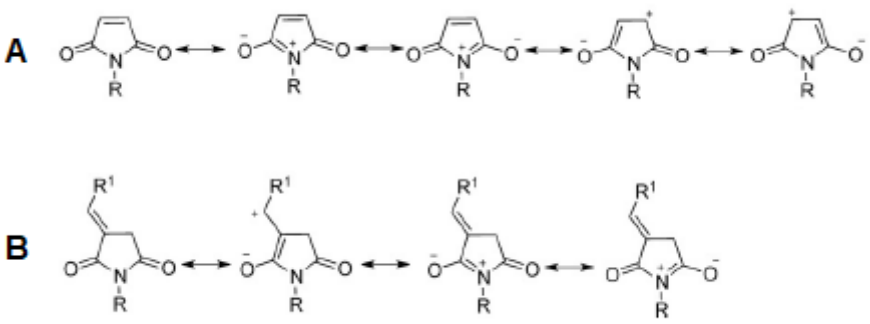


Figure 4: A – resonance stabilization of maleimide; B – resonance stabilization of exocyclic olefinic maleimides; adapted from (17)

Within the most recent studies there is a new proposal to bypass the retro-Michael impact on stabilization of thiosuccinimides, reinforcing the benefit of using N-terminal cysteine as residue for site-selective modifications. After demonstrating that thiosuccinimides undergo transcyclization when they are in the N-terminal position (Fig. 4 A), it was demonstrated that they do not undergo retro-Michael elimination, since the free amine cyclizes with maleimide over time, stabilizing it, unlike the same thiosuccinimides in chain or in C-terminal cysteines. (10)

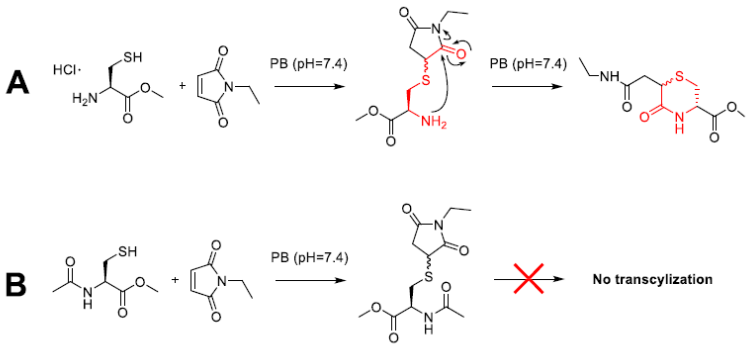
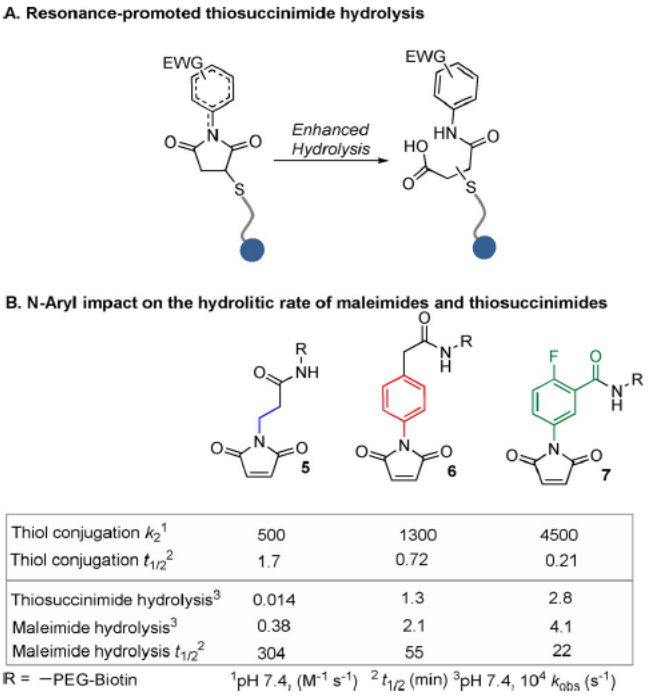


Figure 5: A – Model system for the transcyclization reaction using L-cysteine methyl ester hydrochloride as a Michael Donor and N-ethyl maleimide as Michael acceptor; B – Reaction of N-acetyl protected L-cysteine methyl ester with N-ethyl maleimide served as control; Adapted from (10)

Another important strategy to accelerate the hydrolysis of thiosuccinimide is an N-α-substituent with aromatic systems such as aryl maleimides. The rationale behind this strategy relies on the resonance structures of this family of molecules (Fig. 6 A). Alkyl maleimides have a nitrogen lone pair of electrons that resonate between the nitrogen and carbonyl group, decreasing the reactivity towards hydrolysis. With the addition of phenyl as N-substituent more resonance structures are added to this nitrogen lone pair of electrons, subserving a potential nucleophilic attack by water on the carbonyl group. Furthermore, it was shown that addition of an electron withdrawing group to the phenyl ring contributes to increase the rate of hydrolysis. However, it should be noted that in addition to accelerating the hydrolysis of thiosuccinimide, these substituents also accelerate the hydrolysis of maleimide in solution (Fig. 6 B).(14)



Thiol conjugation k_2[1]	500	1300	4500
Thiol conjugation $t_{1/2}$[2]	1.7	0.72	0.21
Thiosuccinimide hydrolysis[3]	0.014	1.3	2.8
Maleimide hydrolysis[3]	0.38	2.1	4.1
Maleimide hydrolysis $t_{1/2}$[2]	304	55	22

R = −PEG-Biotin [1]pH 7.4, (M^{-1} s^{-1}) [2] $t_{1/2}$ (min) [3]pH 7.4, 10^4 k_{obs} (s^{-1})

Figure 6: Aryl-maleimides as a strategy for more stable thiosuccinimide conjugates; Adapted from (8)

Even though more and more ways are being developed to circumvent the instability problem of thiosuccinimide linkage, unfortunately there are few studies comparing these different methods. Thus, it is difficult to conclude on the relative merit of these approaches. As of now, based on the available data, no clear "best approach" can be identified. All the methods mentioned above have their advantages and disadvantages, and the "best fit" approach, at least for now, must be identified on a case-by-case basis (15).

Thus, it is imperative to carry out comparison studies to better understanding of the advantages and drawbacks between existing strategies and generate new stabilization rationales which can tackle still occurring problems, while introducing a novel functionalization dynamics.

2. Objectives

The objective of this work is the synthesis of several maleimides with different N-α-substituent in order to study the impact that these substituents have on reactivity and stability of the maleimides. Thus, we intend to study the influence of the N-α-substituent on the susceptibility to occur Michael addition and retro-Michael, and also its influence on the hydrolysis of the corresponding thiosuccinimide, in order to inquire whether this maleimides would be suitable for bioconjugation

For our study, we envisioned the synthesis of three analogous maleimides with different N-α-substituents, namely **1**, **2** and **14**.

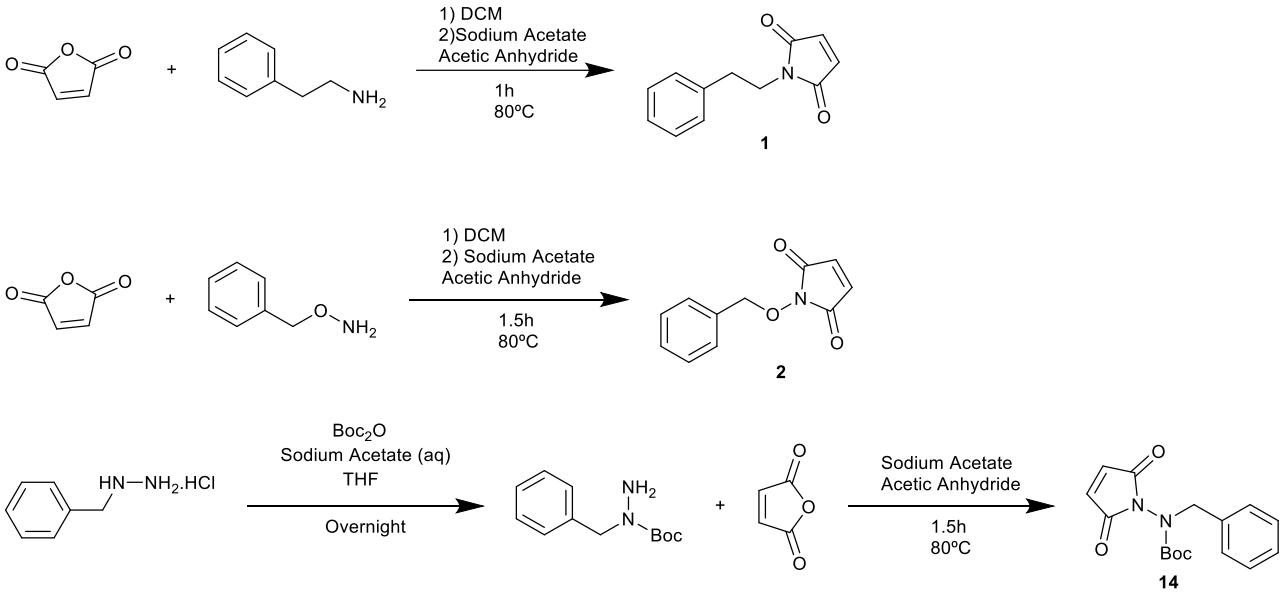


Figure 7: Synthesis of objective molecules

Thus, we intend to make comparative studies between the molecules with withdrawing N-α-substituent, namely **2** and **14**, and N-Phenyl, which is well described in the literature, but also with **1**, as the ethyl group don't have any electron withdrawing proprieties.

3. Materials and Methods

NMR spectra were recorded on a Bruker Fourier 300 NMR spectrometer (^{1}H 300 MHz; ^{13}C 100, 61 MHz). ^{1}H and ^{13}C chemical shifts (δ) are expressed in ppm (parts per million) and are relative to the corresponding resonance of non-deuterated solvent. Sartorius analytic scale was used to weight the materials and Buchii RE11Rotavapor with thermostatic Buchii water bath was used for vacuum evaporation.

Solvents for flash chromatography (hexanes, EtOAc, DCM) were of technical grade and distilled prior to use. THF was purified by distillation from sodium/benzophenone prior to use. DCM was purified by distillation from CaH_2, deuterated solvents for NMR were purchased from Cambridge isotope laboratories.

Reactions and fractions from flash chromatography were monitored by thin layer chromatography using precoated glass plates (Merck, silica 60 F254) and visualized by staining with basic $KMnO_4$ solution or ninhydrin solution.

UV spectra were traced in Thermo Scientific Evolution 201 UV-visible spectrophotometer.

Low resolution ESI mass spectra were carried on an ion trap mass analyser (Thermo Scientific LCQ Fleet Ion Trap LC/MS) equipped with an electrospray interface

4. Experimental procedures

4.1. Chemical Synthesis

4.1.1. Synthesis of 1-phenethyl-1H-pyrrole-2,5-dione (1)

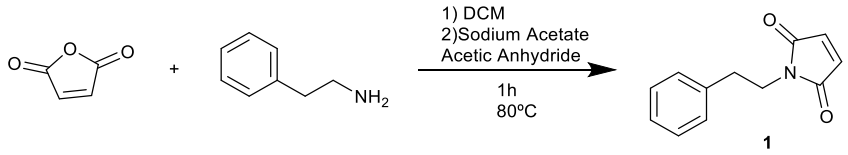


Figure 8: Synthesis of 1-phenethyl-1H-pyrrole-2,5-dione

In a 100 mL round bottom flask, maleic anhydride (1.0 g, 10.2 mmol, 1 equiv.) was dissolved in 40 mL of DCM, followed by slow addition of phenethylamine (1.28 ml, 10.2 mmol, 1 equiv.). After 10 minutes, the solvent was evaporated at low pressure and the solid was resuspended in 20 ml of acetic anhydride. Sodium acetate (1.7 g, 20.4 mmol, 2 equiv.) was added slowly to the reaction, and left stirring for 1 hour at 80˚C, followed by TLC.

After completion, the reaction was quenched with water, basified with $NaHCO_3$ saturated solution, submitted to liquid-liquid extraction with ethyl acetate (3x15 mL) and evaporated in low pressure. Afterwards the compound was purified by column chromatography using a mixture of Hexane/Ethyl acetate 2:1 to afford 223 mg as a white solid (Yield=21.9%).

^{1}H NMR (300 MHz, Chloroform-d): δ 7.37 – 7.12 (m, 3H), 6.65 (s, 2H), 3.84 – 3.65 (m, 2H), 2.90 (dd, J = 8.5, 6.7 Hz, 2H).

^{13}C NMR (75 MHz, Chloroform-*d*) δ 170.6, 134.0, 128.8, 126.7, 77.4, 77.0, 76.6, 39.1, 34.5.

4.1.2. Synthesis of 1-(benzyloxy)-1H-pyrrole-2,5-dione (2)

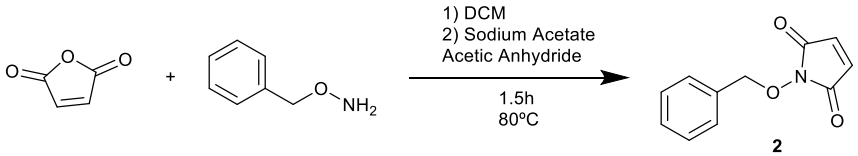


Figure 9: Synthesis of 1-(benzyloxy)-1H-pyrrole-2,5-dione

In a 25 mL round bottom flask, maleic anhydride (160.0 mg, 1.63 mmol, 1 equiv.) was dissolved in 10 mL of DCM, followed by slow addition of *O*-phenethylhydroxylamine (200.9 mg,

1.63 mmol, 1 equiv.). After 10 minutes, the solvent was evaporated at low pressure and the solid was resuspended in 5 mL acetic anhydride, Sodium acetate (267.7 mg, 3.26 mmol, 2 equiv.) was added slowly to the reaction and left stirring for 1 hour at 80˚C.
After completion, the reaction was quenched with water, basified with $NaHCO_3$ saturated solution, submitted to liquid-liquid extraction with ethyl acetate (3x15 mL) and evaporated in low pressure. Afterwards the compound was purified by column chromatography using a mixture of Hexane/Ethyl acetate 2:1 to afford 223 mg as a white solid (Yield=67%)

^{1}H NMR (300 MHz, Chloroform-*d*): δ 7.48 – 7.33 (m, 5H), 6.42 (d, *J* = 5.6 Hz, 1H), 5.23 (s, 2H), 4.97 (s, 2H).
^{13}C NMR (75 MHz, Chloroform-*d*) δ 129.7, 128.7, 128.5, 77.4, 77.0, 76.6, 30.9, 24.9.

4.1.3. Synthesis of Boc protected Benzylhydrazine (3)

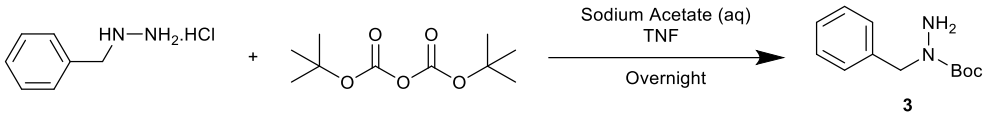


Figure 10: Synthesis of Boc protected Benzylhydrazine

In a 25 mL round bottom flask, Benzylhydrazine monohydrochloride (470 mg, 3 mmol, 1 equiv.) and Boc_2O (720 mg, 3.3 mmol, 1.1 equiv.) were dissolved in 15 mL of THF, sodium bicarbonate (756 mg, 9 mmol, 3 equiv.) was dissolved in 11 mL of water and added dropwise over 2 hours into the flask. Past this addition, the reaction was carried out overnight under room temperature.

After the completion, the solvent was evaporated in low pressure, the crude mixture was redissolved in ethyl acetate, and submitted to liquid-liquid extraction and basification with $NaHCO_3$ saturated solution. Afterwards the compound was purified by column chromatography using a mixture of Hexane/Ethyl acetate 2:1 to afford 220 mg as a white solid (Yield=22%).

^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.24 (td, *J* = 5.8, 4.7, 2.0 Hz, 6H), 4.56 (s, 2H), 2.10 (d, *J* = 0.6 Hz, 1H), 1.42 (s, 9H).

^{13}C NMR (75 MHz, Chloroform-*d*) δ 128.5, 76.6, 28.1.

4.1.4. Synthesis of Boc protected 1(benzylamino)-1H-pyrrole-2,5-diona (4)

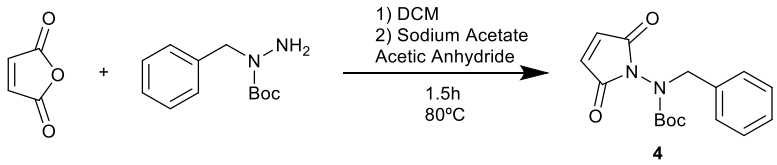


Figure 11: Boc protected 1(benzylamino)-1H-pyrrole-2,5-diona

In a 25 mL round bottom flask, maleic anhydride (100 mg, 1.02 mmol, 1 equiv.) was dissolved in 5 ml of DCM, followed by slow addition of Boc protected Benzylhydrazine (226.69 mg, 1.02 mmol, 1 equiv.). After 10 minutes, the solvent was evaporated at low pressure and the solid was resuspended in 1 mL of acetic anhydride, Sodium acetate (190 mg, 2.04 mmol, 2 equiv.) was added slowly to the reaction, and left stirring for 1 hour at 80ºC, followed by TLC.

After the completion, the reaction was quenched with water, basified with $NaHCO_3$ saturated solution, submitted to liquid-liquid extraction with ethyl acetate and evaporated in low pressure. During the evaporation, the flask fell into the water bath and the compound was lost. The reaction was repeated under the same condition but there was not possible to isolate the product.

4.1.5. Synthesis of Boc protected hydroxylamine (5)

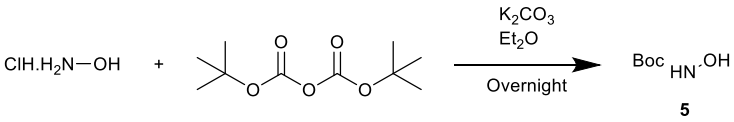


Figure 12: Synthesis of Boc protected hydroxylamine

In a 50 mL round bottom flask hydroxylamine hydrochloride (4.78 g, 68.79 mmol, 1.5 equiv.) was suspended in Et_2O (30 mL) and H_2O (1 mL), K_2CO_3 (9.51 g, 68.79 mmol, 1.5equiv.) was added at room temperature and left stirring for 1 hour, then cooled to 0˚C, Boc_2O (10 g, 42.86 mmol,1equiv.) was dissolved in Et2O (10 mL) and slowly added for 40 minutes, stirring vigorously overnight. After the reaction conclusion, the precipitate was filtered, and the filtrate was wahsed with Brine solution and HCl 1M and the organic phase was evaporated at low pressure to afford 1.338 g as a white solid (Yield=21.9%)

^{1}H NMR (300 MHz, Chloroform-*d*): δ 1.45 (t, *J* = 1.8 Hz, 9H).

^{13}C NMR (75 MHz, Chloroform-*d*) δ 158.9, 82.2, 28.2.

4.1.6. Synthesis of Boc protected O-(mesitylsulfonyl) hydroxylamine (6)

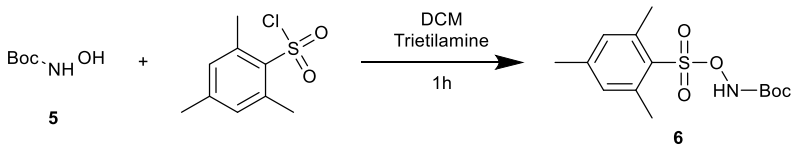


Figure 13: Synthesis of Boc protected O-(mesitylsulfonyl) hydroxylamine

In a 50 mL round bottom flask Boc protected hydroxylamine (502.28 mg, 3.77 mmol, 1.1 equiv.) was dissolved in DCM (30 mL) and TEA (1 mL, 7.2 mmol, 2.1 equiv.), left stirring for 5 minutes, 2,3,4-trimethylbenzenesulfonyl chloride (755 mg, 3.34 mmol, 1 equiv.) was slowly added thereafter. The reaction was left for 50 minutes with magnetic stirring and followed by TLC.

After completion, the reaction was quenched with water and washed with HCl 1M, $NaCO_3$ saturated solution and Brine solution, and the organic phase was evaporated at low pressure.

4.1.7. Synthesis of Boc protected O-(4-nitrobenzoyl) hydroxylamine (7)

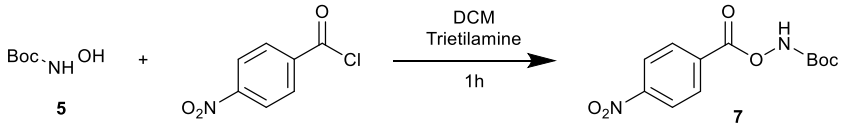


Figure 14: Synthesis of Boc protected O-(4-nitrobenzoyl) hydroxylamine

In a 50 mL round bottom flask Boc protected hydroxylamine (502.28 mg, 3.77 mmol, 1.1 equiv.) was dissolved in DCM (30 mL) and TEA (1 ml, 7.2 mmol, 2.1 equiv.), left stirring for 5 minutes, 4-nitrobenzoyl chloride (635.38 mg, 3.43 mmol, 1 equiv.) was slowly added thereafter. The reaction was left for 50 minutes with magnetic stirring and followed by TLC.

After completion, the reaction was quenched with water and washed with HCl 1M, $NaCO_3$ saturated solution and Brine solution, and the organic phase was evaporated at low pressure.

^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.34 – 8.15 (m, 4H), 1.45 (s, 9H).

4.1.8. Synthesis of Boc protected amine N-α-substituted maleimide using diverse electrophilic nitrogen reagent. (8)

Method A- Via the use of electrophilic nitrogen reagents.

4.1.8.1. Using KOtBu and Boc protected O-(4-nitrobenzoyl)hydroxylamine

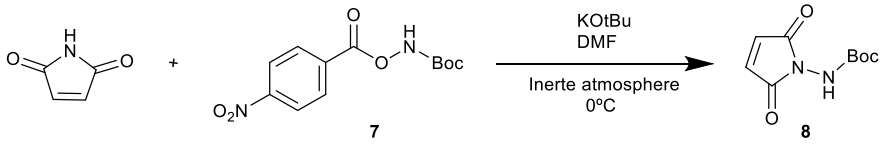


Figure 15: Synthesis of Boc protected amine N-α-substituted maleimide using KOtBu and Boc protected O-(4-nitrobenzoyl)hydroxylamine

In a 25 mL round bottom flask, KOtBu (59.76 mg, 532.59 µmol, 1.1 equiv.) and maleimide (47,00 mg, 484,19 µmol, 1 equiv.) was cooled to 0˚C and stirred for 15 minutes, Boc protected O-(4-nitrobenzoyl) hydroxylamine (148.9 mg, 530.73 µmol, 1.2 equiv.) was suspended in dry DMF (1 mL), and added to the reaction, that was left stirring for 50 min.

The reaction what quenched with 20 mL of water, submitted to liquid extraction with DCM and evaporated at low pressure. Afterwards the compound was purified by column chromatography using mixture of Hexane/Ethyl acetate 2:1.

^{1}H NMR (300 MHz, Chloroform-*d*): δ 6.73 (s, 2H), 6.48 – 6.28 (m, 1H), 1.41 (s, 9H).

^{13}C NMR (75 MHz, Chloroform-*d*): δ 28.20, 102.46.

4.1.8.2. Method B: Synthesis of Boc protected amine N-α-substituted maleimide via maleic anhydride

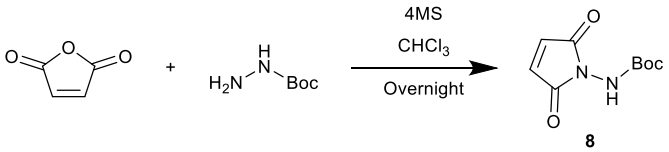


Figure 16: Synthesis of Boc protected amine N-α-substituted maleimide via maleic anhydride

In a 100 mL round bottom flask in inert atmosphere and 1 g of molecular sieves, maleic anhydride (2.45 g, 24.99 mmol, 1 equiv.) was dissolved in 50 mL of chloroform, Boc protected

hydrazine (3.30 g, 24.99 mmol, 1 equiv.) was added slowly to the reaction, that was left at 80˚C overnight.

Afterwards the compound was purified by column chromatography using mixture of Hexane/Ethyl acetate 4:1 to afford 2.75 g as a white solid (yield=52%).

^{1}H NMR (300 MHz, Chloroform-*d*): δ 6.73 (s, 2H), 1.57 – 1.23 (m, 9H).

^{13}C NMR (75 MHz, Chloroform-*d*) δ 140.2, 102.5, 83.7, 28.0.

4.1.9. Synthesis of 3-((4-nitrophenyl)thio)-1-phenylpyrrolidine-2,5-dione using triethylamine as catalyst (9)

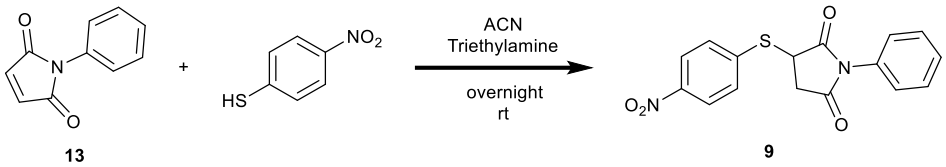


Figure 17: Synthesis of 3-((4-nitrophenyl)thio)-1-phenylpyrrolidine-2,5-dione using triethylamine as catalyst

In a 25 mL round bottom flask, 1-phenyl-1*H*-pyrrole-2,5-dione (50 mg, 288,73 µmol ,1 equiv.) and 4-nitrobenzenethiol (44.80 mg, 288,73 µmol, 1 equiv.) was dissolved in ACN (3 mL), TEA (40 µL) was added slowly and the reaction was left stirring overnight under room temperature.

Afterwards the solvent was evaporated, and the compound was purified by column chromatography using mixture of Hexane/Ethyl Acetate 7:3 to afford 69 mg as a yellow solid (yield=49%).

^{1}H NMR (300 MHz, Acetone-d_6): δ 8.15 – 8.04 (m, 2H), 7.68 (d, J = 26.6 Hz, 2H), 7.42 – 7.23 (m, 3H), 7.23 – 7.13 (m, 2H), 4.79 (dd, J = 9.4, 5.0 Hz, 1H), 3.49 (dd, J = 18.4, 9.4 Hz, 1H), 2.81 (dd, J = 18.4, 5.0 Hz, 1H).

4.1.10. Synthesis of Boc protected 1-amino-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (10)

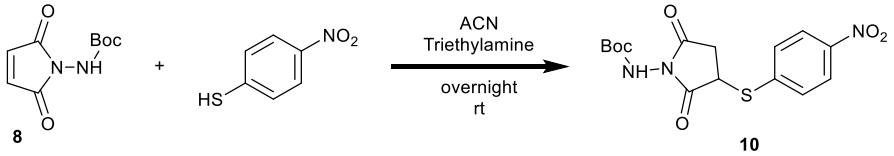


Figure 18: Synthesis of Boc protected 1-amino-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione

In a 50 mL round bottom flask Boc protected 1-amino-1H-pyrrole-2,5-dione (100 mg, 892,16 µmol, 1 equiv.) and 4-nitrobenzenethiol (138.44 mg, 892,16 µmol, 1 equiv.) was dissolved in ACN (6 mL), TEA (80 µL), was added slowly and the reaction was left stirring overnight.

Afterwards the solvent was evaporated, and the compound was purified by column chromatography using mixture of Hexane/Ethyl Acetate 9:1 to afford 29 mg as a white solid (yield=22%).

^{1}H NMR (300 MHz, Acetone-d_6) δ 8.11 (dd, J = 13.7, 8.9 Hz, 2H), 7.78 – 7.57 (m, 2H), 4.75 (s, 1H), 3.43 (dd, J = 18.7, 9.6 Hz, 1H), 2.67 (s, 9H).

4.1.11. Synthesis of 3-((4-nitrophenyl)thio)-1-phenethylpyrrolidine-2,5-dione (11)

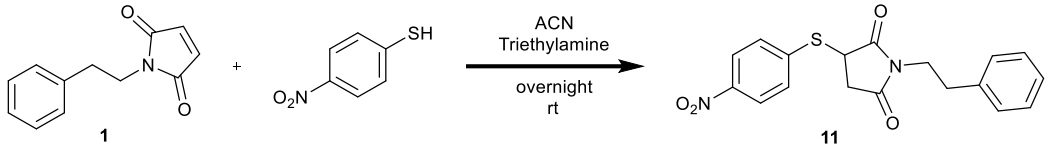


Figure 19: Synthesis of 3-((4-nitrophenyl)thio)-1-phenethylpyrrolidine-2,5-dione

In a 25 mL round bottom flask, 1-phenethyl-1*H*-pyrrole-2,5-dione (50 mg, 248.48 µmol ,1 equiv.) and 4-nitrobenzenethiol (38,56 mg, 248.48 µmol, 1equiv.) was dissolved in ACN (3 mL), TEA (40 µL) was added slowly and the reaction was left stirring overnight under room temperature.

Afterwards the reaction was concentrated, and the compound was purified by column chromatography using mixture of Hexane/Ethyl Acetate 9:1 to afford 54mg as a yellow solid (yield=60%).

^{1}H NMR (300 MHz, Acetone-d_6) δ 8.21 (dd, J = 8.9, 1.4 Hz, 2H), 7.75 (dd, J = 8.8, 1.6 Hz, 2H), 7.40 – 7.10 (m, 5H), 4.72 (ddd, J = 9.3, 4.7, 1.2 Hz, 1H), 3.80 – 3.64 (m, 2H), 3.43 (dd, J = 18.4, 9.2 Hz, 1H), 2.87 (dd, J = 8.7, 6.6 Hz, 2H), 2.69 (dd, J = 18.4, 4.6 Hz, 1H).

4.1.12. Synthesis of 1-(benzyloxy)-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (12)

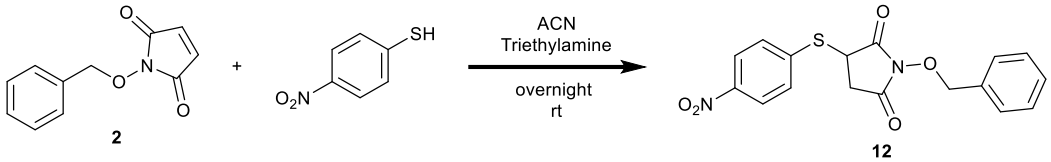


Figure 20: Synthesis of 1-(benzyloxy)-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione

In a 25 mL round bottom flask, 1-(benyloxy)-1H-pyrrole-2,5-dione (50 mg, 246.07 µmol ,1 equiv.) and 4-nitrobenzenethiol (38,18 mg, 246.07 µmol, 1 equiv.) was dissolved in ACN (3 mL), TEA (40 µL) was added slowly and the reaction was left stirring overnight under room temperature.

Afterwards the reaction was concentrated, and the compound was purified by column chromatography using mixture of Hexane/Ethyl Acetate 9:1 to afford 23 mg as a white solid (yield=26%).

^{1}H NMR (300 MHz, Acetone-d_6) δ 8.09 (d, J = 9.0 Hz, 1H), 7.76 – 7.59 (m, 2H), 7.43 – 7.33 (m, 2H), 7.33 – 7.22 (m, 3H), 4.95 (s, 2H), 4.61 (dd, J = 9.2, 4.4 Hz, 1H), 3.33 (dd, J = 18.2, 9.2 Hz, 1H), 2.70 – 2.48 (m, 2H) .

4.2. ESI-MS Assays

General procedure for the base scope

In four different eppendorfs, 1.1 equiv. of K2CO3, KOtBu, DBU or NaH (50 mM) were added to 1 equiv. of maleimide (500 mM) , followed by the addition of 1.2 equiv. of Boc protected O-(4-nitrobenzoyl) hydroxylamine(500 mM). Before each reading, the reaction was diluted to 100 µM. The readings were taken at 15 min and 60 min.

4.3. UV Spectroscopy Assay

General procedure: retro-Michael addition susceptibility study

In three different cuvettes was added 15 µL of **11**, **10**, and **9** (10 mM) previously solubilized in 450 µL of DMF to 2550 µL of buffer at pH=7.8. The readings were taken every 15 minutes in the first hour of the test, followed by hourly readings in the first 5 hours, ending with an overnight reading, at 14 hours.

General procedure: thiol exchange susceptibility study

In three different cuvettes was added 15 µL of **11**, **10**, and **9** (10 mM) previously solubilized in 450 µL of DMF to cysteine, solubilized in 15 µL of water (50 mM), to 2535 µL of buffer at pH=7.8. Readings were taken every 15 minutes in the first hour of the assay, at the 2 and 3 hours of the assay, and after 6 hours.

5. Results and discussion

The rationale behind modulating the N-α-substituent is to inquire about its impact on the reactivity/stability profile, based on their tendency to withdraw or donate electrons. Thus, it was chosen to synthesize maleimides with oxygen (**2**) and nitrogen (**3**) N-α-substituent (heteroatoms as N-α-substituent), but also an ethyl as a N-α-substituent (**1**) and compare them to maleimides with Phenyl as N-α-substituent (**13**).

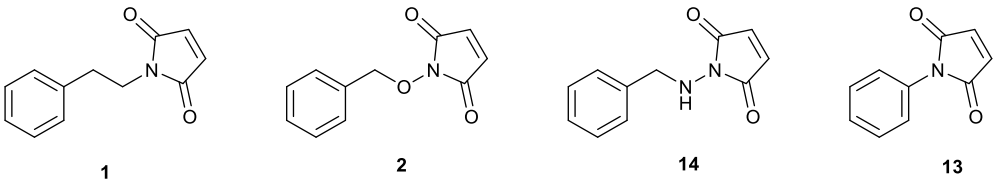


Figure 21: Ethyl maleimide **1**; Oxygen N- α -substituent maleimide **2**; Nitrogen N- α -substituent maleimide **14**; N-Phenyl maleimide **13**

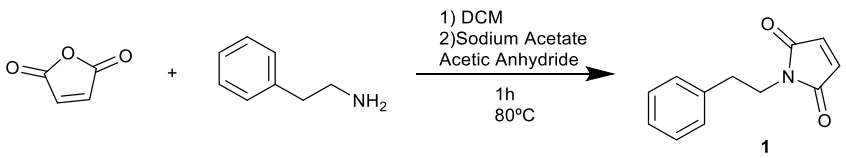
In our study we used **13** as a benchmark for all the assays since it is well described by Christie et.al.(14). It has an electron withdrawing group, that increases the imide electrophilicity, accelerating thiosuccinimide hydrolysis and leading to the more stable thiosuccinimic acid. Thus, we aspire to determine a comparison between this maleimide and the other three we synthesized (**1**, **2** and **14**) in order to determine the impact of different N-α-substituent on hydrolysis of maleimides and their thiossucinimides, but also on the susceptibility to Michael addition and retro-Michael.

As we already had maleimide **13** in hands there was no need to synthesize it.

5.1. Synthesis of Ethyl maleimide (1), Oxygen N-α-substituent maleimide (2) and nitrogen N-α-substituent maleimide (4)

We proceeded to synthesize our maleimides directly from maleic anhydride by using either a primary amine or O-substituted hydroxylamine.

5.1.1. Synthesis of 1-phenethyl-1H-pyrrole-2,5-dione (1)



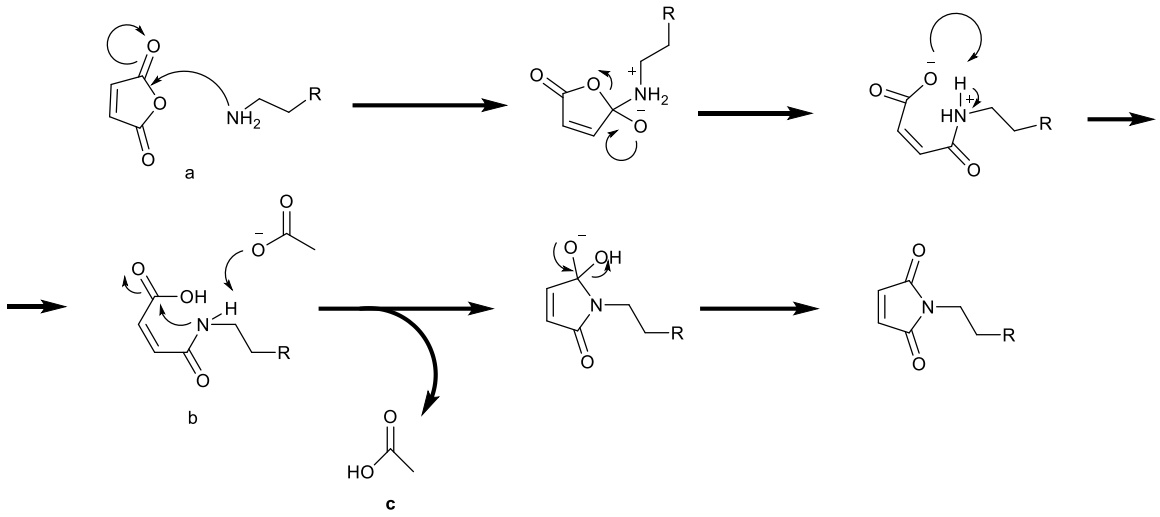


Figure 22: Synthesis of **1** and its mechanism

This reaction consists of two steps. First, the amine is added to the anhydride (which is already solubilized in DCM) and left for 15 min for the nucleophilic attack (Fig. 22- **a**) of the amine to take place on the carbonyl . As a result, the anhydride ring opens. To proceed with the cyclization step we evaporated the DCM, resuspended the solid in acetic anhydride and added sodium acetate (Fig. 22- **b**), and reacted 1 hour at 80ºC with reflux.

The major problem with this synthesis was the formation of acetic acid as a side product (Fig. 22- **c**). Even though the workup procedure included a basification step and several ethyl ether extractions, it was very difficult to evaporate the acetic acid.

The maleimide was synthesized and purified by column chromatography and characterized by NMR (Annex 1-2).

Similarly, the synthesis of **1**, oxygen N-α-substituent maleimide **2**, was also successful.(Annex 5-6).

5.1.2. Synthesis of Boc protected 1(benzylamino)-1H-pyrrole-2,5-dione (4)

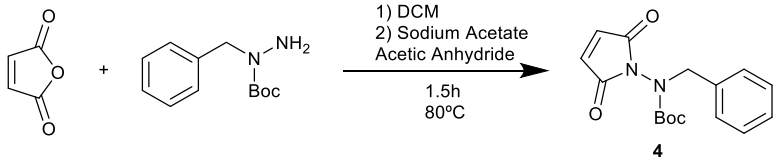


Figure 23: Synthesis of **4**

As in the synthesis of **1**, this reaction consists of the two steps, and has the same problem with the formation of acetic acid. The use of a protected hydrazine reagent was essential for the success of this synthesis. With the unprotected hydrazine , the formation of the six-membered ring was strongly favored, since after the first nucleophilic attack the unoccupied amine can perform one on the other carbonyl of the maleimide.

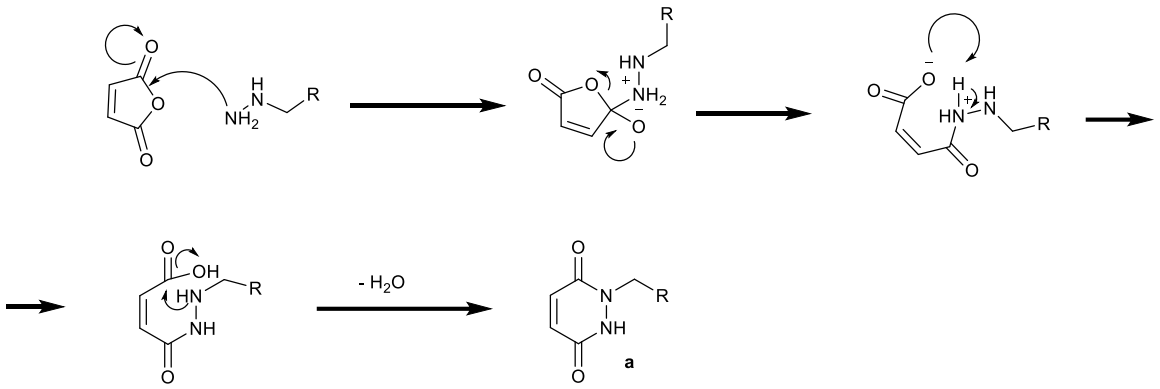


Figure 24: Mechanism of synthesis of the six-membered ring with unprotected hydrazine

As already mentioned in experimental procedure, it was not possible to complete this reaction, so we decided to repeat it. At the second try it was carried out under the same conditions, however it was not possible to isolate the product. Considering that it is difficult to obtain a Boc protection in the intended hydrazine nitrogen, plus the mechanistic difficulties described in the Figure 24 took us to look for synthetic alternatives to obtain the desired maleimide.

Thus, we proceeded to the synthesis of Boc protected amine N-α-substituted maleimide **8** as a starting point to a new synthetic pathway to **4**:

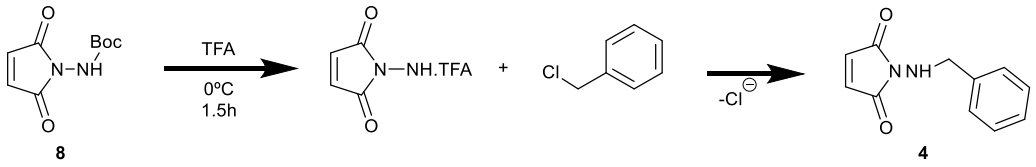


Figure 25: New synthetic pathway to **4**'s synthesis

5.1.3. Synthesis of Boc protected amine N-α-substituted (8)

Method A: Synthesis of Boc protected amine N-α-substituted maleimide using electrophilic nitrogen reagents:

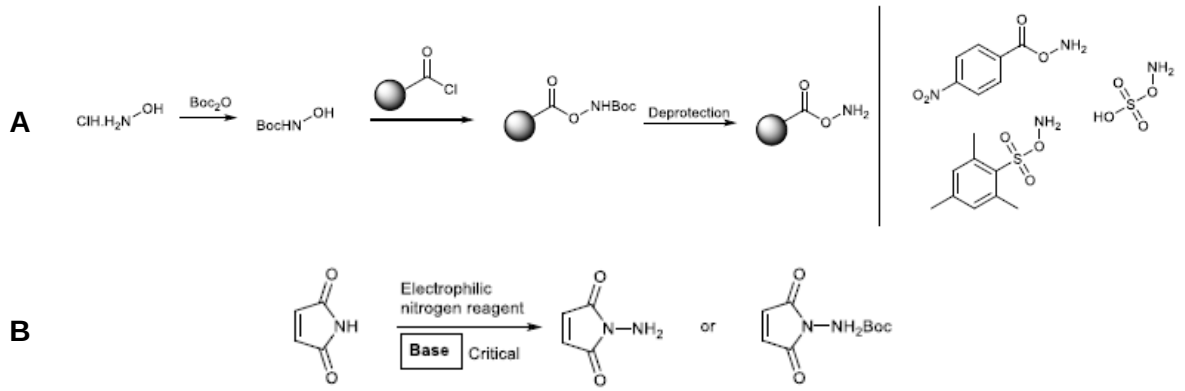


Figure 26: A - General synthesis of electrophilic nitrogen reagents; B - Synthesis of amine N- *α* - substituted maleimide using Method A

In order to synthesize **8** according to Method A (Fig. 26 - **B**), we decided to synthetize nitrogen electrophilic reagents. The use of these reagents is necessary since the direct use of Boc protected hydrazine can also lead to the formation of a 6-membered ring similar to the molecule **a** from Figure 24. The interest of these reagents is their ability to "donate nitrogen" because of their electrowithdrawing functional groups. We decided to synthesize two electrophilic nitrogen reagents to compare their effectiveness in the synthesis of **8**.

Another crucial point for the realization of this method is the choice of a base strong enough to deprotonate the maleimide, however without nucleophilic character, since bases with this characteristic can carry out nucleophilic attacks on carbonyls, opening the ring.

We anticipate that synthesis of **8** involving the use of nitrogen electrophilic reagents is more efficient since there is no ring opening involved in the synthesis, bypassing the mechanistic problem described in Figure 24.

To procced to the synthesis of nitrogen electrophilic reagents, we needed to synthetize Boc protected hydroxylamine (**5**):

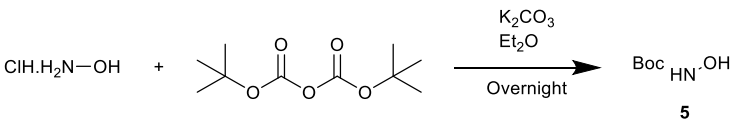


Figure 27: Synthesis of **5**

To protect our amine a Boc protecting group was used, the first step of the mechanism is the amine attack to one of the Boc carbonyl groups, as this function is a comparatively stronger nucleophile that the hydroxyl group, which then releases the rest of the molecule under the form of an unstable carbamate resulting in the formation of CO_2 and tert-butyl alcohol. The excess base was neutralized by the HCl 1M solution and the by-product of the protection, being water-soluble, was removed by liquid-liquid extraction. We were able to purify **5** and characterize it by NMR (Annex 7-8).

Then we proceeded to the synthesis of nitrogen electrophilic reagents: **6** and **7**

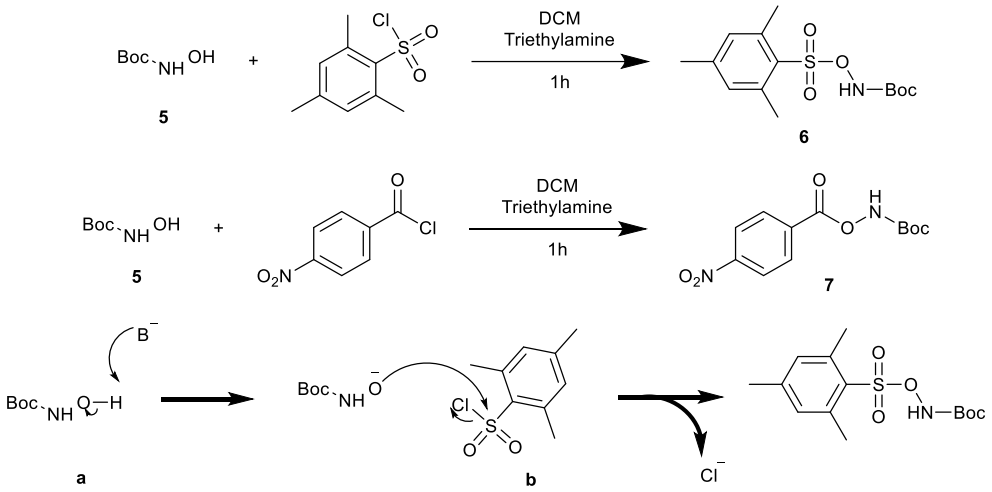


Figure 28: Synthesis of **6** and **7**, with **6**'s mechanism

As a first step we have **5**´s deprotonations (Fig. 28 – **a**), and for this we used triethylamine as a base, as it has enough driving force to capture the proton of the hydroxyl group. In a second step, deprotonated **5** performs nucleophilic attack on **6** (Fig. 28 – **b**) , with Cl as the leaving group. After completion of the reaction, HCl was used to neutralize the excess of triethylamine and $NaCO_3$ saturated solution was used to neutralize any sulfonyl chloride and HCl, allowing a very effective workup in just two washes. As described before, we were able to purify by chromatography column only the **7** (Annex 9).

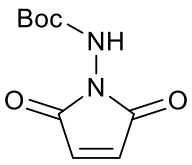
Both reactions were performed under the same conditions, however we did not isolate the product of the synthesis using **6**, which was confirmed after molecular characterization using NMR.

5.1.3.1. Base scope for 8's synthesis

The objective of the test was to understand which base is more adequate to mediate the synthesis of amine maleimide. For that we turn our attention to ESI-MS in order to try to track whether any base was capable of generating a corresponding ESI peak for our desired product. Albeit not ideal, this method was used as the synthetic approach was unusual and would allow us to quickly inquire the viability of the synthetic route.

In four different eppendorfs, 1.1 equiv. of K2CO3, KOtBu, DBU and NaH (50 mM) were added to 1 equiv. of maleimide (500 mM) , followed by the addition of 1.2 equiv. of Boc protected O-(4-nitrobenzoyl) hydroxylamine (500 mM). Before each reading, the reaction was diluted to 100 µM. The readings were taken at 15 min and 60 min

After analyzing the results, we can conclude that for the reaction based on KOtBu, after 15min in, a peak corresponding to the molecular weight of **8** is observed (212.21 g/mol) (Fig. 30). For the reaction that used NaH as a base, after 60 min a peak (252.90 m/z) (Fig. 31) can be observed that may correspond or to the sum of the molecular weights of our product (212.21 g/mol), sodium (22,98 g/mol) and water (18.01 g/mol), or to the sum of molecular weight of our hydrolyzed product (230,22 g/mol) and sodium (22,98 g/mol) . In the reactions that used DBU and K_2CO_3 as bases, no peaks that could correspond to our product were identified.



m/z=212.21, [M+H]=213.1

Figure 29: Molecular weight of **8**

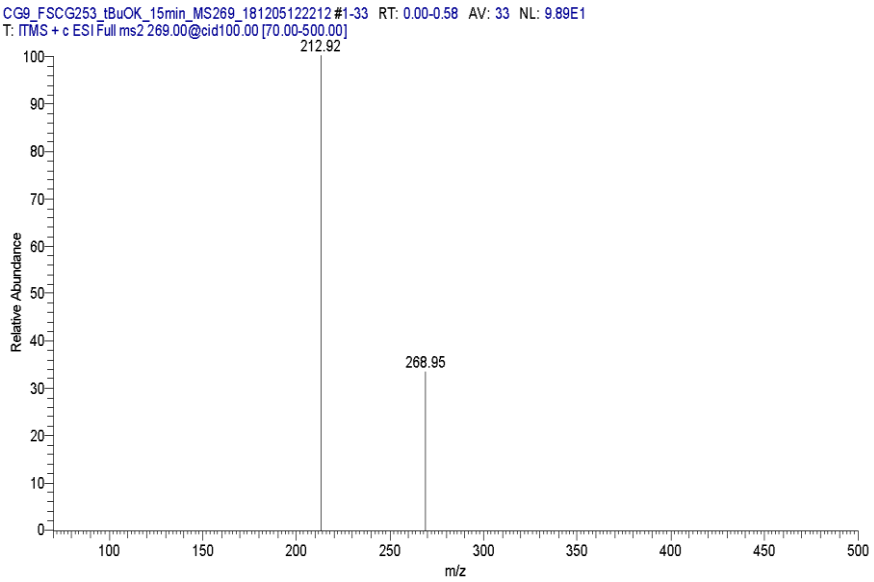


Figure 30:EMS-MS spectrum at 15 minutes using KOtBu as base

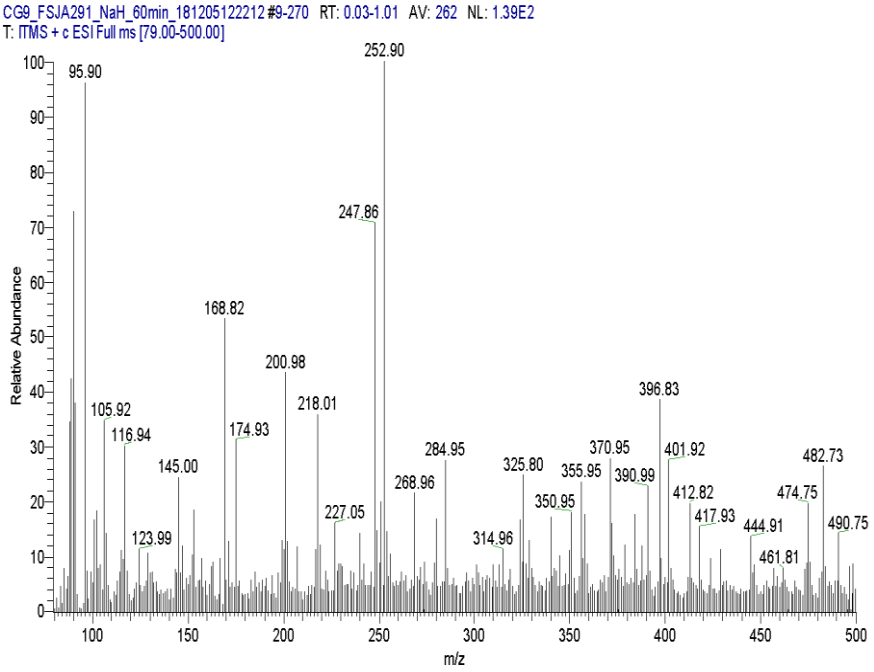


Figure 31: EMS-MS spectrum at 60 minutes using NaH as a base

We repeated the reaction in the laboratory using Sodium Hydride and **7**:

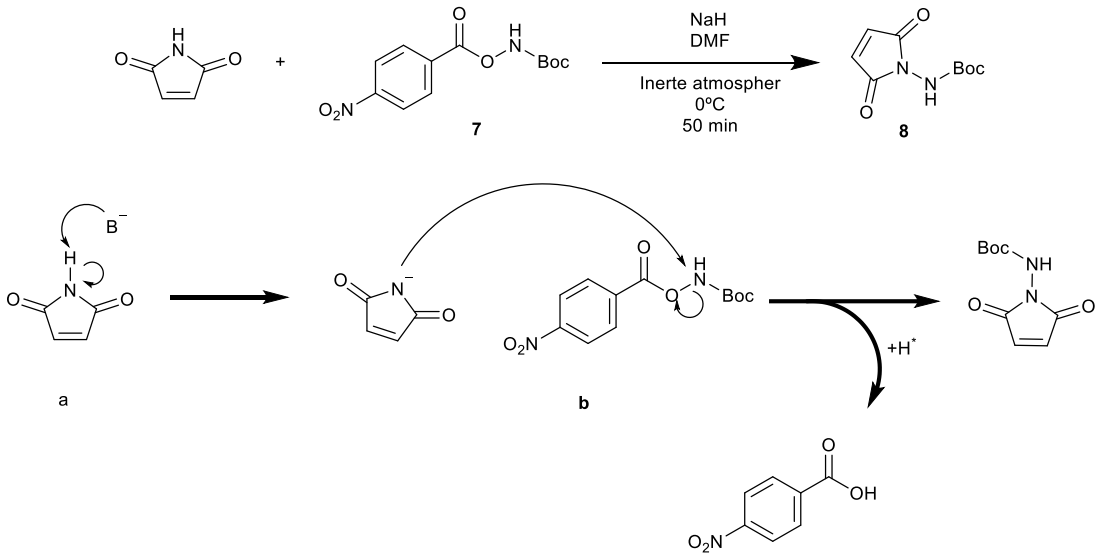


Figure 32: Synthesis of **8** using electrophilic nitrogen reagent and its mechanism

Firstly, the strong non nucleophilic base (sodium hydride) was added to maleimide in order to deprotonate it (Fig. 32 – **a**), leaving the mixture to stir for 15 min at 0 ° C , thereafter was added the electrophilic nitrogen reagent **7**, and the reaction was left in constant stirring and in an ice bath for 50 minutes. Thus, the deprotonated maleimide performs the nucleophilic attack to the electrophilic nitrogen reagent, forming the N-N bond (Fig. 32 – **b**). An inert atmosphere and dry solvents were chosen to have the least amount of interferents possible. Our main concern with this reaction was the formation of by-products resulting from the reaction between the product itself (Aza-Michael reaction) and maleimide hydrolysis, so to avoid this problem the reaction was followed by TLC to halt the end of the reaction.

However, it was mistakenly weighed less sodium hydroxide than the amount predicted by stoichiometry, which is the main cause of the failure of this reaction. The absence of product was later confirmed by molecular characterization obtained by NMR.

We also repeated the reaction in the laboratory using KOtBu and **7** under the same condition as the reaction before:

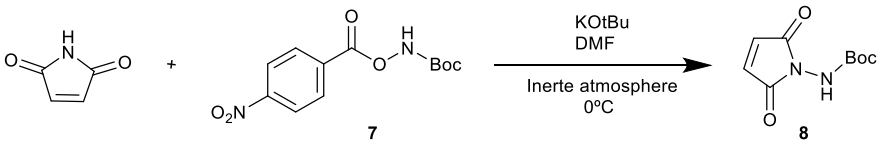


Figure 33: Synthesis of **8** using KOtBu and **7**

We were able to purify **8** by column chromatography and characterize it by NMR. (Annex 10-11)

To obtain more quantity of Boc protected amine N-α-substituted maleimide, we repeated both reaction under the same condition, but none of them had the desired results.

5.1.4. Method B: Synthesis of Boc protected amine N-α-substituted maleimide via maleic anhydride

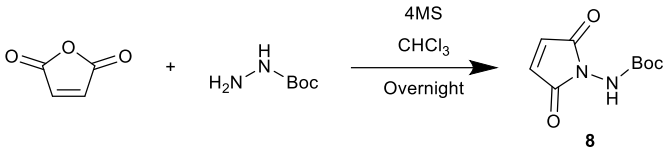


Figure 34: Synthesis of **8** via maleic anhydride

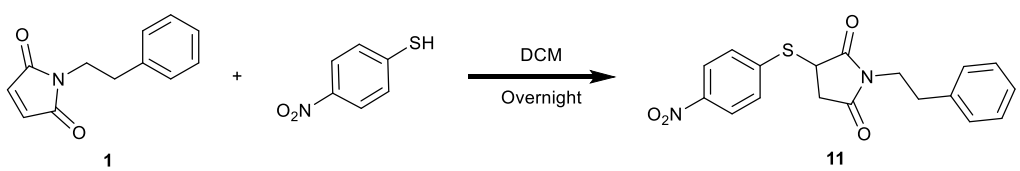
As an alternative method of synthesizing this specific amine N- α -substituted maleimide, this method has been shown to be very efficient as it can be performed in one step with relatively simple reagents. The main precaution is the presence of water in the reaction medium, for this the reaction is done in an inert atmosphere and with addition of molecular sieves, which capture the water that forms during the reaction, not allowing the creation of any biphasic medium and/or undesirable side products.

Even though Method B have theoretical mechanistic disadvantages already here described , it has proved itself more efficient and practical than Method A

Unfortunately, due to lack of resources, we were unable to complete the synthetic route to obtain the **14**. Thus, it was decided that we will proceed with the **8** for our studies, taking into account that the presence of the protective group Boc, being a carbamate, may have an influence on the electro-withdrawing profile of the N-α-substituent.

5.2. Studies of Michael addiction on **1**, **2**, **8** and **13**

To understand the potential influence of N-α-substituent of maleimide in the Michael addition we performed qualitative NMR studies of the reaction of **1**, **2**, **8** and **13** (50mM) with 4-nitrothiophenol (50mM) in DCM.



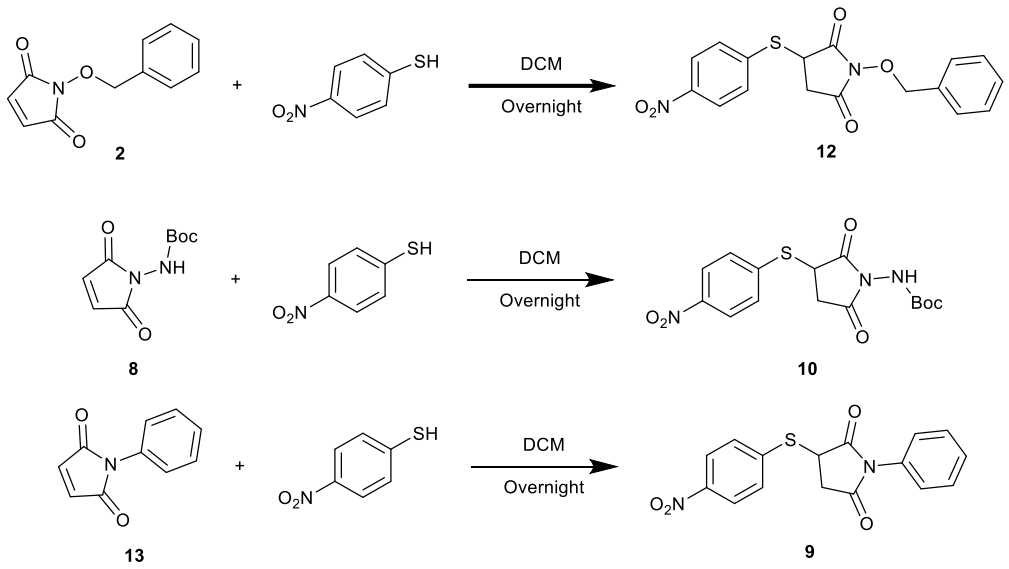


Figure 35 Reaction of **1**, **2**, **8** and **13** with 4-nitrothiophenol for Michel addition study

After following TLC during the first hour of reaction, we concluded that the reaction was not yet over, and decided to leave it overnight. After 24h we evaporated the DCM and analyzed the compounds by NMR.

Firstly, we concluded that after 24h only two of the reactions were complete, namely reactions using **2** and **8**.

To analyze the rate of transformation we integrated the characteristic peak of the starting material and the characteristic peak of the target molecule, and out of the comparation of the extent of the picks we concluded that we had 74% transformation in the reaction using **13** and 80% transformation in the reaction using **1. (** Annex 20 and 13)

From these results it follows that the presence of a heteroatom as a N-α-substituent may facilitate the thiol attack to the alkene moiety of the maleimide. It was expected that the reaction using **2** will be one of the fastest to react because of the synergic electron withdrawing effect of the oxygen and the phenyl group, which has been shown to accelerate both thiol conjugation and hydrolysis of the maleimides.

The fact that the reaction using **8** was concluded allows us to presume that the presence of nitrogen as N-α-substituent may accelerate thiol conjugation. To confirm this presumption, it will be necessary to repeat the assay with nitrogen N-α-substituent maleimide using unprotected **8**, since it is necessary to exclude potential interference stemmed from the protective group.

After this assay, we preceded to synthesize the correspondent thiosuccinimide from each of our maleimides. We find in the literature that triethylamine is used as a catalyst for Michael additions using 4-nitrothiophenol (17), so we proceeded according to the literature.

5.3. Synthesis of thiosuccinimides

5.3.1. Synthesis of 3-((4-nitrophenyl)thio)-1-phenylpyrrolidine-2,5-dione using triethylamine as catalyst (9)

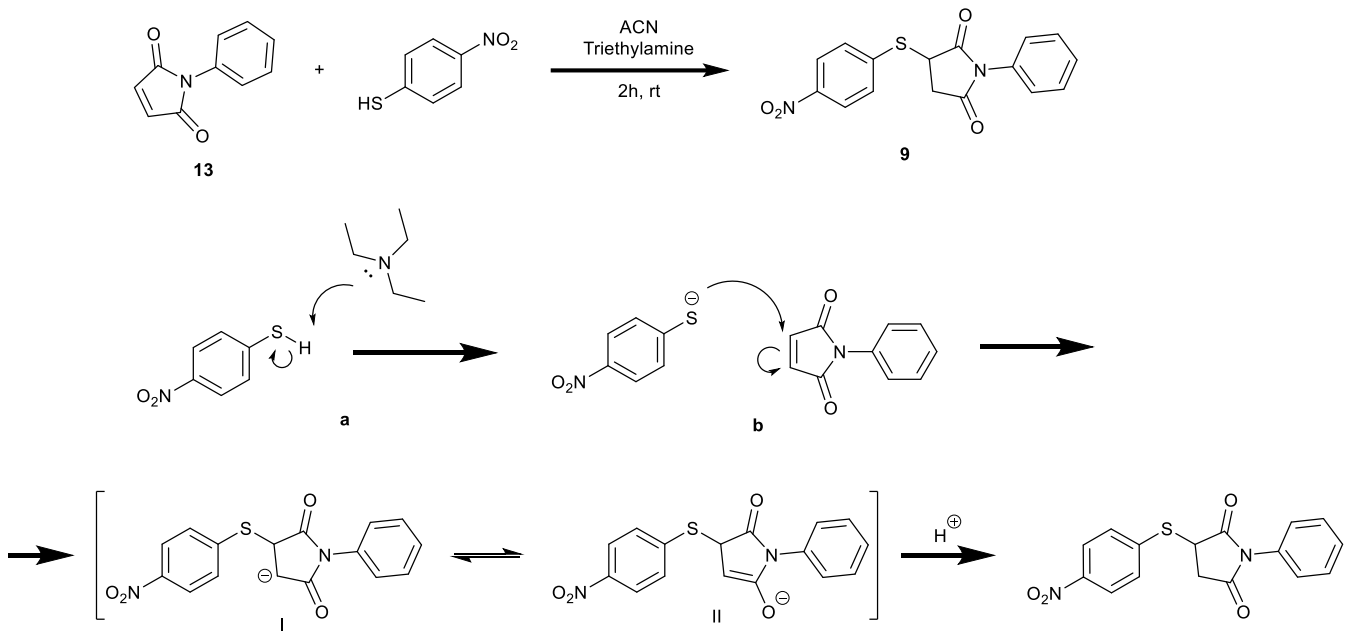


Figure 36: Synthesis and mechanism of **9**

Thus, triethylamine deprotonates the thiol (Fig. 36 - **a**), mediating the Michael addition at room temperature in a relatively short time by facilitating the thiol attack to carbon of the alkene moiety (Fig. 36 - **b**). This step is instantaneous, and can be observed by the changing color to bright red.

By following the reaction by TLC we concluded that the reaction was practically complete, which allowed us to isolate the product without less effort, but after this procedure we noticed a white impurity that was not revealed by TLC and was insoluble in organic solvents. The was observed in all the followed reaction.

The first time that **11**, **12** and **10** were synthesized, the reaction occurred without major complications. However, by repeating the synthesis of **12** for further assays, this synthesis proved to be more complicated than that of the other molecules. At first it seemed like a purification problem since TLC revealed the presence of a product. We tried to change the purification method, choosing a purification using the silica preparative. Increasing the scale

of the reaction resulted in no new spots on the TLC, even by reacting overnight. In an attempt to speed up the reaction we decided to add temperature to the reaction, however we observed that the TLC stains underwent Rf changes, which we interpret as a possible hydrolysis of the starting material or product. Thus, no attempt allowed to obtain **12** in satisfactory quantity and purity to proceed with this molecule in the tests that proceed.

5.4. Studies of retro-Michael elimination by UV-Vis Spectroscopy assay

According to the literature, the electrowithdrawing group as a N-substituents not only accelerate the hydrolysis of thiosuccinimide, but also increases the possibility of the of retro-Michael elimination, due to the general increase in the acidic of the alpha proton in the thiosuccinimide. UV-Vis spectroscopy assays were designed considering that the thiolate resulting from the retro-Michael ion absorbs at a wavelength of 410 nm. The increase in absorbance over time in this wavelength would be indicative that thiosuccinimide thiol release is. However, to be able to state that one species is giving rise to another, it is also necessary an isosbestic point to be present: a wavelength in which the total absorbance of a sample does not change during a chemical reaction. This way, we designed our assay focused on looking for possible comparisons between thiosuccinimides with electrowithdrawing groups as N-α-substituent (**10** and **9**) with a thiosuccinimide without this characteristic (**11**), in order to understand its role in the hydrolysis of thiosuccinimides..

To understand the influence of the N-α substitute for thiosuccinimides on the occurrence of retro-Michael we added each maleimide to a 15% DMF solution in PBS (30 μM). Due to a basic character of the DMF, the final pH of each solution was 7,8.

The readings were taken every 15 minutes in the first hour of the test, followed by hourly readings in the first 5 hours, ending with an overnight reading, at 14 hours.

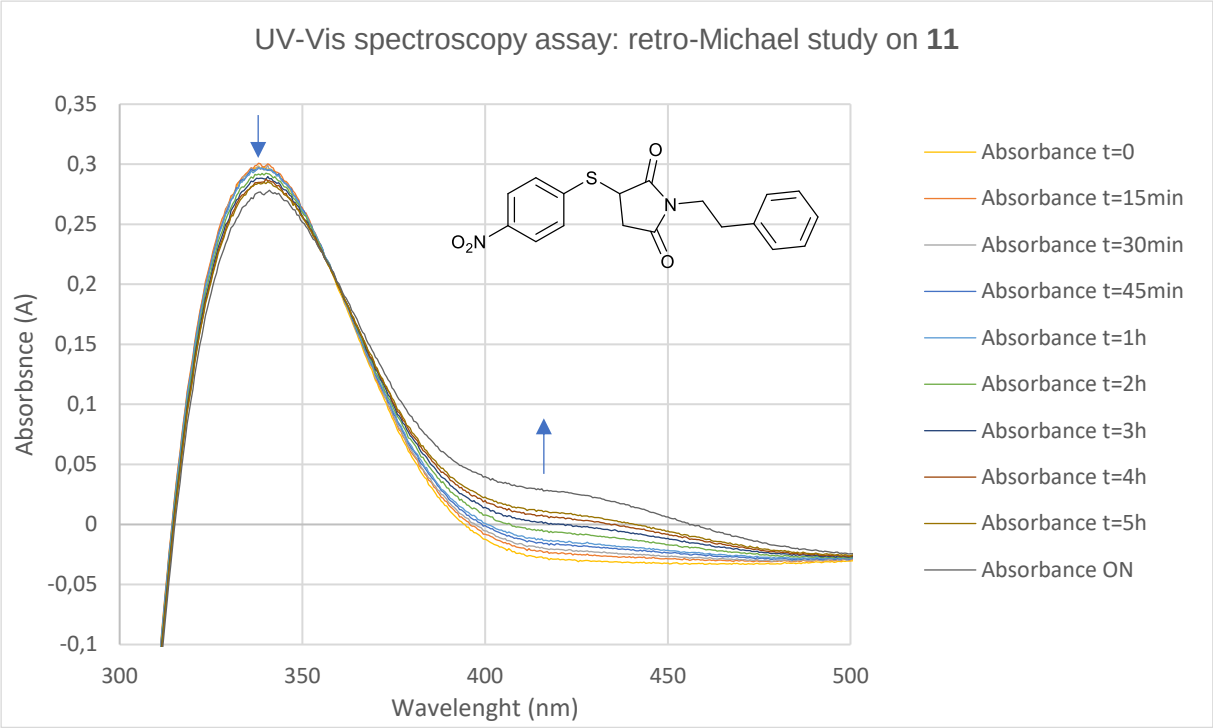


Figure 37:UV-Vis spectroscopy assay: retro-Michael study on **11**

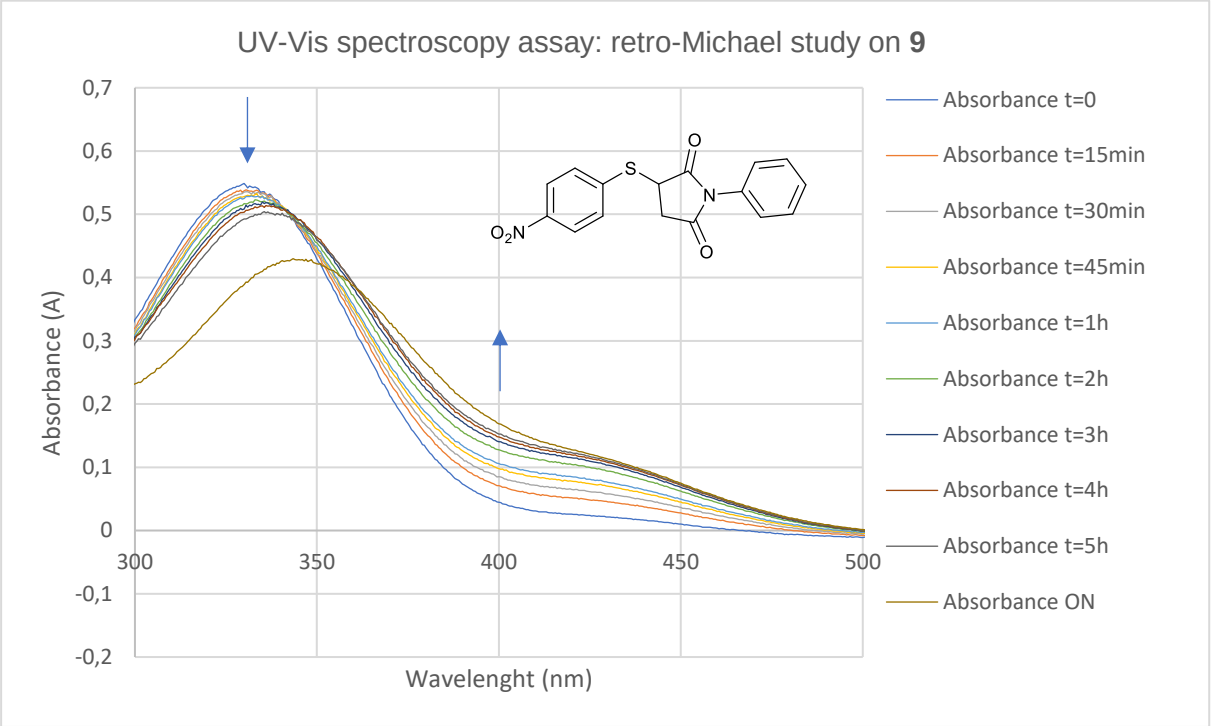


Figure 38: UV-Vis spectroscopy assay: retro-Michael study on **9**

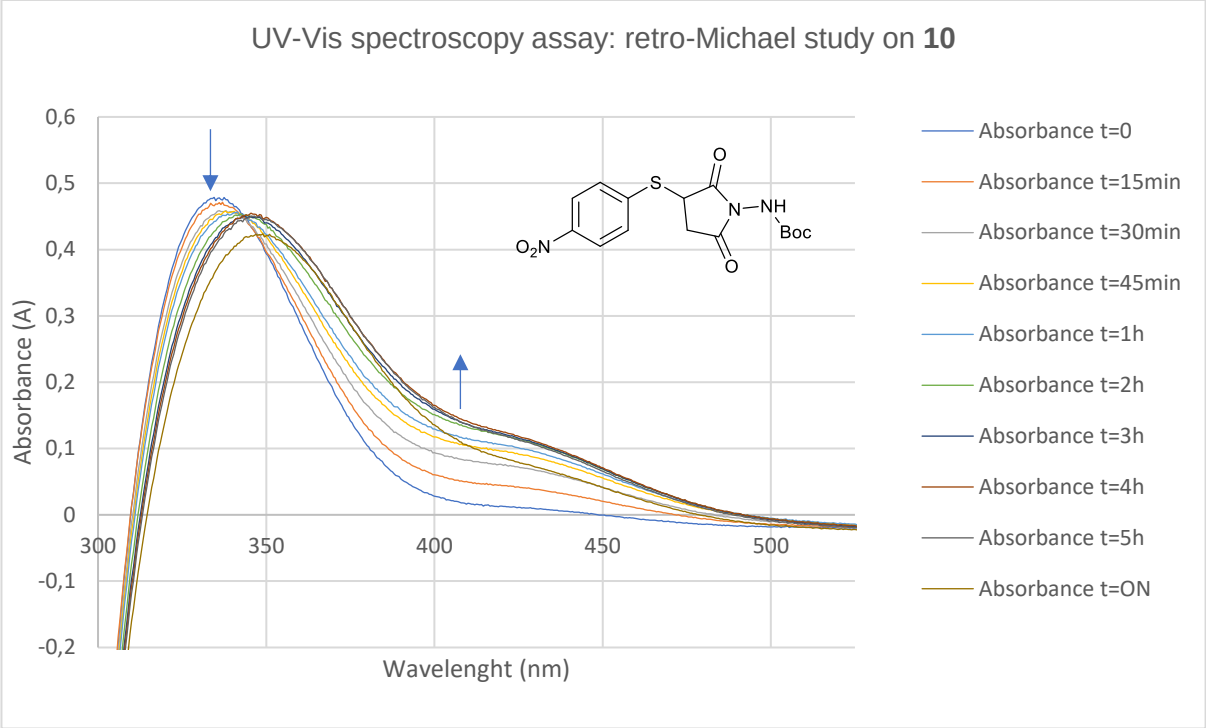


Figure 39:UV-Vis spectroscopy assay: retro-Michael study on **10**

As we can see in the graphs (Fig. 36; Fig. 37; Fig. 38) the three thiosuccinimides have been shown to underwent retro-Michael elimination to different extents. All of them have a defined isosbestic point and an increase in absorbance at 410 nm over time, which means the presence of free thiol in solution.

According to the work of Christie et. al. (14), molecules similar to **9** undergo hydrolysis about 100 times faster than **11** (Fig. 6).Thus it would be expected that the molecule that underwent hydrolysis more quickly would be unavailable to undergo retro-Michael addition. However, what we see in our essays in Figure 38 and 39 just the opposite. We can observe that thiosuccinimides with electrowithdrawing as N-α-substituent, namely **10** (Fig. 39) and **9** (Fig. 38), were more susceptible to suffer retro-Michael, since they are the ones with the greatest increase in absorbance at the wavelength of 410 nm.

It is worth noting that it was also in the assays with **10** (Fig. 39) and **9** (Fig.38) that a reading appeared that did not pass through the isosbestic point, both taken overnight. This data can be interpreted as the formation of a new species in the reaction medium; however, we cannot associate it with certainties to the succinamic acid thioethers resulting from hydrolysis.

No less surprising was the test result using **11**. N-alkyl thiosuccinimides are known for their slow hydrolysis and are therefore more apt to undergo Retro-Michael. However, **11** (Fig. 37) appears to have undergo retro-Michael to the same extent as **9** (Fig. 38).

5.5. Studies of thiol exchange by UV-Vis Spectroscopy assay

As a main weakness of maleimides use is a possible thiol exchange (*e.g.* with glutathione; GSH) of the formed thiosuccinimide, induced by a retro-Michael reaction, that results in the loss of targeting properties and therefore promotes off-target activity, (10) it is of great interest to carry out comparative studies of **11**, **10** and **9** to the susceptibility to this phenomenon.

To understand the influence of the N-α substitute for maleimide on the occurrence of retro-Michael we added each maleimide (30 µM) and cysteine (150 µM) to a 15% DMF solution in PBS. Due to a basic character of the DMF, the final pH of each solution was 7,8. Readings were taken every 15 minutes in the first hour of the assay, at the 2 and 3 hours of the assay, and after 6 hours. We also performed a reading with only the cysteine solubilized in the solution of 15% DMF in PBS.(Annex 27)

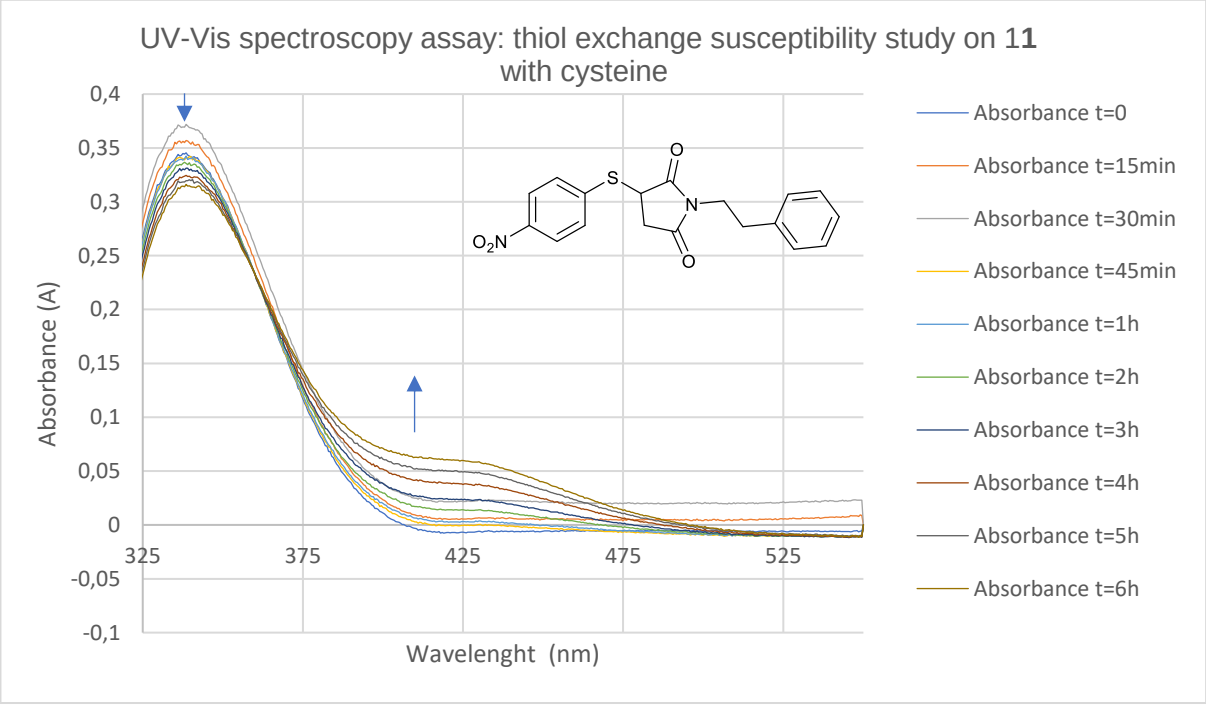


Figure 40:UV-Vis spectroscopy assay: thiol exchange susceptibility study on **11** with cysteine

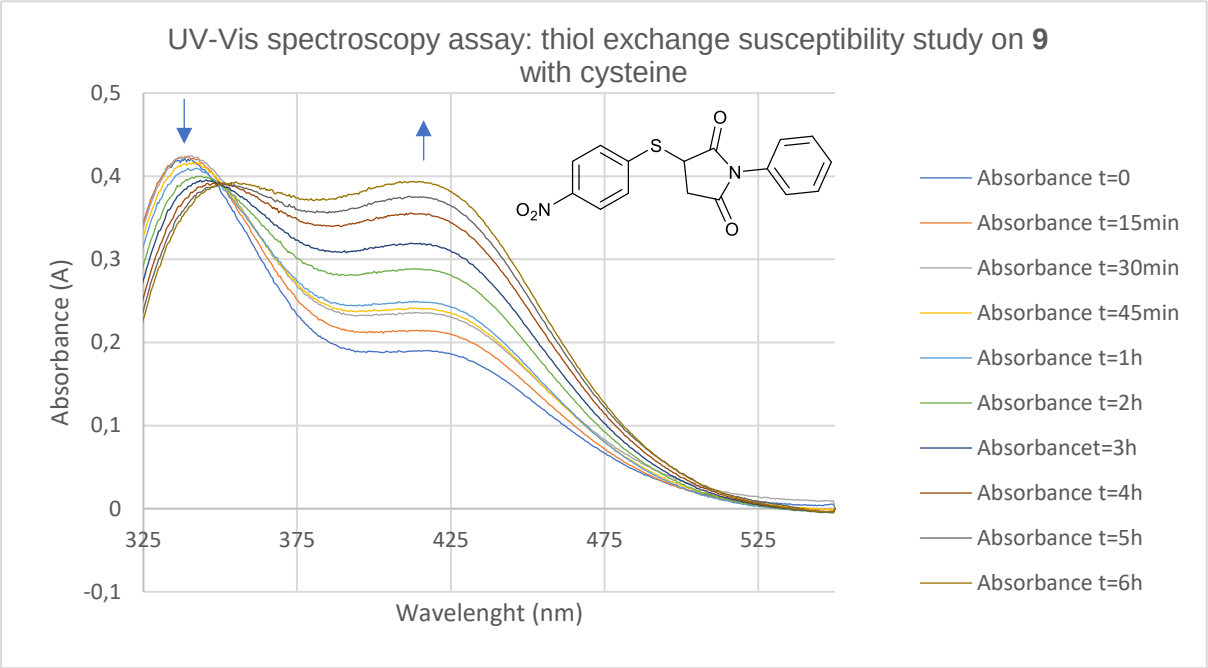


Figure 42:UV-Vis spectroscopy assay: thiol exchange susceptibility study on **9** with cysteine

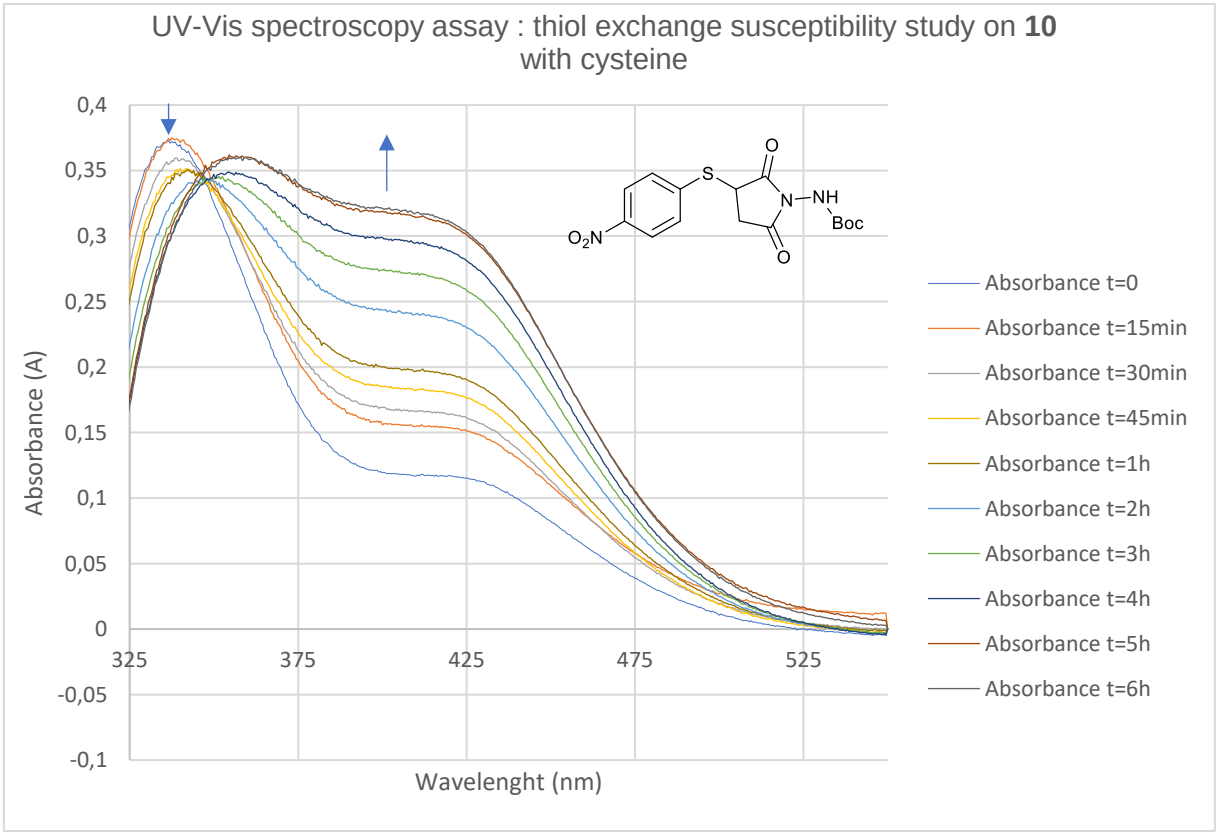


Figure 41: UV-Vis spectroscopy assay : thiol exchange susceptibility study on **10** with cysteine

As we can see in the graphs, the three thiosuccinimides have been shown to underwent retro-Michael addition to different extents. All of them have a defined isosbestic point and an increase in absorbance at 410 nm over time, which means the presence of free thiol in solution.

Firstly, it should be noted that readings at T = 0 of both **10** and **9** solution seem to indicate that there was already free thiol in the medium. We associate this to the fact that the DMF solutions of the three thiosuccinimides were prepared the day before, which, due to its basic character, could cause the retro-Michael even before the test.

Just like on the previous assay, we can conclude that thiosuccinimides with electrowithdrawing group as N-α-substituent, namely **10** (Fig. 41) and **9** (Fig. 40**)**, were more susceptible to suffer retro-Michael elimination, consequently, potential thiol exchange. These data allow us to suspect that the hydrolysis of these two thiosuccinimides is either not as fast as expected, which may facilitate the occurrence of processes that we are trying to avoid, or the speed of this hydrolysis is competitive with the speed of the occurrence of retro-Michael elimination, the two reactions are happening simultaneously.

We can also see that **11** (Fig. 39) was surprisingly stable to thiol exchange in the same way that it was the most resistant to retro-Michael in the previous assay.

Thus, it is necessary to repeat these assays at least twice more under the same conditions and add more tests focused on the quantitative study of the hydrolysis of each of the thiosuccinimides in order to be able to have more robust conclusions.

6. Conclusion

The objective of this work was the synthesis of several maleimides with different N-α-substituent, namely ethyl, Oxygen and Nitrogen in order to study the impact that these substituents have on reactivity and stability of the maleimides.

We succeeded at the synthesis of **1** and **2** but faced some struggle at the synthesis of N-N maleimide **14**, which led us to focus on an alternative: amine N-α-substituted maleimide **8**. Thus, we tried two different methods to synthetize **8.** For the first one, we were able to successfully synthesize electrophilic nitrogen reagents and carry out studies of the influence of different bases on the synthesis of Boc protected amine N-α-substitute maleimide. Thus, we conclude that the use of **7** as an electrophilic nitrogen reagent and KOtBu as base were the most effective reagents for the synthesis of Boc protected amine N-α-substituted maleimide from maleimide, using electrophilic nitrogen reagents. However, as the yield of this synthetic pathway proved to be low, we opted to try a different pathway. Although the mechanism of this synthesis involves the opening of the ring, with the potential formation of an unwanted 6-membered molecule, it proved to be much more efficient than the previous method, and should therefore be considered a good alternative for synthesis of this type of maleimides.

The influence of a heteroatom as a N-α-substituent on the thiol conjugation on maleimides also came to light in the NMR qualitative reactivity studies, as only the reaction with these maleimides were complete after 24 hours in DCM. To confirm this influence on the Michael addition in the context of bioorthogonal chemistry, it is necessary to repeat this assay in aqueous medium and physiological pH.

The UV-Vis assays allowed us to suspect that thiosuccinimide with electrowithdrawing N-α-substituent like **9** and **10** hydrolysis is either not as fast as expected, which can facilitate the occurrence of thiol exchange, or the speed of this hydrolysis is competitive with the speed of occurrence of retro- Michael elimination, both reactions are happening simultaneously.

This project allowed to demonstrate the impact of the electrowithdrawing a N-α-substituent, and although it may pave a way, more comprehensive studies are still required.

7. References

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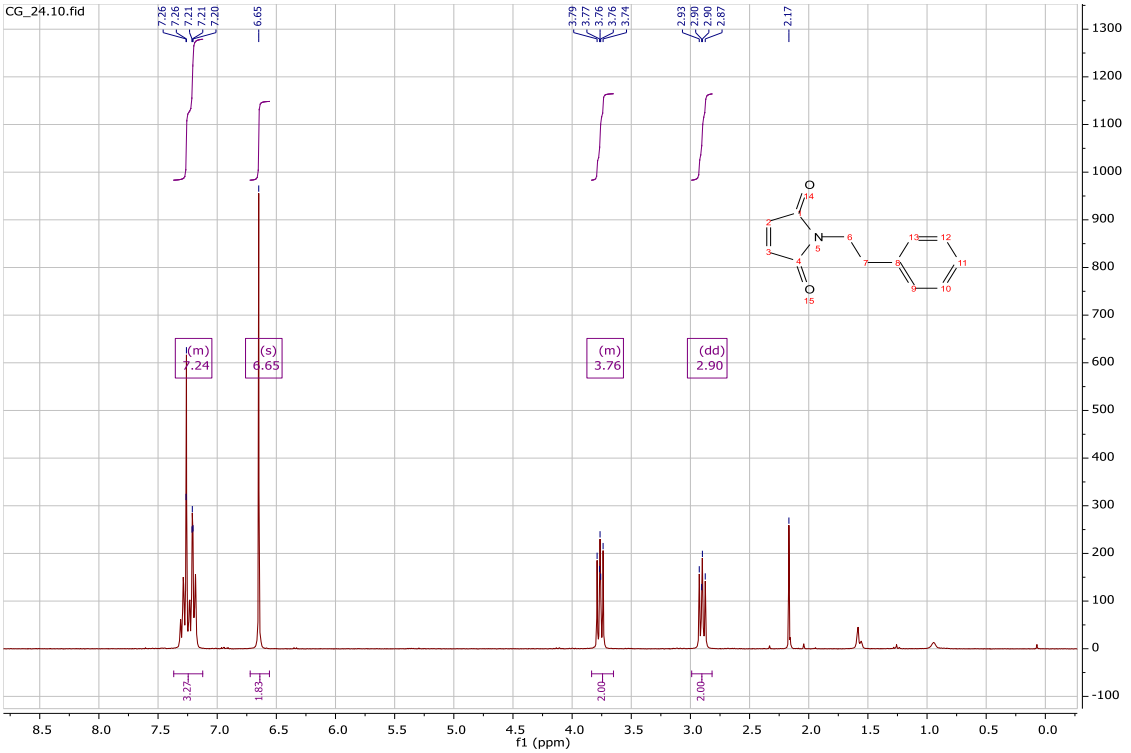
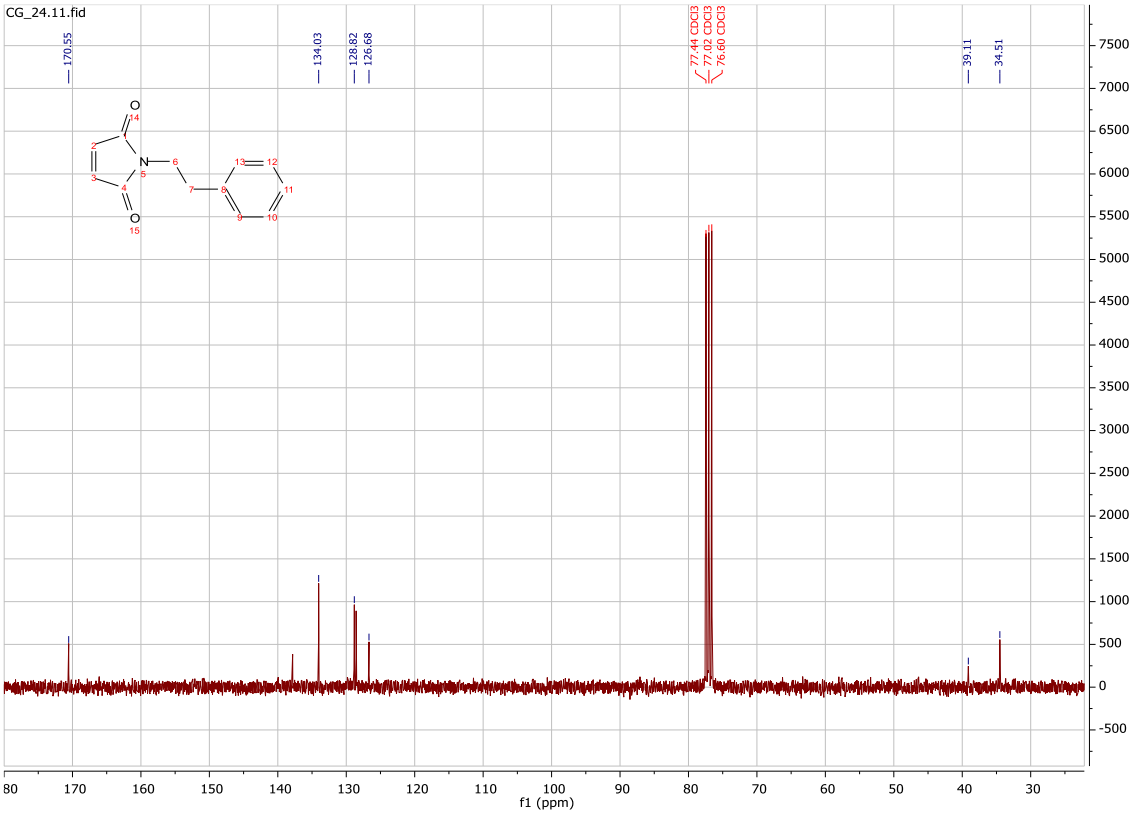
Annexes

NMR Spectra

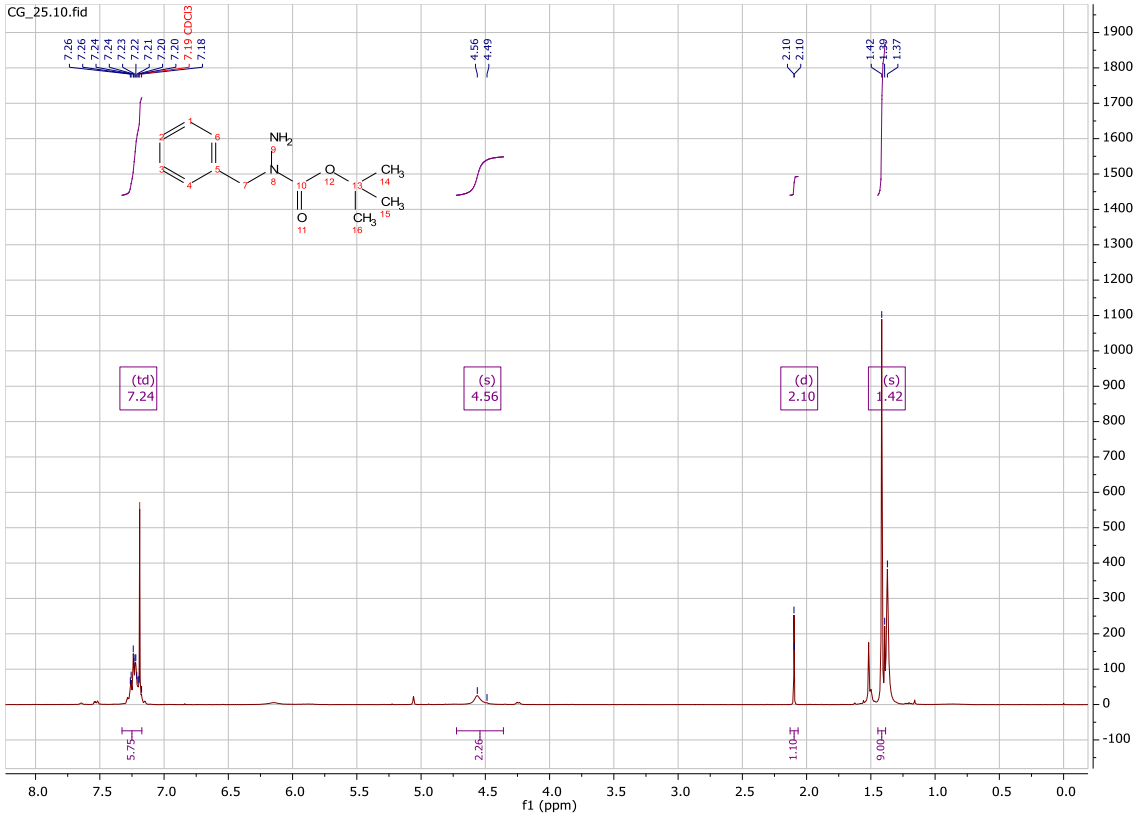
Annex 1- ^{1}HNMR of 1-phenethyl-1H-pyrrole-2,5-dione (1) ^{1}H NMR (300 MHz, Chloroform-*d*):



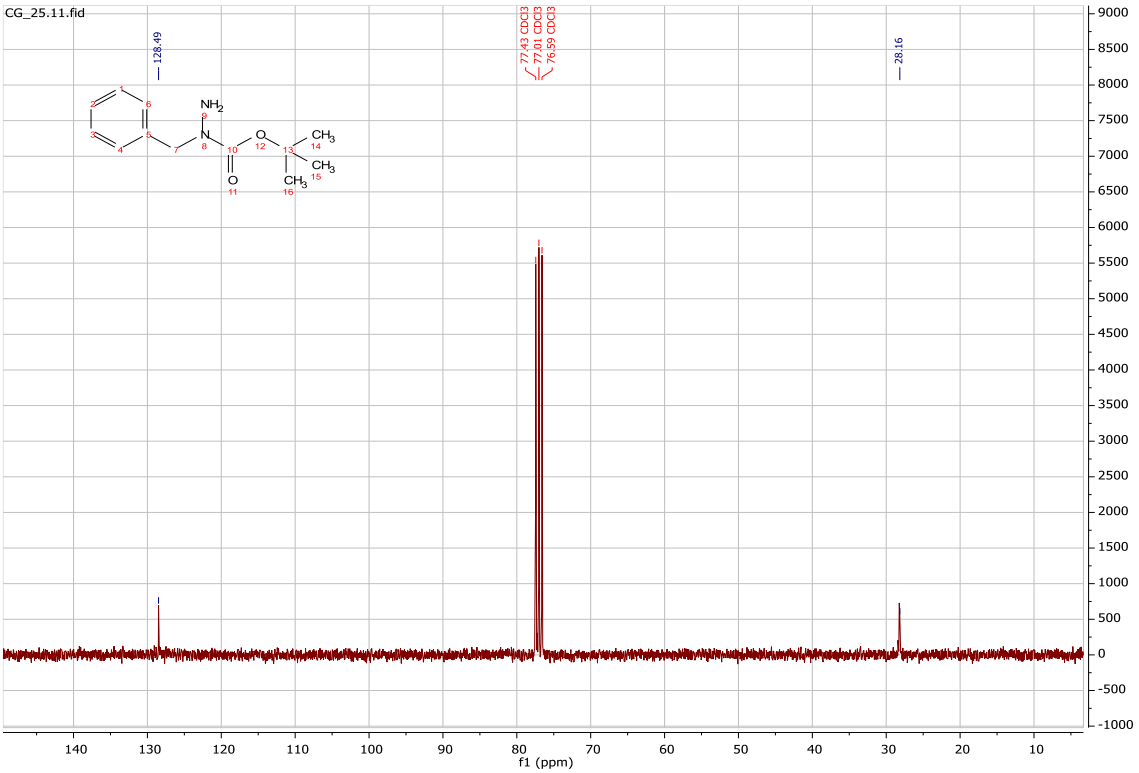
Annex 2- 13CNMR of 1-phenethyl-1H-pyrrole-2,5-dione (1) ^{13}C NMR (75 MHz, Chloroform-*d*):



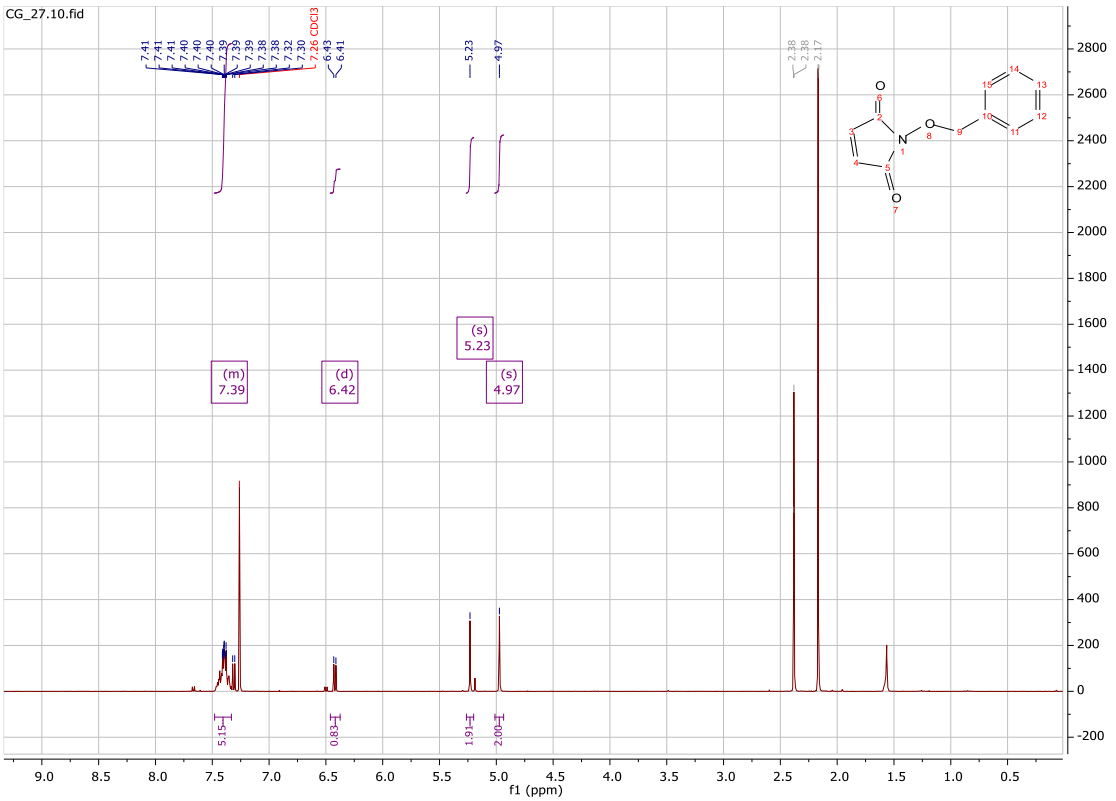
Annex 3- Boc protected Benzylhydrazine (3) ^{1}H NMR (300 MHz, Chloroform-*d*)



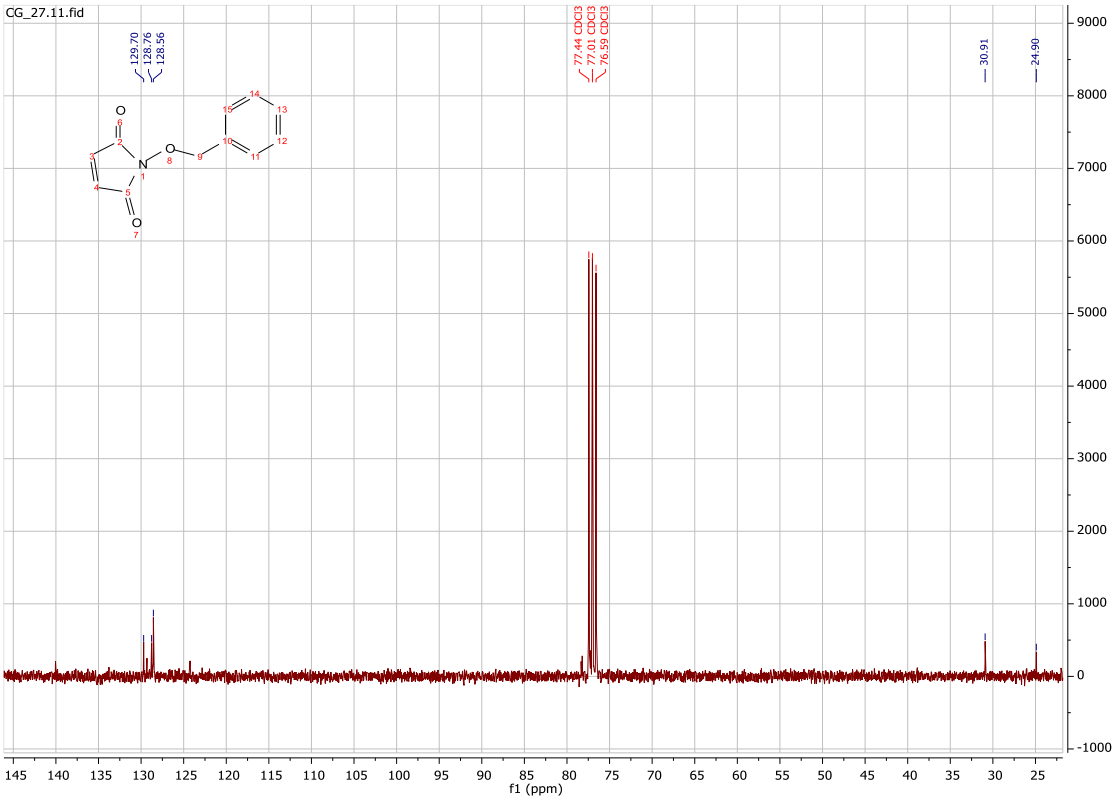
Annex 4- Boc protected Benzylhydrazine (3) 13C-NMR^{13}C NMR (75 MHz, Chloroform-*d*):



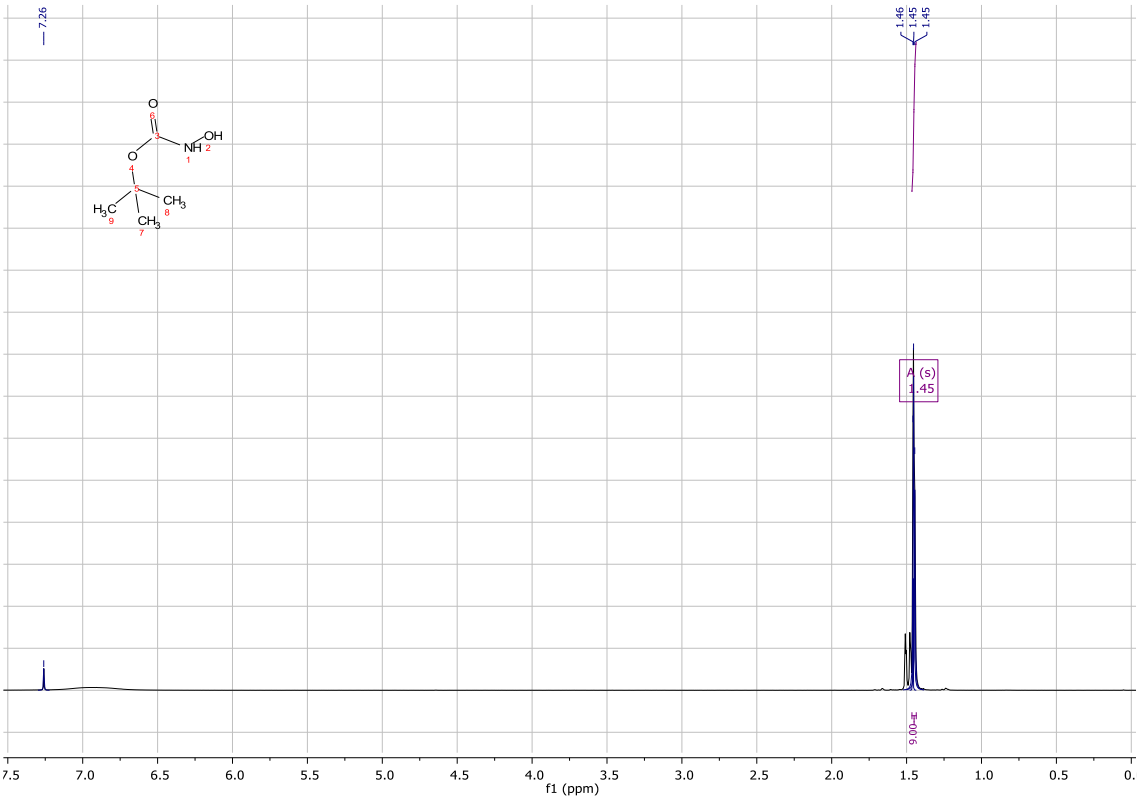
Annex 5: 1-(benzyloxy)-1H-pyrrole-2,5-dione (2) ^{1}H NMR (300 MHz, Chloroform-*d*):



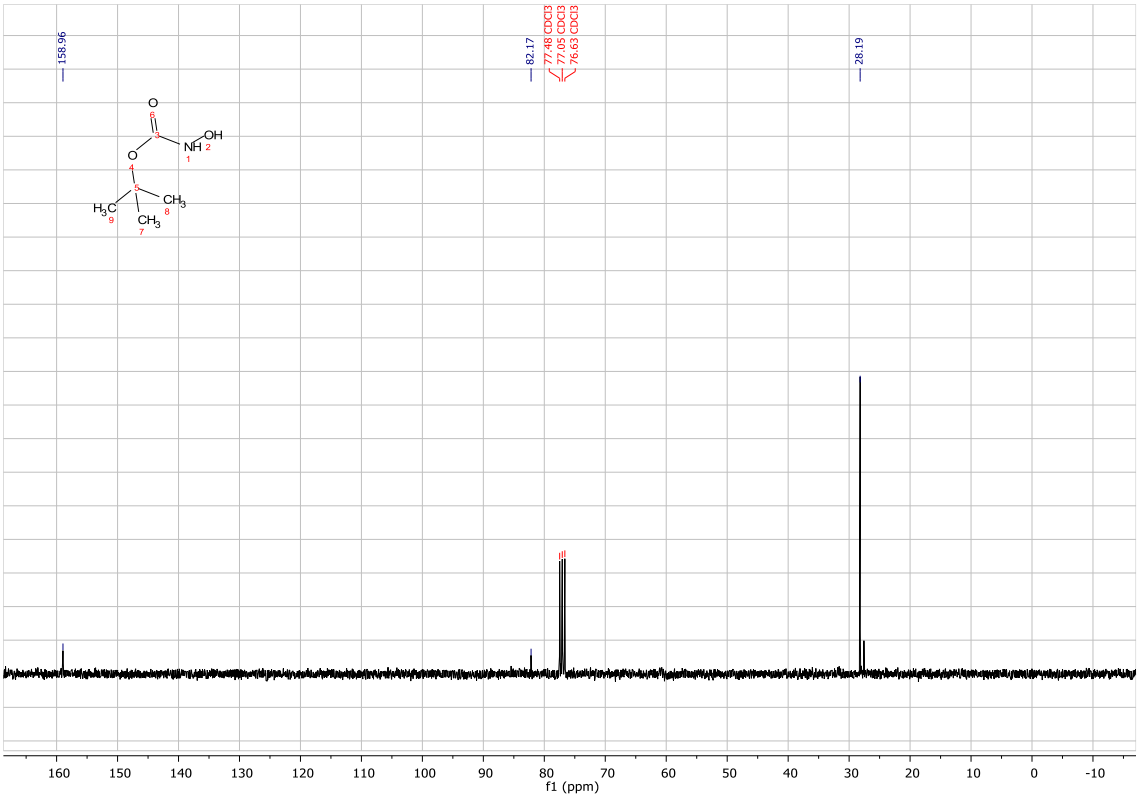
Annex 6-1-(benzyloxy)-1H-pyrrole-2,5-dione (2) ^{13}C NMR (75 MHz, Chloroform-*d*):



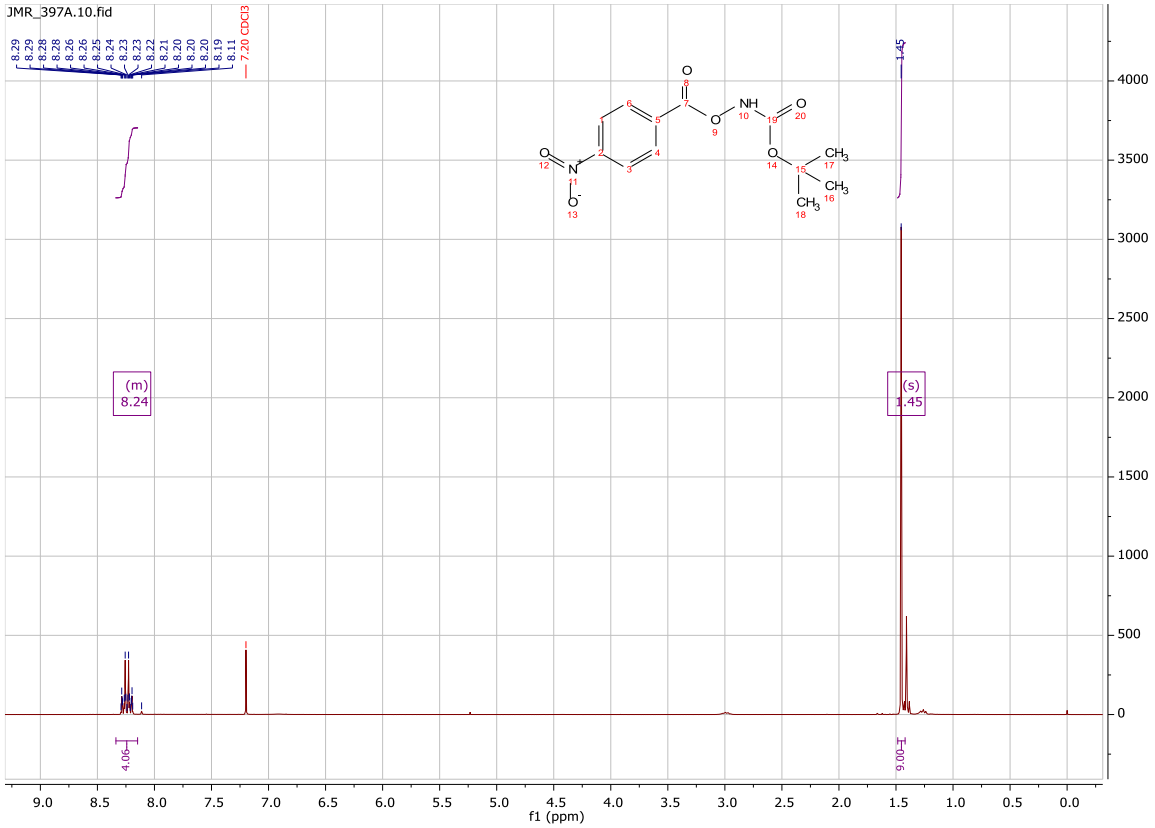
Annex 7- Boc protected hydroxylamine 1H-NMR (5) ^{1}H NMR (300 MHz, Chloroform-*d*):



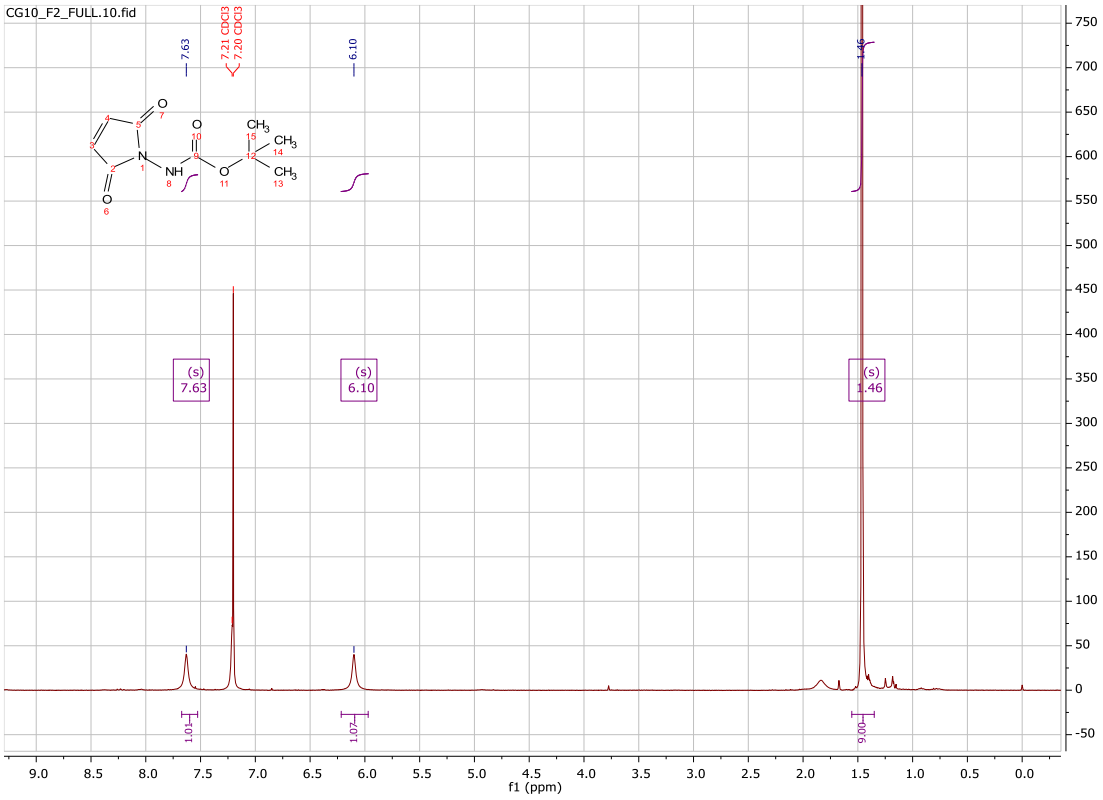
Annex8- Boc protected hydroxylamine (5) ^{13}C NMR (75 MHz, Chloroform-*d*):



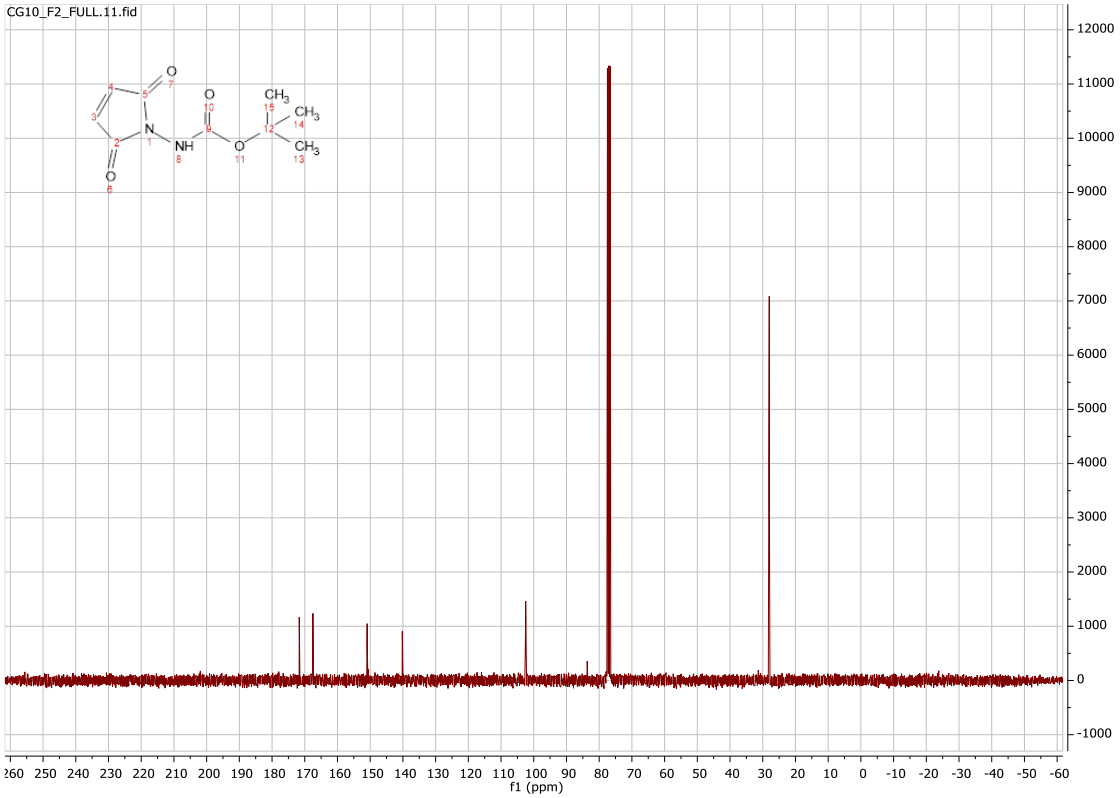
Annex 9- Boc protected O-(4-nitrobenzoyl) hydroxylamine) (7) ^{1}H NMR (300 MHz, Chloroform-*d*):



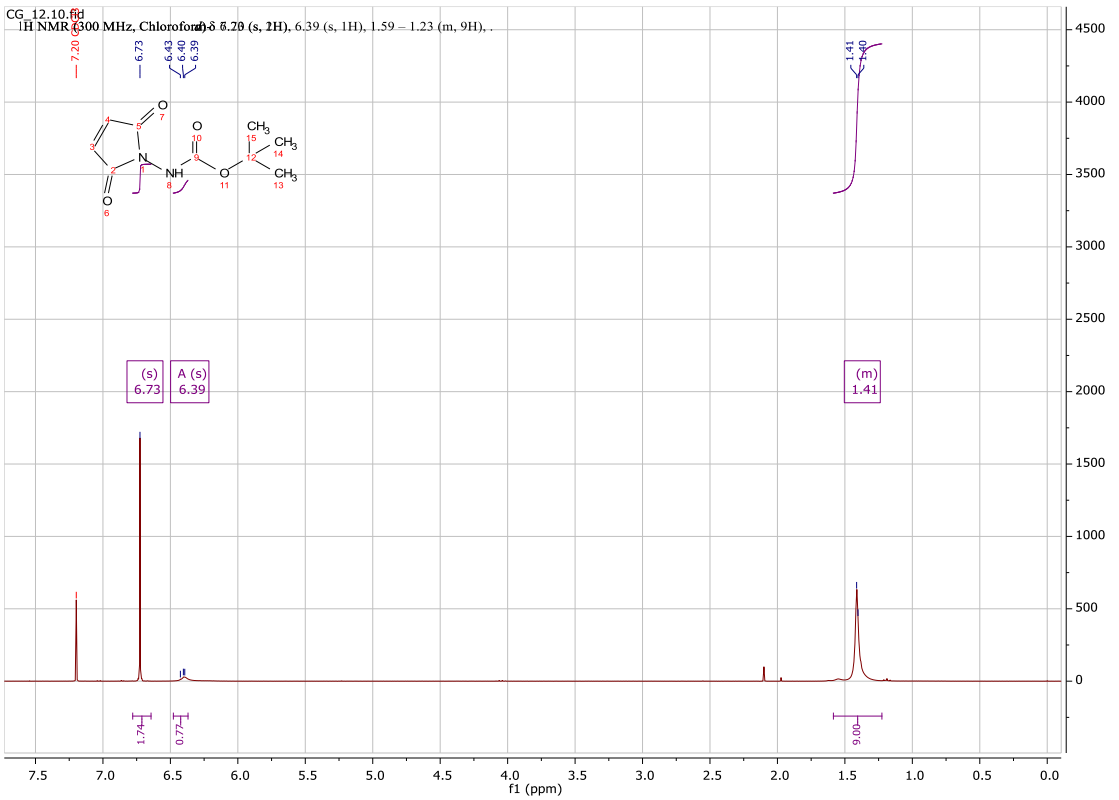
Annex 10- Boc protected 1-amino-1H-pyrrole-2,5-dione (8) ^{1}H NMR (300 MHz, Chloroform-*d*):



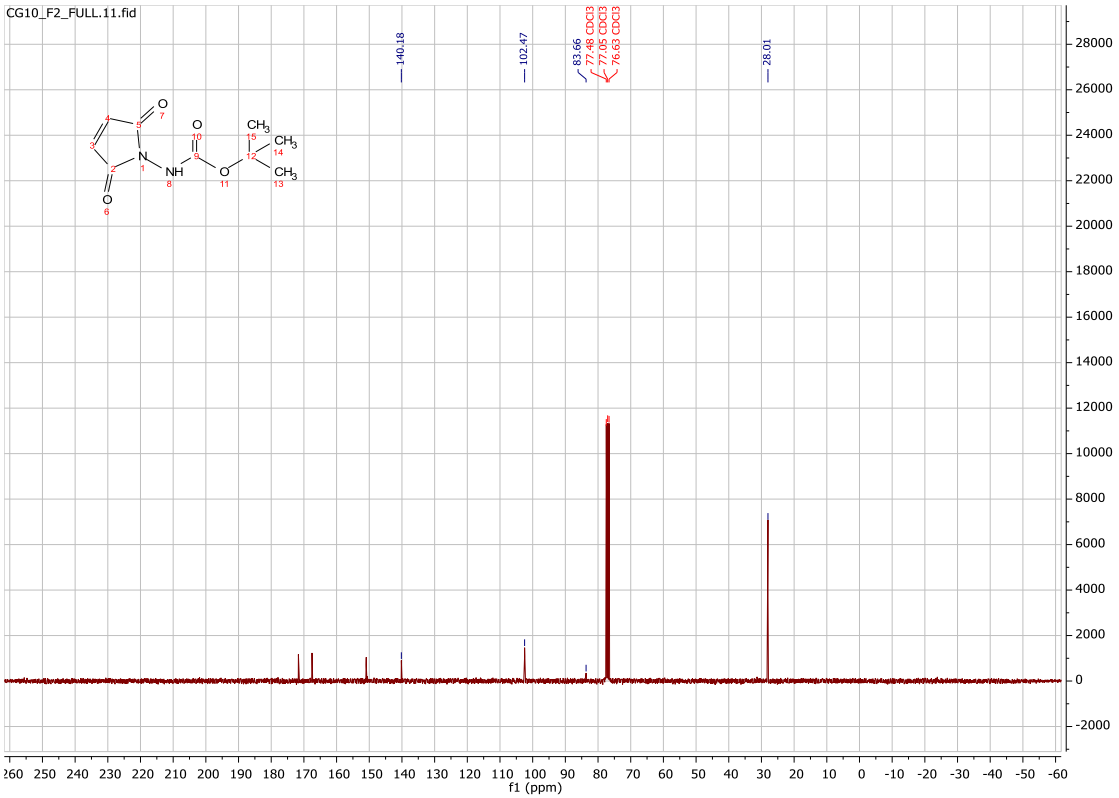
Annex 11- Boc protected 1-amino-1H-pyrrole-2,5-dione (8) ^{13}C NMR (75 MHz, Chloroform-*d*)



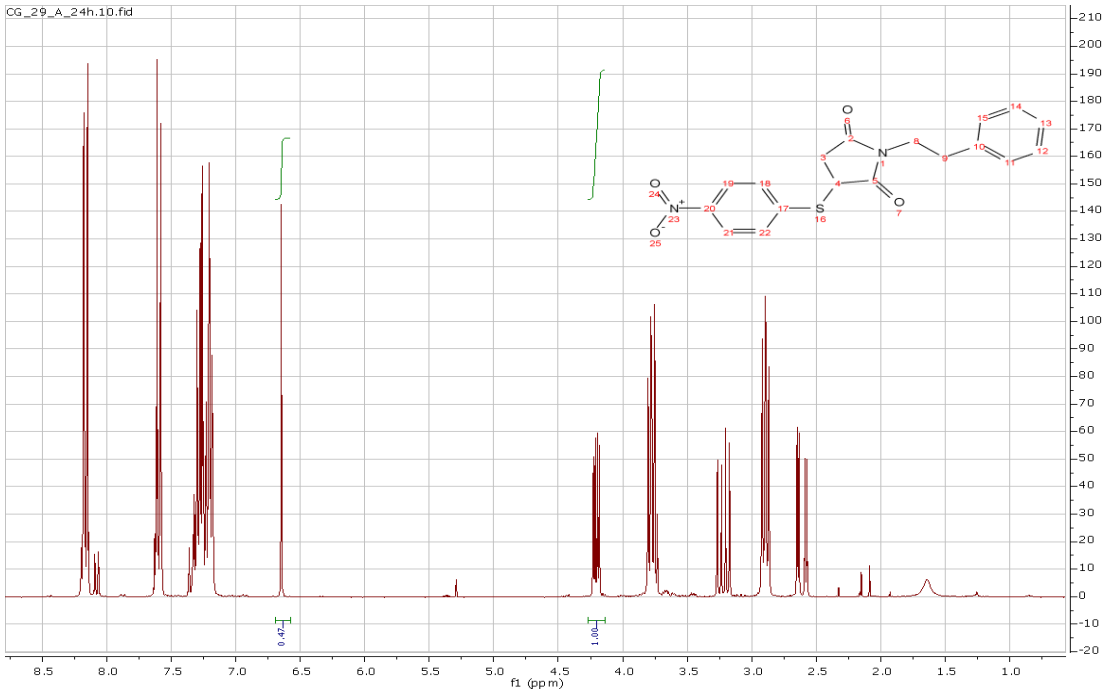
Annex 12: Boc protected 1-amino-1H-pyrrole-2,5-dione (8) ^{1}H NMR (300 MHz, Chloroform-*d*)– Method B



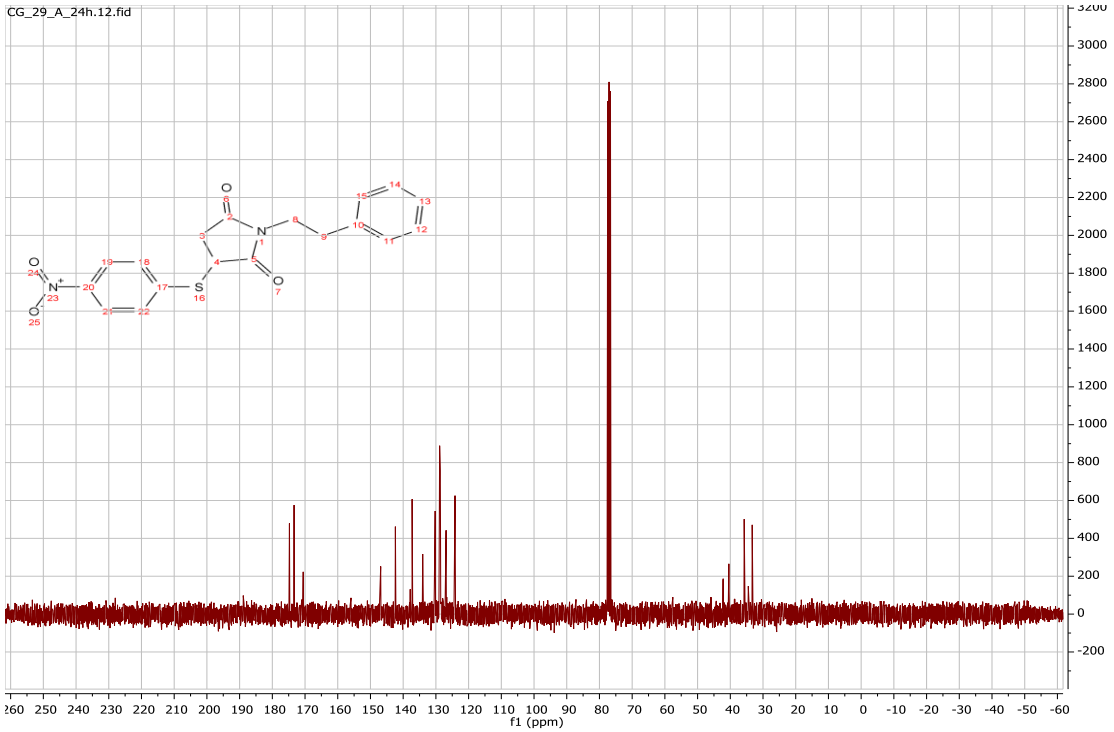
Annex 13- Boc protected 1-amino-1H-pyrrole-2,5-dione^{13}C NMR (75 MHz, Chloroform-*d*)– Method B)



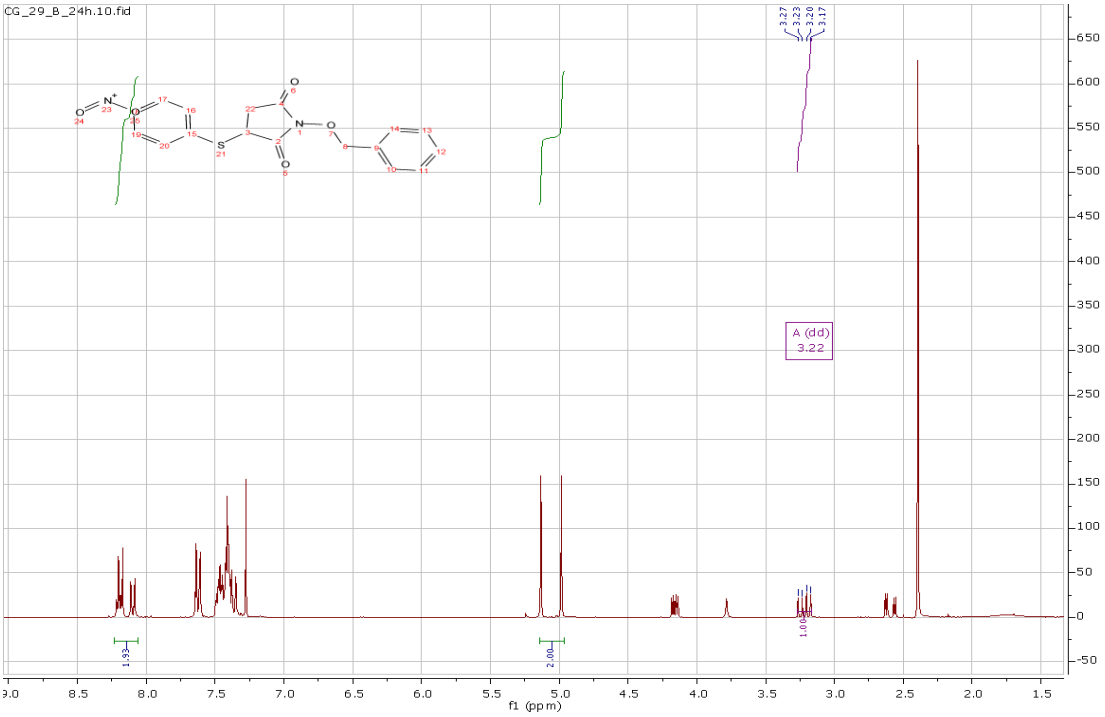
Annex 14- 3-((4-nitrophenyl)thio)-1-phenethylpyrrolidine-2,5-dione (11) ^{1}H NMR (300 MHz, Chloroform-*d*) crude



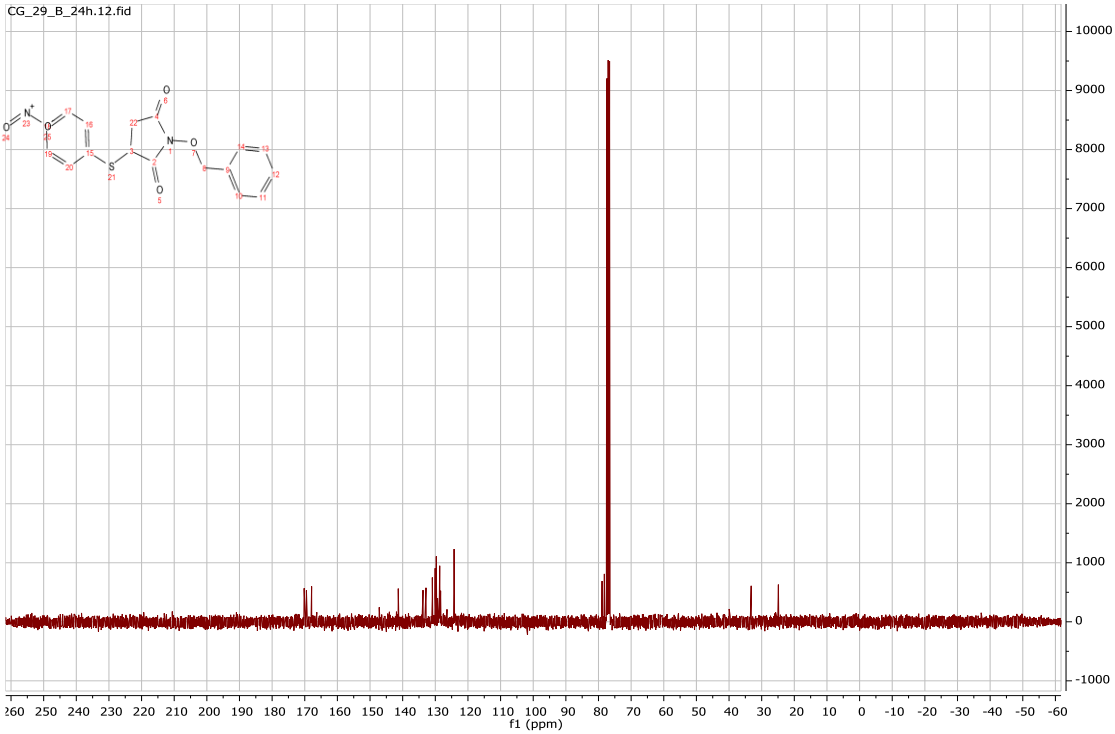
Annex 15-3-((4-nitrophenyl)thio)-1-phenethylpyrrolidine-2,5-dione (11^{13}C NMR (75 MHz, Chloroform-*d*)) crude



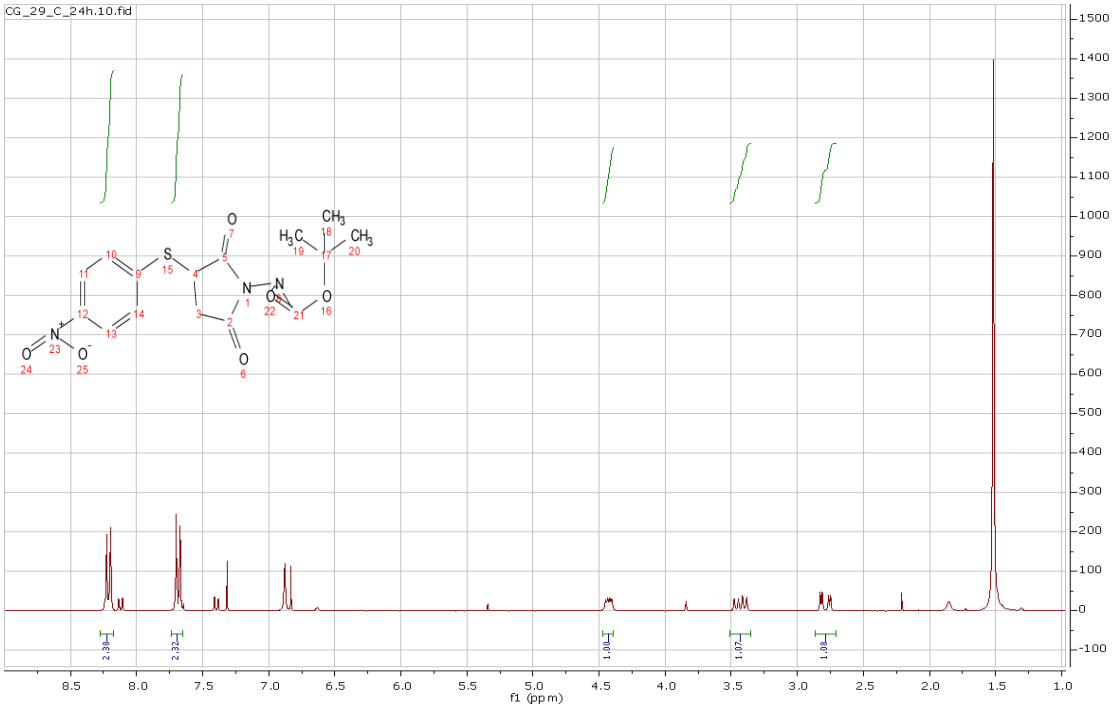
Annex 16- 1-(benzyloxy)-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (12) ^{1}H NMR (300 MHz, Chloroform-*d*) crude



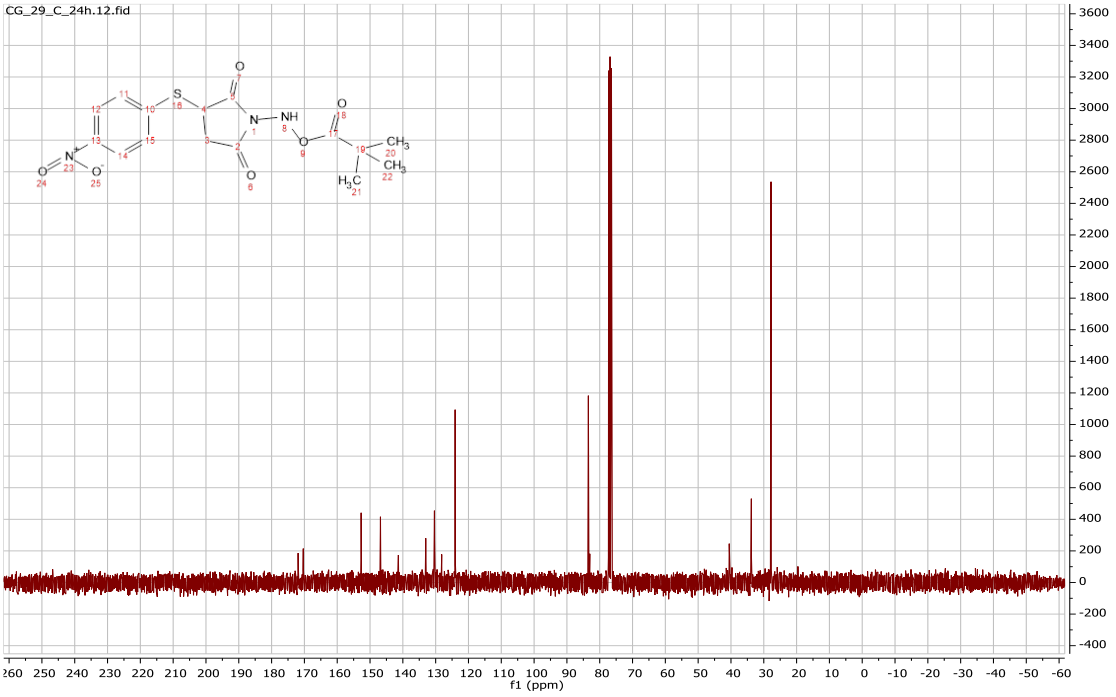
Annex 17- 1-(benzyloxy)-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (12) ^{13}C NMR (75 MHz, Chloroform-*d*) crude



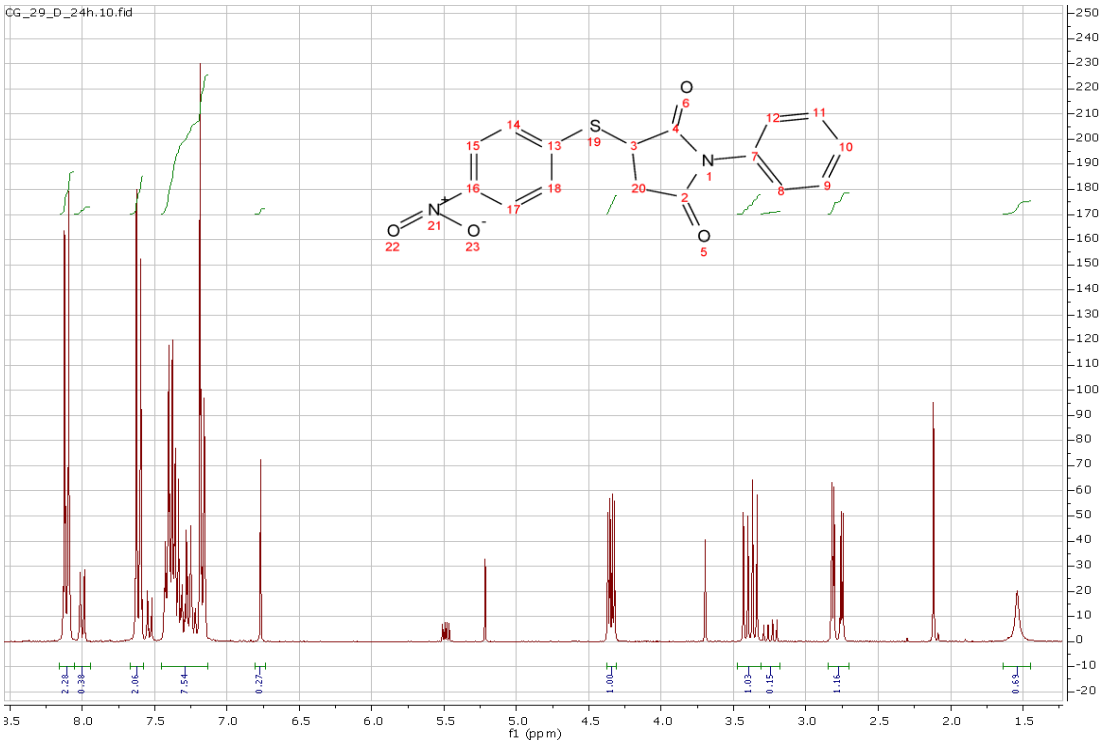
Annex 19- Boc protected 1-amino-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (10) ^{1}H NMR (300 MHz, Chloroform-*d*) crude



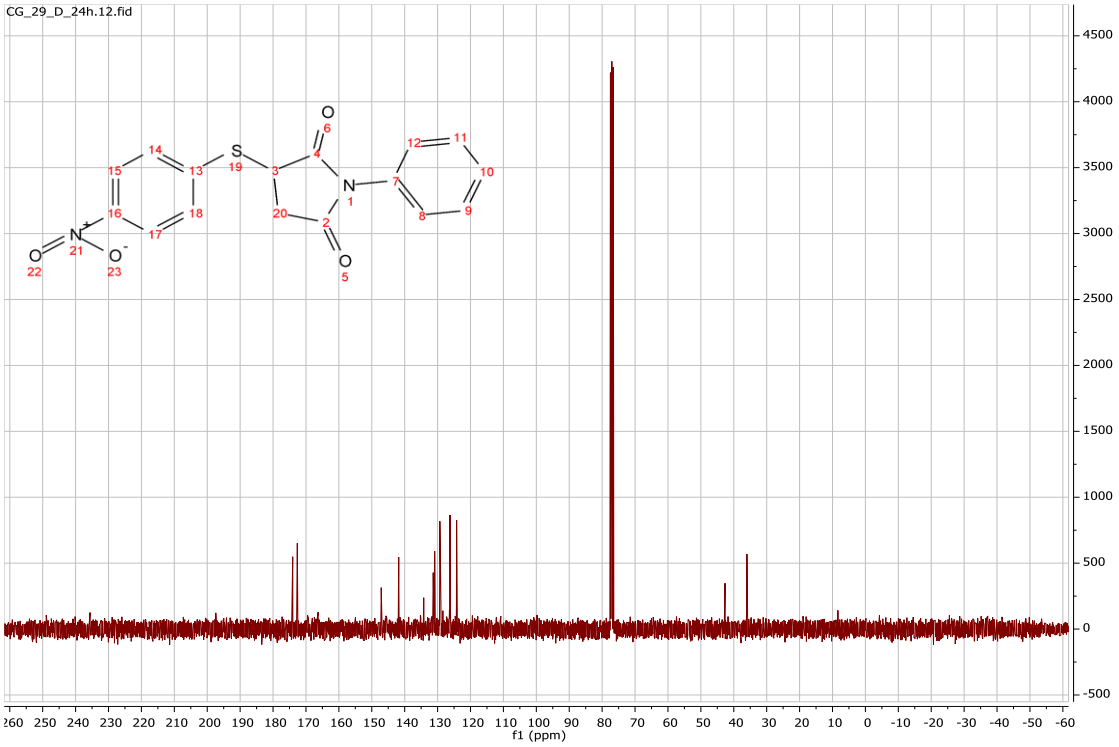
Annex 20- Boc protected 1-amino-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (10) ^{13}C NMR (75 MHz, Chloroform-*d*) crude



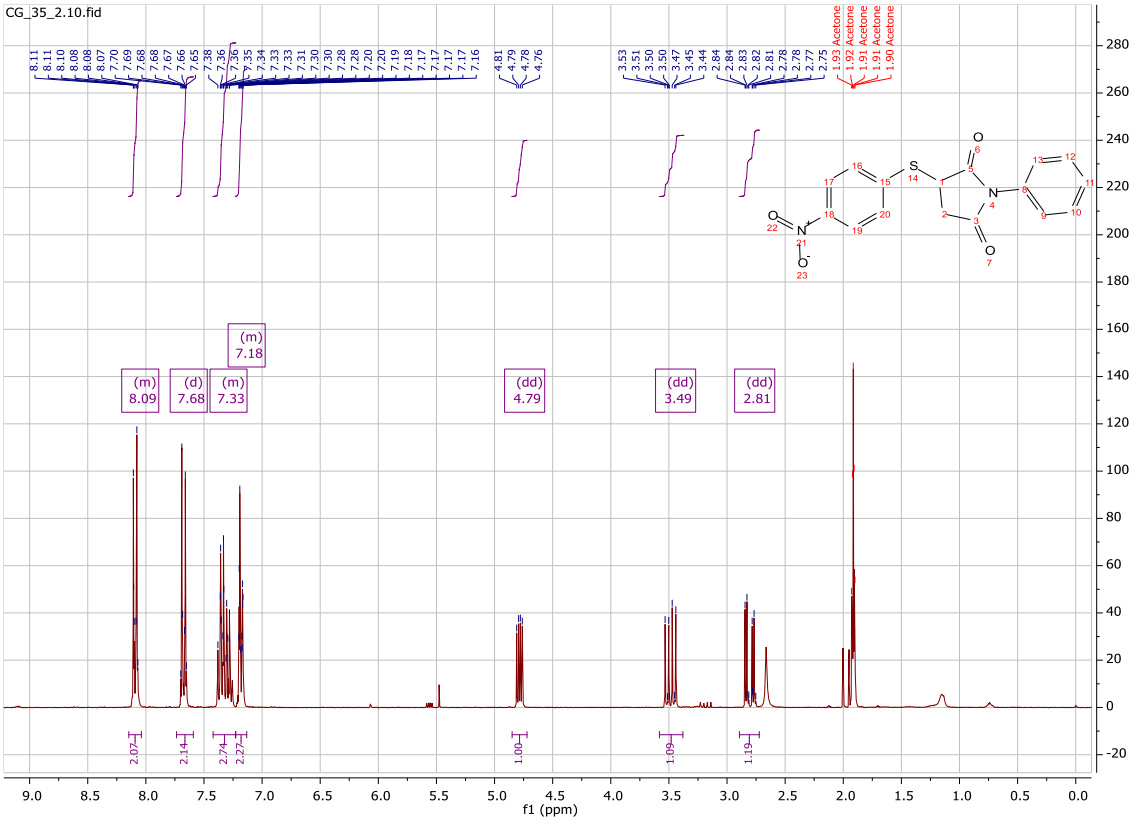
Annex 21- 3-((4-nitrophenyl)thio)-1-phenylpyrrolidine-2,5-dione (9) ^{1}H NMR (300 MHz, Chloroform-*d*) crude



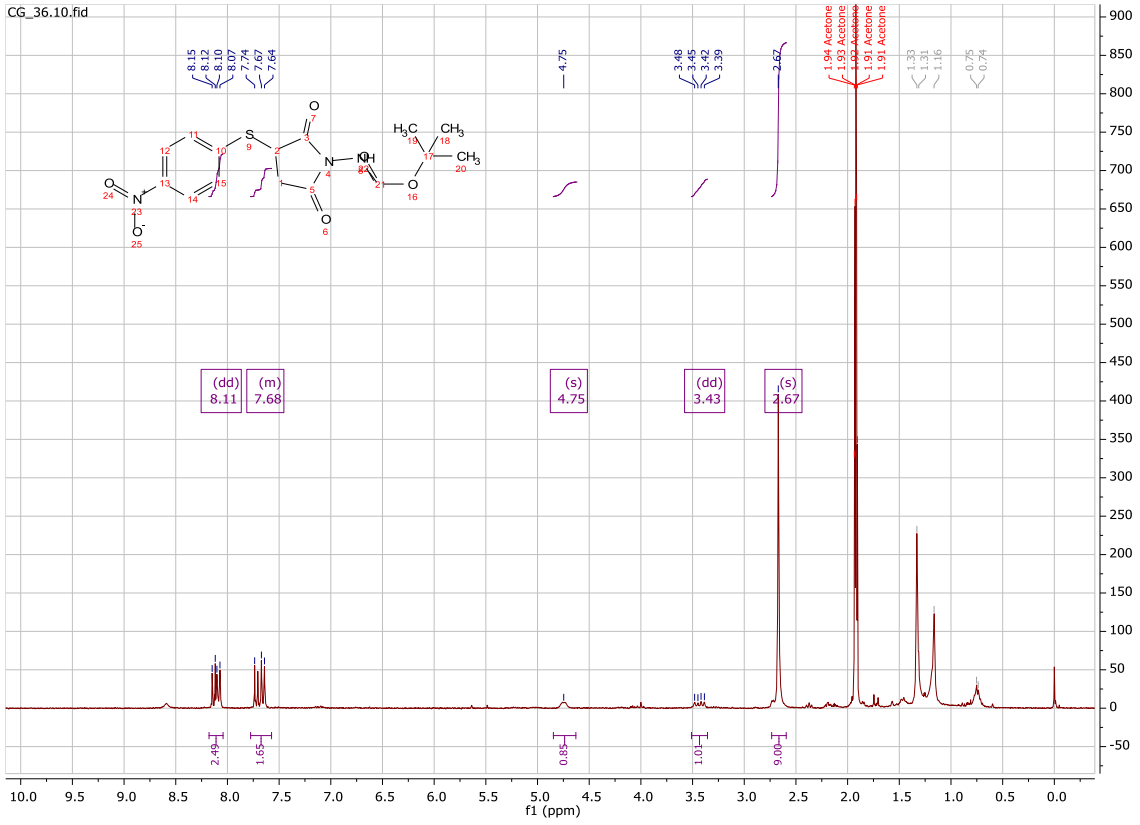
Annex 22- 3-((4-nitrophenyl)thio)-1-phenylpyrrolidine-2,5-dione 13C-NMR (9) ^{13}C NMR (75 MHz, Chloroform-*d*) crude



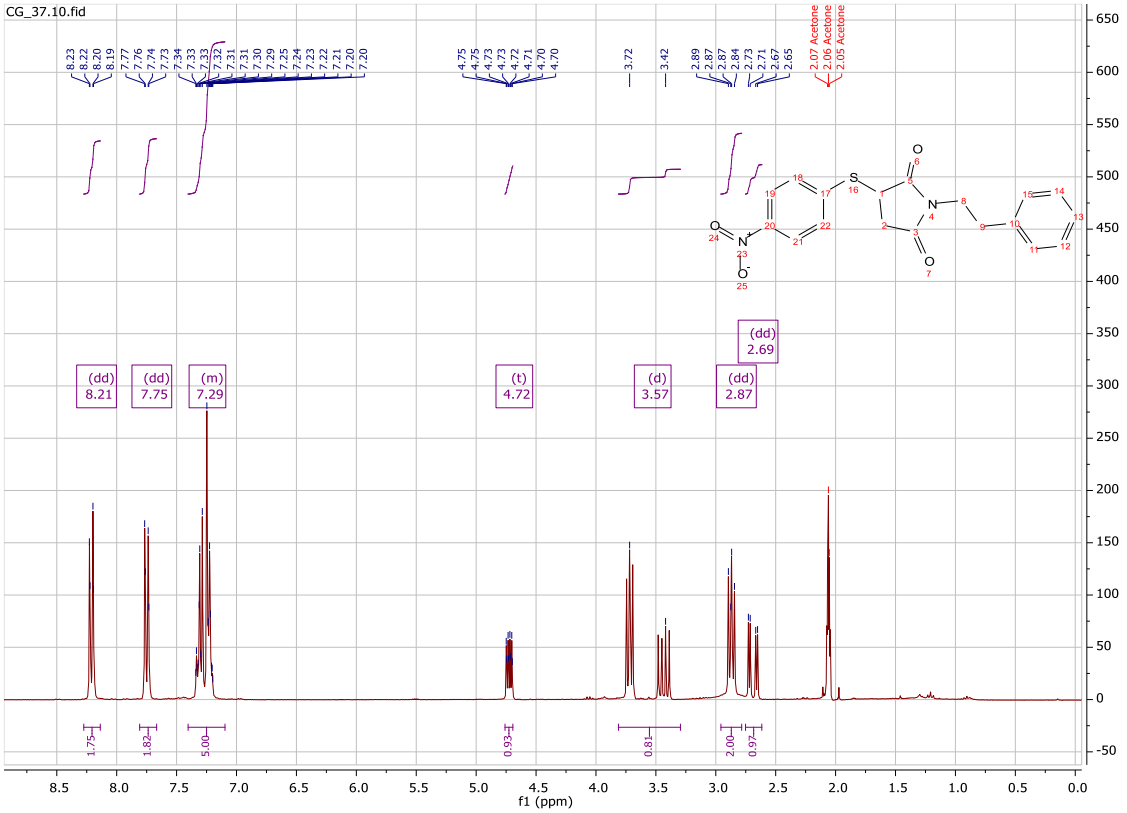
Annex 23- 3-((4-nitrophenyl)thio)-1-phenylpyrrolidine-2,5-dione (9) ^{1}H NMR (300 MHz, Acetone-d_6)



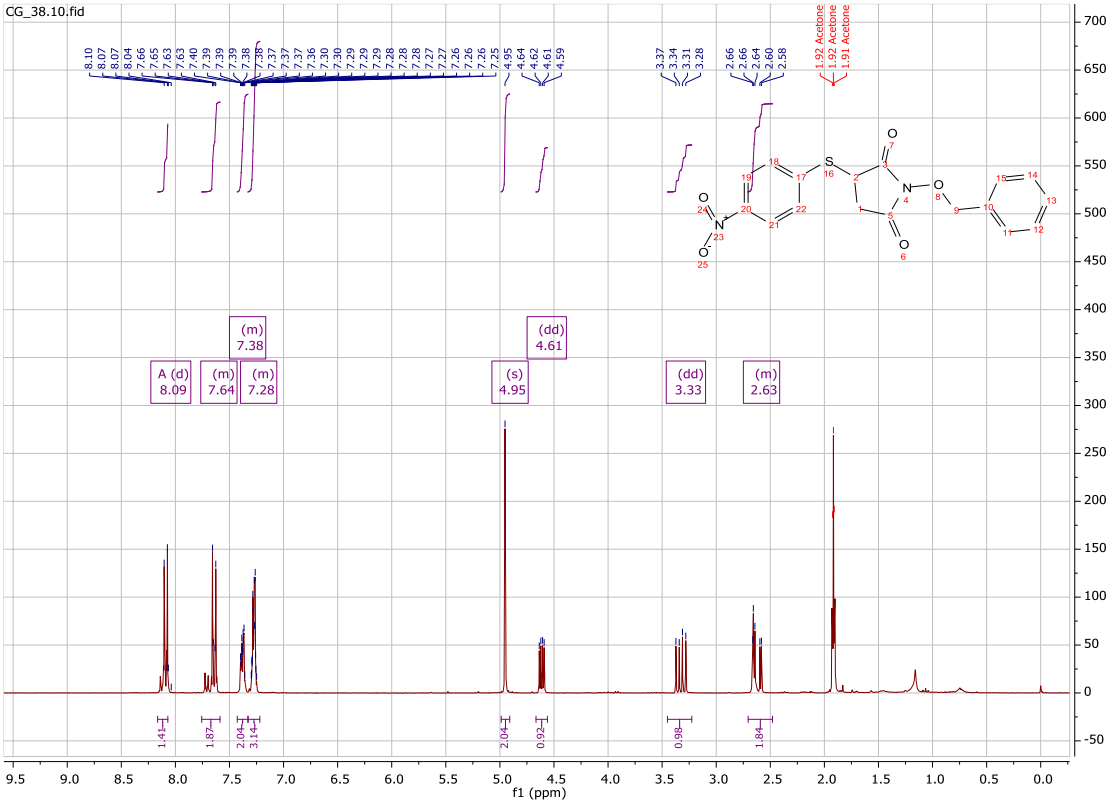
Annex 24- Boc protected 1-amino-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (10) ^{1}H NMR (300 MHz, Acetone-d_6):



Annex 25- 3-((4-nitrophenyl)thio)-1-phenethylpyrrolidine-2,5-dione (11) ^{1}H NMR (300 MHz, Acetone-d_6):



Annex 26: 1-(benzyloxy)-3-((4-nitrophenyl)thio)pyrrolidine-2,5-dione (12) ^{1}H NMR (300 MHz, Acetone-d_6)



UV-Vis Spectra

Annex 27: Cysteine's absorbance