DEVELOPMENT OF SARS-COV-2 INHIBITORS BY FRAGMENT BASED DRUG DISCOVERY

Thesis about the synthesis of NMR spin labeled fragments and spacers

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Internship period: 01-09-2020 t/m 01-06-2021
Date of thesis defense: 16-06-2021
Date of entry: 02-06-2021

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Samenvatting

De COVID-19 pandemie vormt nog steeds een ernstige bedreiging voor de wereldwijde volksgezondheid. Daarom is de wetenschappelijke wereld op zoek naar geschikte vaccins en farmacologische behandelmogelijkheden. Deze scriptie vormt de afsluiting van de stage bij Specs Compound Handling BV en beschrijft de resultaten van een deel van het COVID-19-NMR-project dat is gestart aan de Goethe Universiteit in Frankfurt.

In dit project is de methode van fragment-based drug discovery (FBDD) gebruikt, welke een belangrijk hulpmiddel is geworden in het opsporen van hit compounds voor moeilijk te identificeren doelwitten. Het eerste doel van de stage was de synthese van spin-gelabelde analogen van 19 kleine moleculen (fragmenten). Deze fragmenten vertonen interacties van hoge kwaliteit met het severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA, zoals eerder bepaald was door middel van first-site NMR-screening door de Goethe Universiteit van een bibliotheek van 768 fragmenten. Deze spin-gelabelde analogen maken second-site NMR-screening mogelijk, dat wil zeggen, de detectie van fragmenten die binden in de nabijheid van het eerste gelabelde fragment. Drie verschillende organische reacties (Buchwald-Hartwig aminering, aromatische nucleofiele substitutie en amidevorming) werden gebruikt om met succes een TEMPO-spinlabel te introduceren bij 16 first-site hit fragmenten. Van deze reacties vertoonde aromatische nucleofiele substitutie een lage opbrengst of helemaal geen producten.

De gelabelde fragmenten werden ter beschikking gesteld voor second-site NMR-screening. De voorlopige resultaten van deze screening zijn veelbelovend omdat er een belangrijke second-site hit werd gevonden in de aanwezigheid van gelabelde verbinding 19, wat te zien was door het uitdoven van het NMR-signaal.
Het tweede doel van de stage was het covalent koppelen van first-site en second-site fragmenten door middel van een spacer. Omdat de resultaten van de second-site screening recent naar voren kwamen, was het niet mogelijk om deze toe te passen bij de synthese van gekoppelde fragmenten. Daarom werd als proof of principle een 8-staps chemische route ontwikkeld om twee verschillende fragmenten met succes aan elkaar te koppelen. De lengte van de spacer tussen de twee fragmenten was 9 atomen, dit werd bepaald door moleculaire modellering.
De resultaten tonen aan dat de huidige FBDD-aanpak veelbelovend kan zijn bij de ontwikkeling van remmende geneesmiddelen die zich richten op het SARS-CoV-2-RNA.

Summary

The coronavirus disease-19 (COVID-19) pandemic still poses serious threats to global public health. Therefore, the scientific community is desperately looking for suitable vaccines and pharmacological treatment options. This thesis completes the internship at Specs Compound Handling BV and describes the results of a part of the COVID-19-NMR project initiated at the Goethe University in Frankfurt.

In this project, the method of fragment-based drug discovery (FBDD) was used, which has become an important tool for finding hit compounds for difficult targets. The first goal of the internship was the synthesis of spin labeled analogues of 19 small molecules (fragments). These fragments show high quality interactions with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA as previously detected by first-site NMR screening by the Goethe University of a library of 768 fragments. These spin labeled analogues allow for second-site NMR screening, that is, the detection of fragments that bind in the vicinity of the first labeled fragment. Three different organic reactions (Buchwald-Hartwig amination, aromatic nucleophilic substitution and amide formation) were used to successfully introduce a TEMPO spin label for 16 first-site hit fragments. Of these reactions, aromatic nucleophilic substitution showed low yield or no products at all.

The labeled fragments were submitted for second-site NMR screening. The preliminary second site screening results are promising because a major second-site hit was found in the presence of labeled compound 19, as seen by quenching of the NMR signal.

The second goal of the internship was to covalently link first-site and second-site fragments by means of a spacer. Because the second-site screening results emerged very recently, it was not possible to apply them in the synthesis of linked fragments. Therefore, as a proof of principle, an 8-step chemical route was developed to successfully link two different fragments. The length of the spacer between the two fragments was 9 atoms as determined by molecular modeling.
The results show that the present FBDD approach may be promising in the development of inhibitor drugs targeting the SARS-CoV-2 RNA.

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List of abbreviations

BMRZ	Biological Magnetic Resonance Center
Boc	Tert-butyloxycarbonyl
Boc_2O	Di-tert-butyl decarbonate
BuOH	Butanol
COVID-19	Coronavirus disease-19
DavePhos	2-Dicyclohexylphosphino-2'-(N,N-dimethylamino)biphenyl
DCM	Dichloromethane
DIPEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EtOAc	Ethyl acetate
EtOH	Ethanol
FBDD	Fragment-based drug discovery
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HOBt Hydate	1-Hydroxybenzotriazole hydrate
iNEXT	Infrastructure for NMR, EM and X-rays for translational research
i-PrOH	Iso-propanol
LC-MS	Liquid chromatography mass spectrometry
MeOH	Methanol
NaOtBu	Sodium tert-butoxide
NMR	Nuclear magnetic resonance
PE	Petroleum ether (40-60)
PyBOP	Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SRL	Specs Research Laboratory
TEMPO	2,2,6,6-tetramethylpiperidine 1-oxyl free radical
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
XPhos	2-Dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl

1. Introduction

1.1. Specs Compound Handling BV

Specs, headquartered in Zoetermeer The Netherlands, was founded in 1987. Its laboratory, the Specs Research Laboratory (SRL) was founded in 2001. At that time the primary focus of the company was the purchase of a wide array of different chemicals, to store and sell to the Life Science Industry. Nowadays, Specs is an internationally known name, and the world's leading provider of compound management services as well as being the main supplier of screening compounds and building blocks. Compound management involves the logistics, storing, tracking, dispensing, and preparation of compounds to be tested and analyzed in drug discovery processes. SRL is able to synthesize a large variety of molecules including new classes of compounds, natural products, building blocks and scaffolds for further synthesis, synthesize reference compounds and perform patent marker synthesis as well as parallel synthesis of focused libraries for lead discovery and hit-to-lead optimization.

Specs takes part in a number of collaborations with different small biotech and pharmaceutical companies, as well as universities. These collaborations combine the strength and expertise of small biotech and pharmaceutical companies and the academic world[1].

1.2. COVID-19-NMR project

The coronavirus disease-19 (COVID-19) pandemic still poses serious threats to global public health. Therefore, the scientific community is desperately looking for suitable vaccines and pharmacological treatment options. The COVID-19-NMR project was initiated at the Goethe University in Frankfurt in March 2020 and has since quickly grown into an international consortium[2]. Today, scientists from all over the world are collaborating in a unique effort to investigate the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) using NMR spectroscopy, based on open science principles. The aim of the international COVID-19-NMR project is to determine RNA and protein structures of SARS-CoV-2 and to investigate the drugability of those structures by small molecules[3]. The present investigation is a collaboration between Specs and the Goethe University, specifically the department Biological Magnetic Resonance Center (BMRZ). The BMRZ was founded in 2002 and is a research infrastructure facility for high-end nuclear (NMR) and electron magnetic resonance spectroscopy. The research is dedicated to the elucidation of structure and functional mechanism of biomolecules ranging from RNA and RNA-protein complexes via large soluble protein complexes to membrane proteins[4].

1.2.1. Fragment-based drug discovery

The traditional drug discovery process will take too long to effectively counter the current pandemic outbreak. Therefore, in this project a newly developed method of fragment-based drug discovery (FBDD) was used, which allows for the development of drugs in a more structural and efficient way than before. In FBDD a library of relatively small fragments is used to synthesize new drugs. Infrastructure for NMR, EM and X-rays for translational research (iNEXT), is a European Commission-funded Horizon 2020 network to facilitate trans-national user access to several high-end structural biology infrastructures, such as NMR facilities. iNEXT has assembled and validated a library of 768 fragments for FBDD. However, the number of compounds in the library is still increasing[5,6].

1.2.1.1. First-site NMR screening

In this project, the first step in FBDD is the first-site screening of the fragment library against the target molecule (see figure 1)[7].

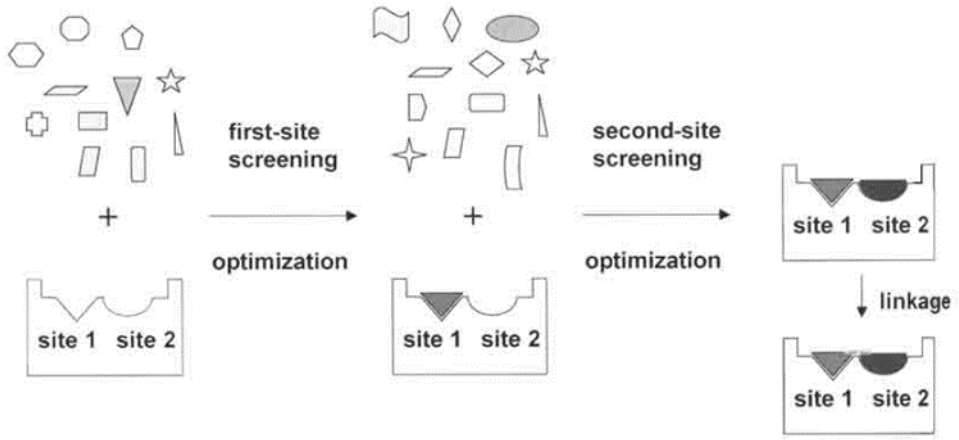


Figure 1: fragment-based drug discovery.

A major challenge of first-site fragment screening is that, although fragment sized compounds make high quality interactions with their targets and bind with high ligand efficiency, overall binding affinity is typically weak due to their small size. Therefore, the techniques used to detect fragment binding must be sensitive on the μM to mM scale. NMR allows the detection of weak interactions between ligand and target and can be used to quantify binding affinities in order to establish structure-activity relationships. Using ^{1}H-NMR, the Goethe University screened 768 fragments from the iNEXT library on 10 selected COVID RNA target molecules. Hereby, 212 hits and 140 selective hits were found. Out of these 140 selective hits, 19 fragments, so-called promiscuous hits were found. This means that each of these fragments binds to different binding sites on the RNA. The area where the fragments bind to the RNA is relatively large and consists of several subdomains where fragments can bind simultaneously.

1.2.1.2. Second-site NMR screening using spin labeled first ligands

The next goal is to identify a second binding site (second-site) right next to the binding site found (first-site). The method used for this is second-site NMR screening by means of saturation-transfer difference NMR. In second-site NMR screening, the first-site fragment is labeled with a spin label such as 2,2,6,6-tetramethylpiperidine 1-oxyl free radical (TEMPO) (see figure 2 and 3). The presence of such a spin label leads to drastically increased spin-spin (R2) relaxation rates of neighboring protons. In extreme cases this effect is so large that the resonances of neighboring protons are completely quenched and invisible in the NMR spectrum. In second-site NMR screening using spin labeled first ligands, the quenching effects of the spin label onto the second ligand are observed. These quenching effects occur if and only if the second ligand is bound to the RNA target simultaneously and in the vicinity of the spin labeled first ligand. In extreme cases, protons from the second fragment become invisible in the NMR spectrum. Since protons oriented towards the spin label experience a larger spin-spin relaxation than protons on the other side of the second ligand, these spin label experiments also will render information on the relative orientation of both ligands towards each other, which is valuable for the design of a spacer between both ligands[7,8].

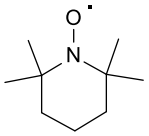


Figure 2: TEMPO-radical with an unpaired electron.

1.2.1.3. Synthesis of linked fragments

In case of a "second hit", the next aim is to covalently link the first-site and second-site fragment by means of a spacer. Both fragments are typically weak ligands for the target molecule, which is why NMR is a useful experimental approach to realize fragment-based screening. However, affinity can increase dramatically upon linkage of the two ligands[7]. In the design of the linker, the distance between the two fragments is important for maintaining the affinity of both fragments. Also, the flexibility of the linker may be of importance.

1.3 Goals of the internship

The objectives of this internship are listed as followed:

- Synthesis of TEMPO-analogues of the active first-site fragments.
  All the active first-site fragments found by the Goethe University contain an aromatic unit to which a cyclic aliphatic group is attached, either directly linked or tethered by a small spacer. The first step in the project is to replace these aliphatic groups with the TEMPO spin label (see figure 3)
  and submit them for testing in the COVID-19-NMR project.

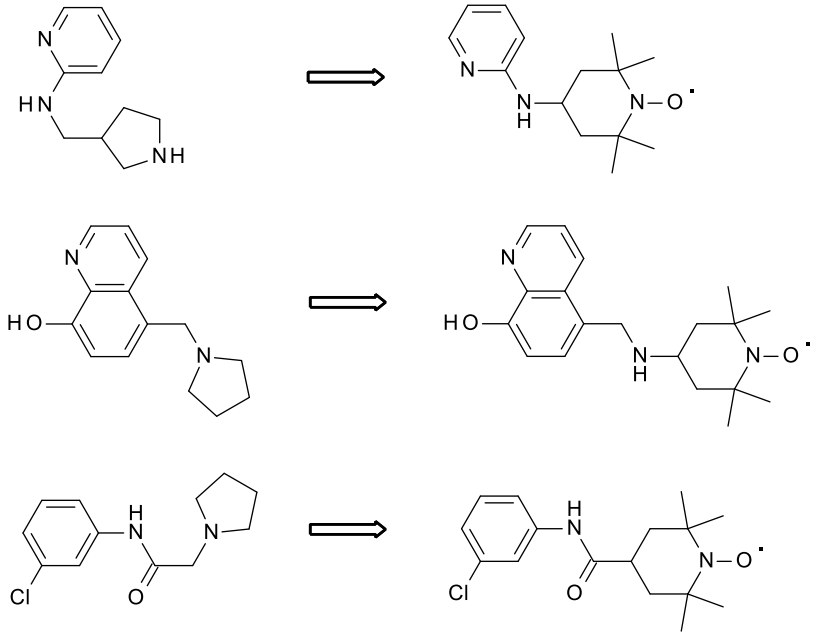


Figure 3: Several active fragments and their proposed spin labeled analogues.

- Synthesis of linked fragments.
  Based on the results of second-site screening, synthetic routes have to be devised to chemically link both fragments with a variety of spacers.
- Purify and characterize the intermediates and final products.
- Participate in the research group and report to the project leaders.
- Write a full report that is up to the standards of the internship provider (Specs) and the student's institution (Hogeschool Leiden).

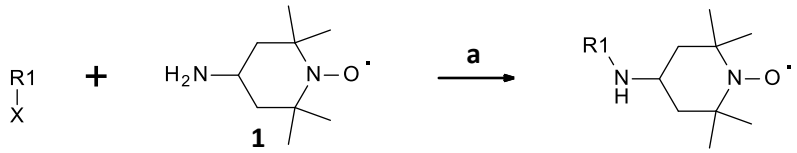
1.4. Different types of reactions in the synthesis of TEMPO-analogues of first-site fragments

The following chemical coupling reactions were performed with 4-amino-TEMPO (compound **1**) or 4-carboxy-TEMPO (compound **2**) and purchased or synthesized compounds (see table 1, 2 and 3).

- Aromatic nucleophilic substitution
- Buchwald-Hartwig amination
- Amide formation

1.4.1. Aromatic nucleophilic substitution

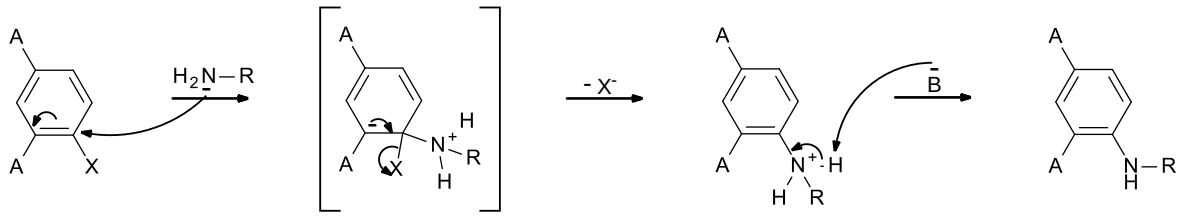
In the used aromatic nucleophilic substitution and Buchwald-Hartwig amination reactions, the functional groups are the amino group in 4-amino-TEMPO (compound **1**) and a halide (X) as present on the aromatic ring (R1) of the compounds (see table 1 on page 11). The general reaction scheme for these reactions is depicted in scheme 1.



Scheme 1: Aromatic nucleophilic substitution with a halide (X) as functional group present on the aromatic ring (R1). Standard reagents and conditions: (a) Et_3N, DMF, 125 °C Buchwald-Hartwig amination. Standard reagents and conditions: (a) $Pd_2(dba)_3$, DavePhos, NaOtBu, dioxane, 90 °C

The compounds used in the reactions contain aromatic rings, which can participate in substitution reactions. Aryl halides with a strong electron-withdrawing substituent(s) on the ring can undergo aromatic nucleophilic substitution.

A general mechanism for the aromatic nucleophilic substitution is depicted in scheme 2[9,10]. To simplify the scheme, A stands for any electron withdrawing group and **1** as R-NH_2. R-NH_2 acts as the nucleophile attacking the electron-deficient aryl halide, forming a resonance-stabilized intermediate called a Meisenheimer complex. The halide ion acts as a leaving group and is eliminated. Next, the amine is deprotonated by the base[9]. Aromatic nucleophilic substitution occurs if the aromatic ring has an electron-withdrawing substituent in a position ortho or para to the leaving group to stabilize the intermediate through resonance.



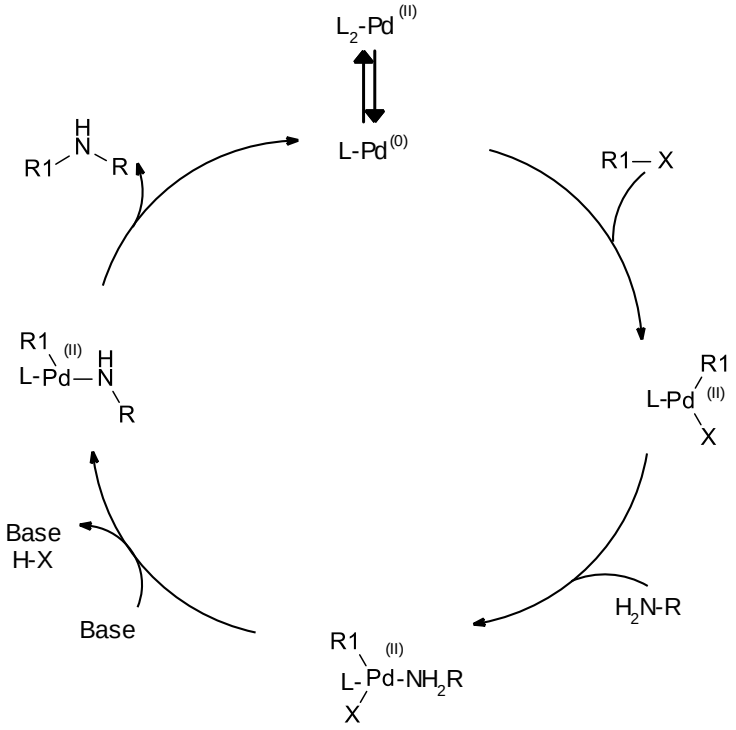
Scheme 2: Aromatic nucleophilic substitution general mechanism

In case of a pyridine ring, the electronegativity of the N atom makes the C atoms susceptible to attack by nucleophiles. Attack at atoms C(2) and C(4) is more favourable than at the C(3) position because of resonance stabilization for the intermediate in each reaction[11]. For the pyridine ring aromatic nucleophilic substitution is favored by strong negatively charged nucleophiles and good leaving groups such as Cl or Br.

1.4.2. Buchwald-Hartwig amination

The Buchwald-Hartwig amination uses a palladium $^{(0)}$ catalyst. A plausible mechanism for the Buchwald-Hartwig amination is depicted in scheme 3[12]. To simplify the scheme, the organohalide (aromatic halide) is abbreviated as R1-X (depicted in table 1 on page 11) and **1** as R-NH_2.

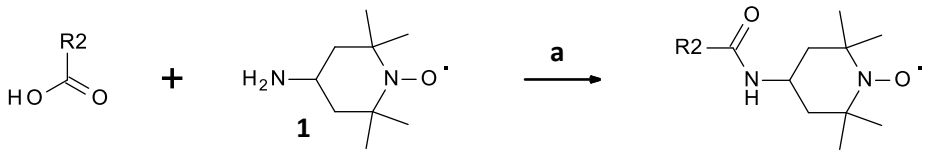
The first step in the mechanism is the oxidative addition of R1-X to the $Pd^{(0)}$ to form an organopalladium intermediate $Pd^{(II)}$ complex. The next step is a coordination of R-NH_2 where another intermediate is formed. Deprotonation of the amine occurs, the base and H-X are expelled. Reductive elimination, gives the final compound and converts the catalyst back to its initial $Pd^{(0)}$. In some cases, β-hydride elimination competes with reductive elimination. However, in the here performed experiments no proof has been found for this to occur.



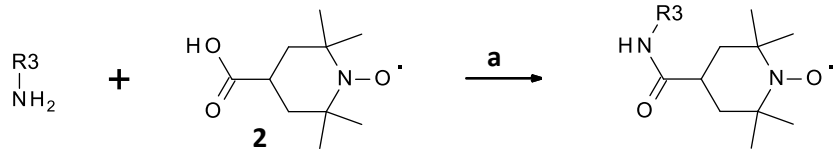
Scheme 3: Buchwald-Hartwig amination mechanism

1.4.2. Amide formation

The general reactions of the amide formation are depicted in scheme 4 and 5. The different R2 and R3 groups are later in the report depicted in table 2 on page 17.

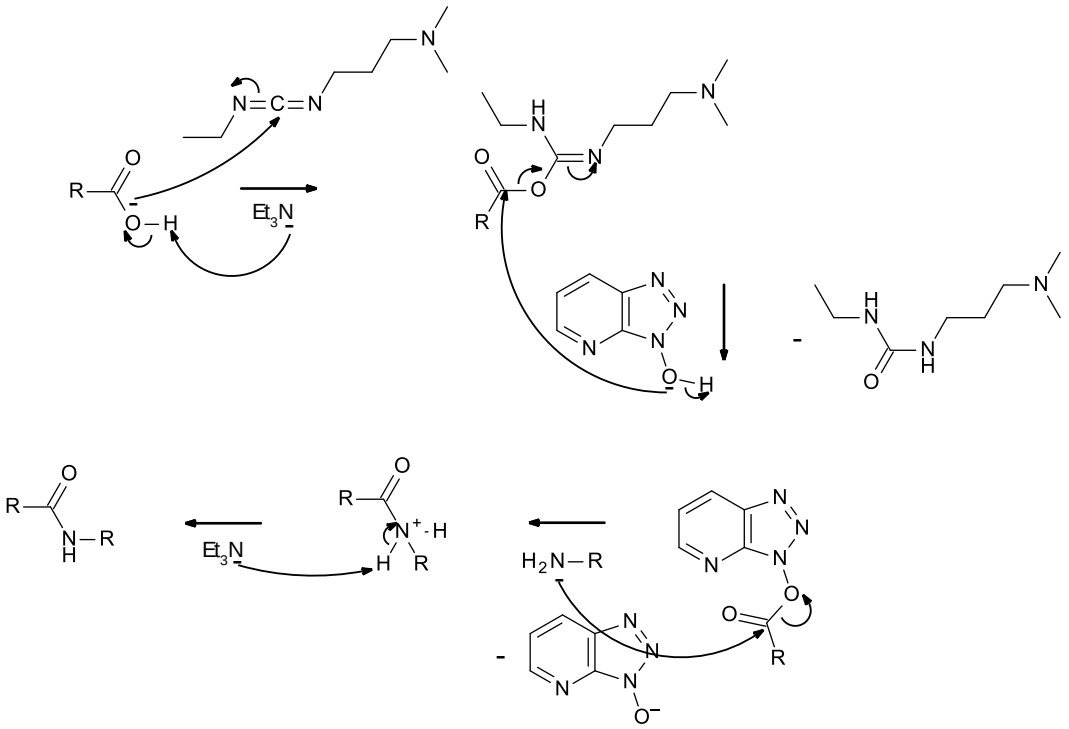


*Scheme 4: Amide formation R2 group with **1**. Reagents and conditions: (a) EDCI, HOBt, Et_3N, DCM or THF, rt*



*Scheme 5: Amide formation R3 group with **2**. Reagents and conditions: (a) EDCI, HOBt, Et3N, DCM or THF, rt*

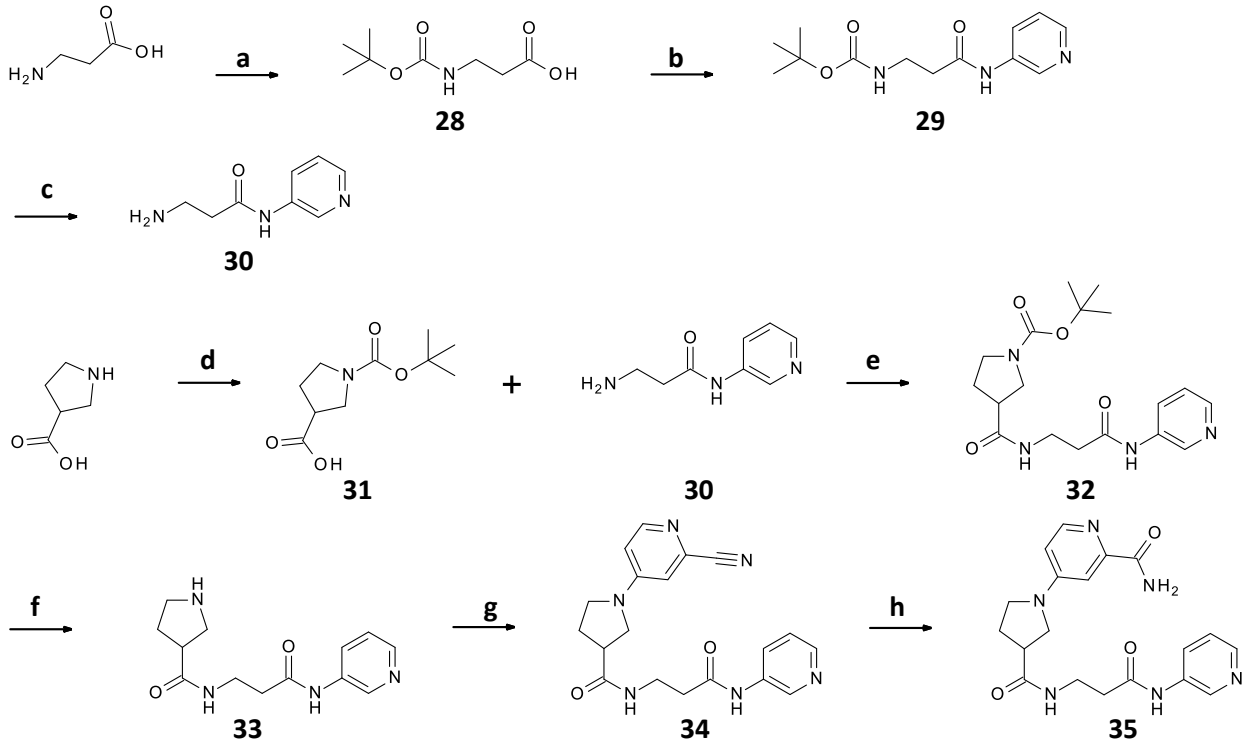
The general mechanism is depicted in scheme 6[13]. For the first step, the carboxylic acid is deprotonated by Et_3N. The anion of the carboxylic acid attacks 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI). Next, the oxygen from hydroxybenzotriazole (HOBt) attacks the carbonyl carbon, which removes the corresponding urea. The R-NH_2 attacks the carbonyl carbon, which removes the HOBt anion. Finally, the base (Et_3N) deprotonates the amine to form the end product.



Scheme 6: General amide formation mechanism. – describes a lone electron pair in this and following mechanisms.

1.5. A method to synthesize linked fragments

The synthesis of two fragments linked by a spacer was performed by an 8-step reaction sequence. The synthesis pathway of the spacer is depicted in scheme 7.



*Scheme 7: Synthesis pathway of compound **35**. Reagents and conditions: (a) Boc_2O, NaOH, THF, 0°C-rt , 23 h, quantitative yield; (b) pyridine-3-amine, HATU, Et_3N, DCM, rt; (c) TFA, DCM, rt; (d) Boc_2O, 1:3 water: acetone, 0°C-rt, 22 h, quantitative yield; (e) DIPEA, PyBOP, DMF, rt); (f) TFA, DCM, rt; (g) 4-bromopyridine-2-carbonitrile, Et_3N, DMSO, 130 °C, 2 h, 57%; (h) K2CO3, H2O2 35%, DMSO, rt, 22 h, 43%.*

The first step (**a**) was the protection of the primary amine with a tert-butyloxycarbonyl (Boc) protecting group by forming a carbamate compound **28**. Next (**b**) pyridine-3-amine was coupled with compound **28** by amide formation to give compound **29**. In the third step (**c**) the amine was deprotected with trifluoroacetic acid (TFA) to give compound **30**. In a second reaction sequence (**d**), the secondary amine of pyrrolidine-3-carboxylic acid was protected with a Boc protecting group to produce compound **31**. Amide formation (**e**) between compound **30** and **31** gave the Boc-protected compound **32**. Deprotecting the Boc group (**f**) from the secondary amine with TFA gave compound **33**, which was reacted with a4-bromopyridine-carbonitrile in an aromatic nucleophilic substitution reaction (**g**) to give compound **34**. Finally (**h**), the nitrile was oxidized to the amide using 35% H_2O_2 to give the final compound **35**.

2. Results and discussion

2.1. Introduction

The aims of this thesis are the synthesis of the different TEMPO-analogues of the active first site fragments and to chemically link first- and second site fragments by means of a spacer. The results of the syntheses that were performed are discussed in this chapter. The synthesis of the 18 TEMPO analogues were performed by reactions of starting compounds **1** and **2**. Since the starting compounds contain different functional groups, corresponding reactions were tested and applied.

2.2. TEMPO labelling experiments of mono arylhalides

2.2.1. Comparison of aromatic nucleophilic substitution and Buchwald-Hartwig amination

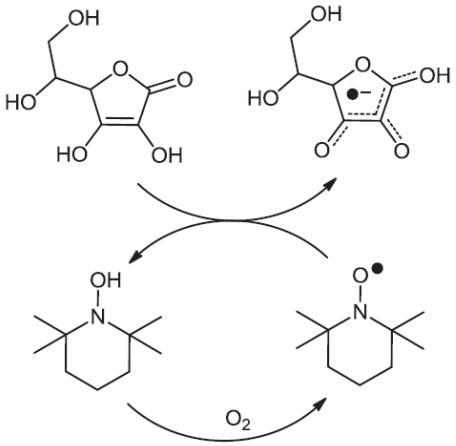
Since the aromatic nucleophilic substitution and the Buchwald-Hartwig amination share common starting compounds, in theory, both reactions could be used. In order to establish which of these reactions is most appropriate, 2-bromo-pyridine was reacted with **1** using both reactions (see table 1, compound **3**). The reaction is depicted in scheme 1 on page 5. All compound numbers with their yields are depicted in appendix 7.5.

For testing aromatic nucleophilic substitution, Et_3N was used as a base with THF or DMF as solvents. Using THF with a reaction time of 2 h (at 66 °C) no reaction products were observed, and both starting materials were detected by LC-MS. Using DMF at 100 °C for 4 h and at 125 °C for 68 h, however, resulted in a black reaction mixture. This indicates degradation products, presumably from compound **1**. Indeed, **1** is easily degraded at high temperatures. With a shorter reaction time of 46 h (100 °C for 22h and 130 °C for 24 h), LC-MS indicated that multiple products were formed, but not the desired compound **3**.

Buchwald-Hartwig amination did result in the desired compound **3** affording 66% yield after purification by column chromatography. Moreover, LC-MS indicated a purity of 100%. Also, ^{1}H-NMR confirmed compound **3**.

For the NMR measurements of the TEMPO labeled fragments, the radical-scavenger ascorbic acid was added. In appendix 7.1 and 7.2 the ^{1}H-NMR spectra of compound **27** are shown without and with ascorbic acid. The free radical of the TEMPO label quenches most protons in the ^{1}H-NMR spectrum. Only small signals from the aromatic hydrogens, which are most distant fromt the spin label are visable. By the addition of ascorbic acid the radical is scavenged, thereby no protons are quenched.

In appendix 7.3 the ^{1}H-NMR spectrum of ascorbic acid in DMSO is depicted. This spectrum is compaired with the spectrum in appendix 7.2. In appendix 7.2, the ^{1}H-NMR signals of ascorbic acid are absent, because TEMPO reacts with ascorbic acid (see scheme 8)[14]. Instead, new signals caused by the formation of TEMPO-H appeared in the ^{1}H NMR spectrum. The ratio of the chemical shift at around 1.4–1.0 ppm was 4:12, and designated as the hydrogen of methylene (4H) and methyl (12H) of TEMPO-H. The reaction between TEMPO and ascorbic acid creates small peaks in the NMR spectrum[14].

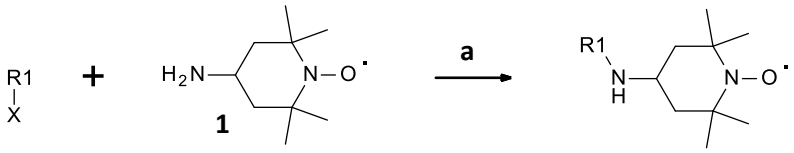


Scheme 8: Mechanism for the reaction between TEMPO and ascorbic acid.

According to different articles[15,16], aromatic nucleophilic substitution with amines does not occur with a pyridine under mild conditions. Aromatic nucleophilic substitution only occurs with harsh reaction conditions (high pressure and/or high temperatures). However, the used compound **1** is susceptible to degradation under these conditions. This was confirmed by the aromatic nucleophilic substitution reactions at high temperatures and long reaction times, in an attempt to obtain compound **3**. This resulted in many degradation products. The palladium-catalyzed amination (Buchwald-Hartwig amination), however, can be performed at lower temperatures and is therefore a better approach for amination. Therefore, Buchwald-Hartwig amination was chosen as default method for further TEMPO labelling of aryl monohalides. Only in cases where the Buchwald-Hartwig amination did not work, the aromatic nucleophilic substitution was tried.

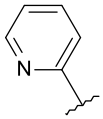
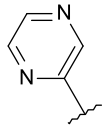
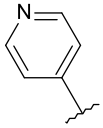
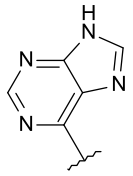
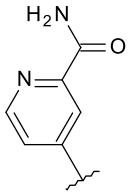
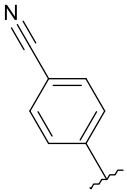
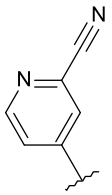
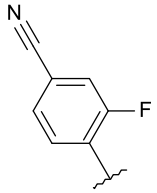
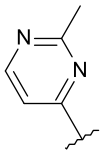
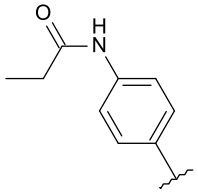
2.2.2. TEMPO labelling results of various aryl monohalide compounds

The results of the reaction of different starting compounds and **1** are summarized in table 1. The reaction is depicted in scheme 9.



Scheme 9: Aromatic nucleophilic substitution with a halide (X) as functional group present on the aromatic ring (R1). Standard reagents and conditions: (a) Et_3N, DMF, 125 °C Buchwald-Hartwig amination. Standard reagents and conditions: (a) $Pd_2(dba)_3$, DavePhos, NaOtBu, dioxane, 90 °C

Table 1: TEMPO labelling experiments performed on various aryl monohalides using the Buchwald-Hartwig amination and aromatic nucleophilic substitution.

Compound nr.	R1 group	X	Buchwald-Hartwig amination yield	Aromatic nucleophilic substitution Yield	Compound nr.	R1 group	X	Buchwald-Hartwig amination yield	Aromatic nucleophilic substitution yield
3		Br	66%	0%	**8**		Br	25%	-
4		Cl	21%	-	**9**		Br	0%	11%
6		Br	0%	0%	**10**		Br	40%	-
5		Br	10% and 7%	-	**11**		Br	42%	-
7		Cl	28%	-	**13**		Br	15%	-

Compound 3:
Has been described above (see page 9).

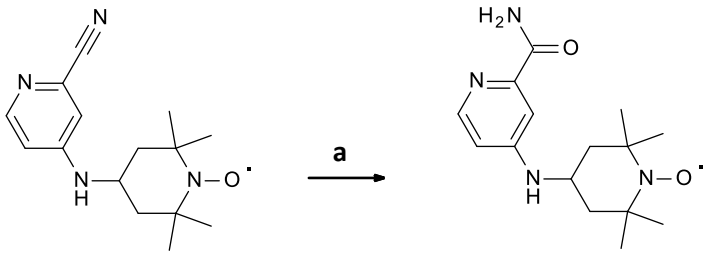
Compound 4:
The procedure used for the synthesis of compound **4** consisted of a Buchwald-Hartwig amination with 4-chloropyridine hydrochloride. The crude product was purified using column chromatography to give compound **4** in 21% yield. LC-MS indicated a purity of 96%. ^{1}H-NMR confirmed the identity of compound **4**. In the aromatic region, there are two small signals left. These signals indicate that there is still some starting material (4-chloropyridine hydrochloride) left.

Compound 6:
For the synthesis of compound **6** with either the Buchwald-Hartwig amination or aromatic nucleophilic substitution, 4-bromopyridine-2-carboxamide was reacted with **1**.
For the Buchwald-Hartwig amination, both starting materials were still present after 8.5 h stirring at 90 °C. LC-MS showed no product. Also, an over the weekend reaction at 100 °C for 3 h and at 90 °C for 67 h, formed no product. For aromatic nucleophilic substitution, DIPEA was used as a base. This reaction mixture was first stirred at 140 °C for 6 h and then stirred at rt for 17 h. LC-MS showed no product.

Compound 5:
Another method to synthesize compound **6** consisted of a two-step reaction. First, compound **5** was synthesized twice using a Buchwald-Hartwig amination with 4-bromopyridine-2-carbonitrile as starting materials with **1**. First, the reaction mixture was stirred at 90 °C for 10 h. The crude product was purified using column chromatography to give compound **5** in 7% yield. LC-MS indicated a purity of 97%. Because of the low yield, the reaction mixture was stirred at 95 °C for 28 h and purified using column chromatography to yield 10%. For both experiments, following the reaction by TLC indicated that all starting material was consumed, however, after the workup TLC showed that there was still starting material **1** left.

The absence of the formation of compound 6 with the Buchwald-Hartwig amination, may be explained by the amide group acting as a ligand for palladium[17]. In addition, the amide group is able to rotate and orient itself out of the aromatic plane and to the palladium.

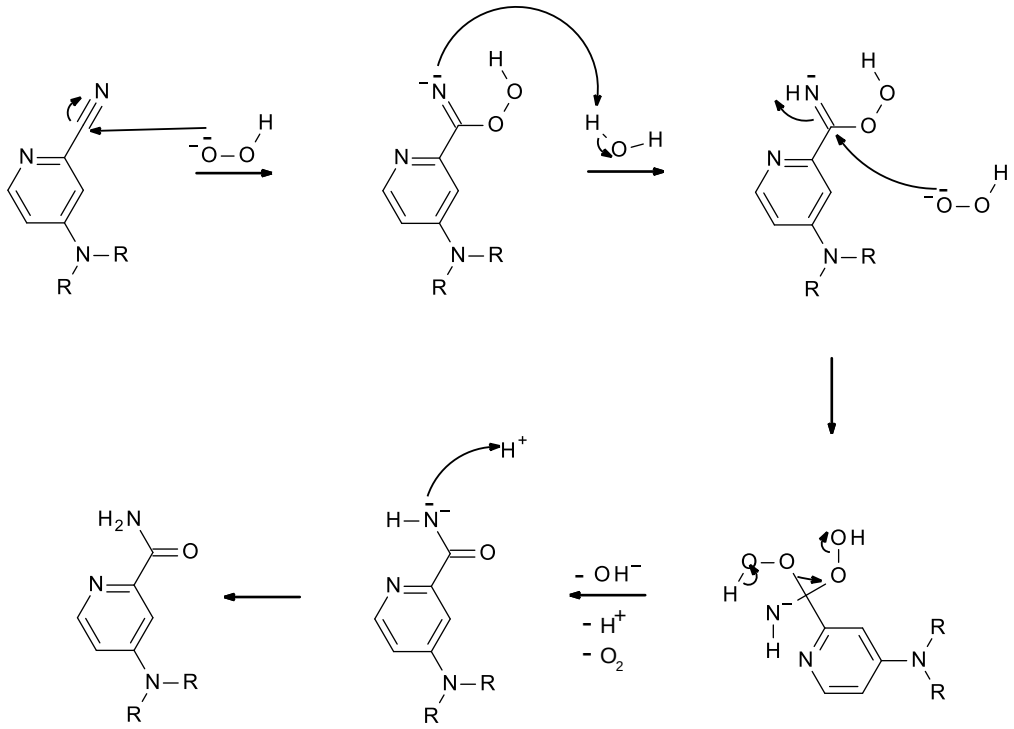
To achieve the desired product **6**, which was not successfully synthesized in a direct coupling reaction, the cyano group of compound **5** was oxidized (see scheme 10).



5 **6**

*Scheme 10: Nitrile oxidation of compound **5** to obtain compound **6**.*
Reagents and conditions: (a) K_2CO_3, H_2O_2 (35%), rt, 48 h, 24%

A general mechanism of the formation of compound **6** is depicted in scheme 11[18]. To simplify the mechanism, R_2N is used as an NH-TEMPO for compound **1**. This reaction is in a basic environment, so the hydrogen peroxide is deprotonated. A rate determining nucleophilic attack of the anion of hydrogen peroxide on the nitrile carbon takes place. The negatively charged nitrogen atom is protonated, followed by a rapid reaction of the intermediate peroxycarboximidic acid with the anion of hydrogen peroxide. The next step results in the loss of OH^-, O_2 and H^+. The negatively charged nitrogen atom is protonated to form compound **6**.



*Scheme 11: Nitrile oxidation mechanism obtaining compound **6** and **35**.*

The procedure used for the synthesis of compound **6** consisted of a nitrile oxidation of compound **5**. According to TLC, only a part of compound **5** reacted. An option was to add more H_2O_2, however this could result in oxidation of the amine (NR_2). The crude product was purified using column chromatography to give compound **6** in 24% yield. LC-MS indicated a purity of 94%.

Compound 7:
The procedure used for the synthesis of compound **7** consisted of a Buchwald-Hartwig amination with 4-chloro-2-methyl-pyrimidine and **1**. The crude product was purified using column chromatography to give compound **7** in 28% yield. LC-MS indicated a purity of 100%. ^{1}H-NMR confirmed compound **7** with few impurities.

Compound 8:
The procedure used for the synthesis of compound **8** consisted of a Buchwald-Hartwig amination with 2-bromopyrazine. The crude product was purified using column chromatography to give compound **8** in 25% yield. LC-MS indicated a purity of 100%.

Compound 9:
The synthesis of compound **9** consisted of two different reaction types: Buchwald-Hartwig amination and aromatic nucleophilic substitution, both using 6-bromo-9H-purine and **1** as starting materials.

For the Buchwald-Hartwig amination, both starting materials were still observed after 9 h stirring at 88 °C. LC-MS showed that there was no product formed. In the reference[19] Davephos was used as ligand and a Suzuki-Miyaura coupling instead of a Buchwald-Hartwig amination was described. The difference here is that according to a Suzuki-Miyaura coupling there is no primary amine involved but an organoboron. 1,2-Dimethoxyethane was used as solvent for this reaction[19]. For most Buchwald-Hartwig aminations used here, dioxane or toluene were used as solvent. So, the different solvent may be a explanation. A recommendation for a different method is described in recommendations (5.1.).

For aromatic nucleophilic substitution reaction Et_3N was used as a base. This reaction mixture was first stirred at 125 °C for 4.5 h and then stirred at rt for 18 h. LC-MS showed that after work-up the crude consisted of 69% compound **9**. Next, the crude product was purified by filtration achieving compound **9** in 11% yield. LC-MS of the residue indicated a purity of 92%. The filtrate, however, also contained some product, as shown by TLC. Therefore, recrystallization was performed resulting in 21% yield, but a purity of only 87% by LC-MS. An additional recrystallization failed since no crystals were formed.

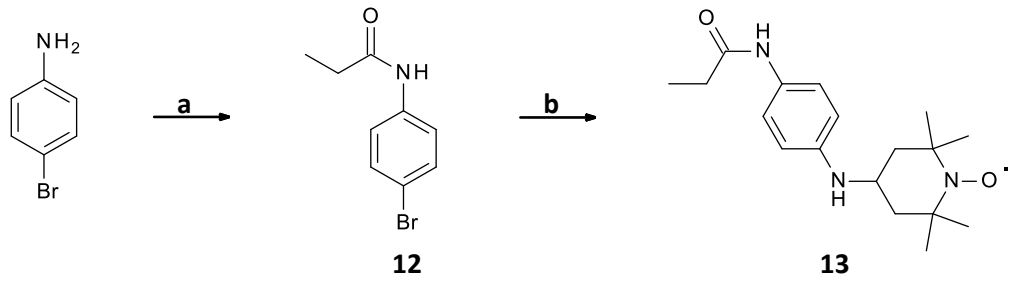
The low yield (11%) of compound **9** may be explained due to loss of product in the filtrate. The reason compound **9** was collected through filtering is because it did not dissolve well in the solvent mixture used for column chromatography. An attempt was made to purify the filtrate by column chromatography, but no good separation was obtained.

Compound 10:
The procedure used for the synthesis of compound **10** consisted of a Buchwald-Hartwig amination with 4-bromobenzonitrile. The crude product was purified using column chromatography to give compound **10** in 40% yield. LC-MS indicated a purity of 100%.

Compound 11:
The procedure used for the synthesis of compound **11** consisted of a Buchwald-Hartwig amination with 4-bromo-3-fluoro-benzonitrile. The crude product was purified using column chromatography to give compound **11** in 42% yield. LC-MS indicated a purity of 100%.

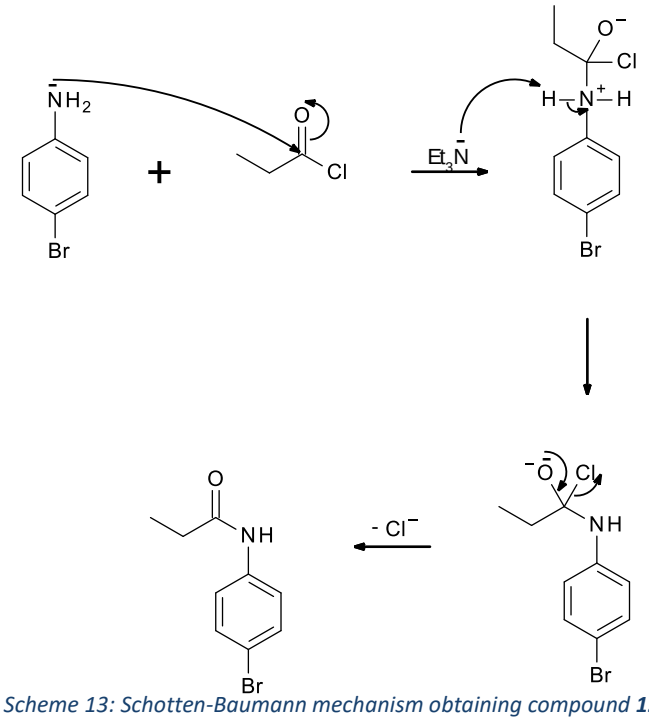
Compound 12 and 13:

The starting material used for obtaining compound **12** was first synthesized. The procedure used for the synthesis of compound **12** consisted of an amide formation, to be more precise, a Schotten-Baumann reaction between 4-bromoaniline and propanoyl chloride. An overview of the total synthesis is depicted in scheme 12.



*Scheme 12: 2 step synthesis of compound **13**. Reagents and conditions: (a) propanoyl chloride, Et_3N, DCM, rt, 3 h, 98%; (b) compound **1**, $pd_2(dba)_3$, XPhos, K_3PO_4, dioxane, 91 °C, 3.5 h, 15%.*

The mechanism of the formation of compound **12** is depicted in scheme 13[11]. First, a nucleophilic attack of the nitrogen to the carbonyl carbon takes place. Which leads to the addition of the aniline. This attack does not result in the direct loss of the chlorine ion, causes the carbonyl carbon bond to open up. The amine is deprotonated by the base (Et_3N). Next, a carbonyl bond is formed, which results in the loss of the chlorine ion.



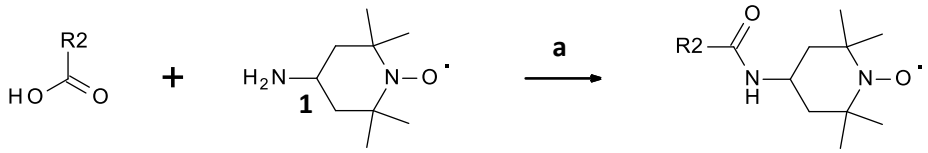
*Scheme 13: Schotten-Baumann mechanism obtaining compound **12**.*

The product was not purified, compound **12** was collected with 98% yield. ^{1}H-NMR confirmed that compound **12** formed. However, at 1.40 ppm a triplet and at 3.07 ppm a quartet is visible, what indicates that some propanoyl chloride is left.

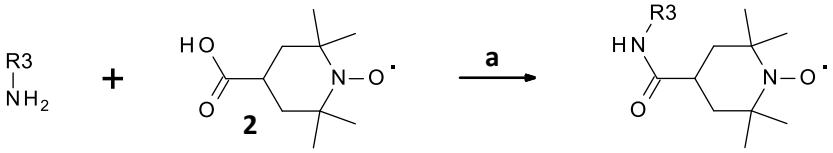
The procedure used for the synthesis of compound **13** consisted of a Buchwald-Hartwig amination with compound **12**. The crude product was purified using column chromatography to give compound **13** in 15% yield. LC-MS indicated a purity of 95%.

2.2.3. TEMPO labelling results using amide formation

To form the amides, reaction between carboxylic acids or acid chlorides and primary amines are used. In scheme 14 and 15 the reactions between R2-COOH and **1** or R3-NH_2 and **2** are depicted. All the different R-X groups are depicted in table 2. The reaction mechanism of the amide formation is depicted in scheme 6 on page 7.

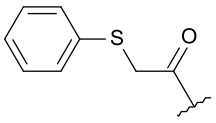
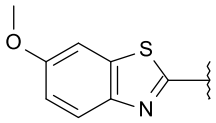
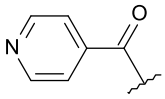
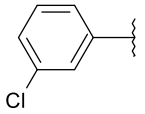
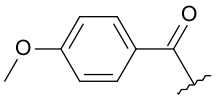
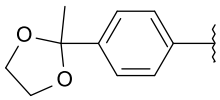
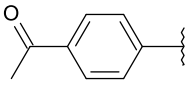
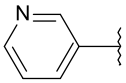


*Scheme 14: Amide formation R2 group with **1**. Reagents and conditions: (a) EDCI, HOBt, Et_3N, DCM or THF, rt*



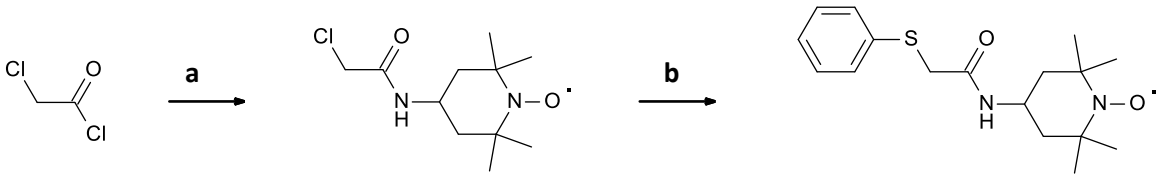
*Scheme 15: Amide formation R3 group with **1**. Reagents and conditions: (a) EDCI, HOBt, Et_3N, DCM or THF, rt*

*Table 2: Overview compounds **16** tm **25** and their different R-groups and yield amide formation*

Compound nr.	R2 group	Functional group	Amide formation yield	Compound nr.	R3 group	Functional group	Amide formation yield
16		OH	47%	**19**		NH_2	52%
17		OH	69%	**20**		NH_2	15%
18		Cl	35%	**23**		NH_2	44%
				24		NH_2	0% and 3%
				25		NH_2	19%

Compound 14, 15 and 16:

An overview of the initial method to synthesize compound **16** is depicted in scheme 16.



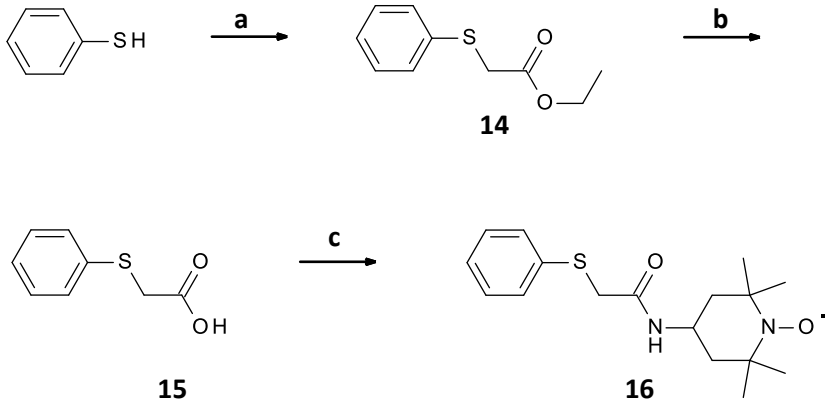
16

*Scheme 16: 2 step reaction obtaining compound **16**. Reagents and conditions: (a) compound **1**, Et_3N, DCM, 2 h; (b) benzenethiol, K_2CO_3, acetone, 70 °C, 20 h.*

First a Schotten-Baumann reaction between 2-chloroacetyl chloride and **1** was performed. The crude product was purified using column chromatography to yield 54% product. Nucleophilic substitution of the formed compound and benzenethiol gave compound **16**. The crude product was purified using column chromatography to give compound 16 in 12% yield. LC-MS indicated only 24% purity. Due to the low yield and purity, the method in scheme 12 is chosen. The low yield may be explained by steric hindrance in the

S_N2 reaction because of the bulky substrate. As a consequence, the back of the substrate hinders the nucleophilic attack.

An overview of the total synthesis to obtain compound **16**, is depicted in scheme 17. The procedure used for the synthesis of compound **14** consisted of a nucleophilic substitution between benzenethiol and ethyl 2-chloroacetate.



*Scheme 17: 3 step reaction obtaining compound **16**. Reagents and conditions: (a) ethyl 2-chloroacetate, K_2CO_3, acetone, 70 °C, 2.5 h, 82%; (b) NaOH, EtOH/H_2O, rt-68 °C, 22 h, quantitative yield; (c) compound **1**, EDCI, HOBt, Et_3N, DCM, rt, 3 h, 47%.*

The mechanism of the formation of compound **14** is depicted in appendix 7.2.1. The crude product was purified using column chromatography to give compound **14** in 82% yield. ^{1}H-NMR indicated that compound **14** was pure. An alternative way to produce compound **15** was the nucleophilic substitution with benzenethiol and chloroacetic acid as starting compounds. In this case, the mixture was not further purified.

The mechanism of the formation of compound **15** is depicted in appendix 7.2.2. The procedure used for the synthesis of compound **15** consisted of the ester hydrolysis of compound **14**. The reaction proceeded quantitatively. ^{1}H-NMR confirmed the presence of compound **15** with a minor impurity. The signal at 1.19 ppm (indicated CH_3 from the ester) was almost absent and at 4.13 ppm (indicated CH_2 from the ester) completely absent, which indicate the formation of compound **15**.

The mechanism is depicted in scheme 6 on page 7. The procedure used for the synthesis of compound **16** consisted of an amide formation between compound **15** and **1**. The crude product was purified using column chromatography to give compound **16** in 47% yield. LC-MS indicated a purity of 100%. ^{1}H-NMR spectrum confirmed the identity of compound **16**.

Compound 17:
The procedure used for the synthesis of compound **17** consisted of an amide formation with isonicotinic acid and **1**. The crude product was purified using column chromatography to give compound **17** in 69% yield. LC-MS indicated a purity of 97%.

Compound 18:
The mechanism of the formation of compound **18** is depicted in appendix 7.2.3. The procedure used for the synthesis of compound **18** consisted of an Schotten-Baumann reaction with 4-methoxybenzoyl chloride and **1**. For this reaction only Et_3N was used instead of EDCI, HOBt hydrate and Et_3N used in the other amide formation reactions. The crude product was purified using column chromatography to give compound **18** in 35% yield. LC-MS indicated a purity of 93%.

Compound 19:
The procedure used for the synthesis of compound **19** consisted of an amide formation between 6-methoxy-1,3-benzathiazol-2-amine and **2**. The crude product was purified using column chromatography to give compound **19** in 52% yield. LC-MS indicated a purity of 97%. ^{1}H-NMR confirmed the identity of compound **19**.

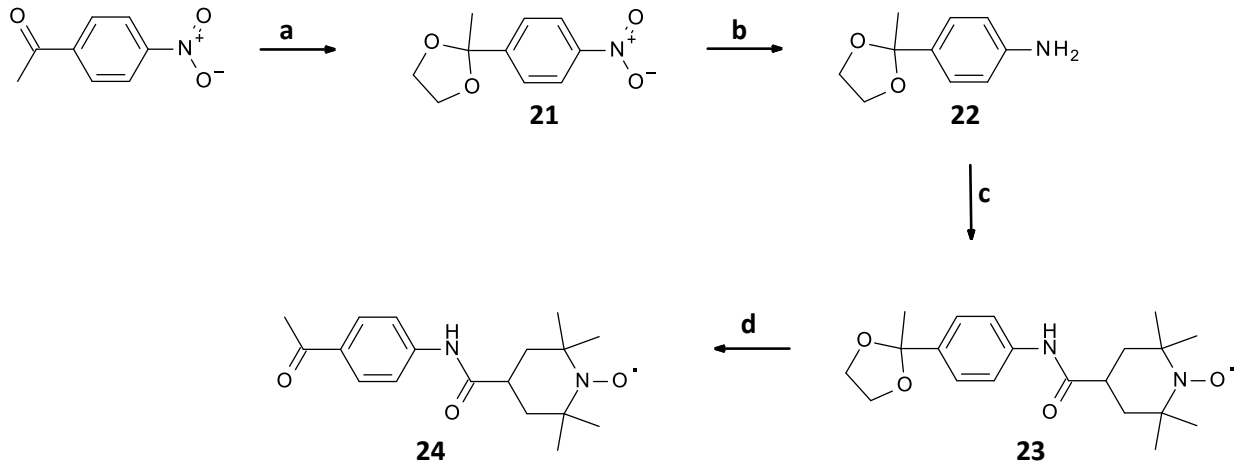
Compound 20:
The procedure used for the synthesis of compound **20** consisted of an amide formation between 3-chloroaniline and **2**. The crude product was purified using column chromatography to give compound **20** in 15% yield. LC-MS indicated a purity of 100%.

One possible explanation for the low yield is that the starting material is meta substituted. Chlorine is electronegative and thus withdraws electrons through the sigma bond with carbon. It pulls the electrons away from the ring and from NH_2, which reduces its nucleophilicity[9].

Compound 24:
The procedure used for the synthesis of compound **24** consisted of an amide formation between 4-aminoacetophenone and **2**. The reaction mixture in THF was stirred at rt for 22.5 h. Using column chromatography, two fractions were collected. Fraction 1 yielded 3% of compound **24**. LC-MS showed a purity of 74%. A mixed fraction of 4-aminoacetophenone and compound **24** was also collected. LC-MS of this fraction indicated 13% of compound **24** and showed that there was still 4-aminoacetophenone left.

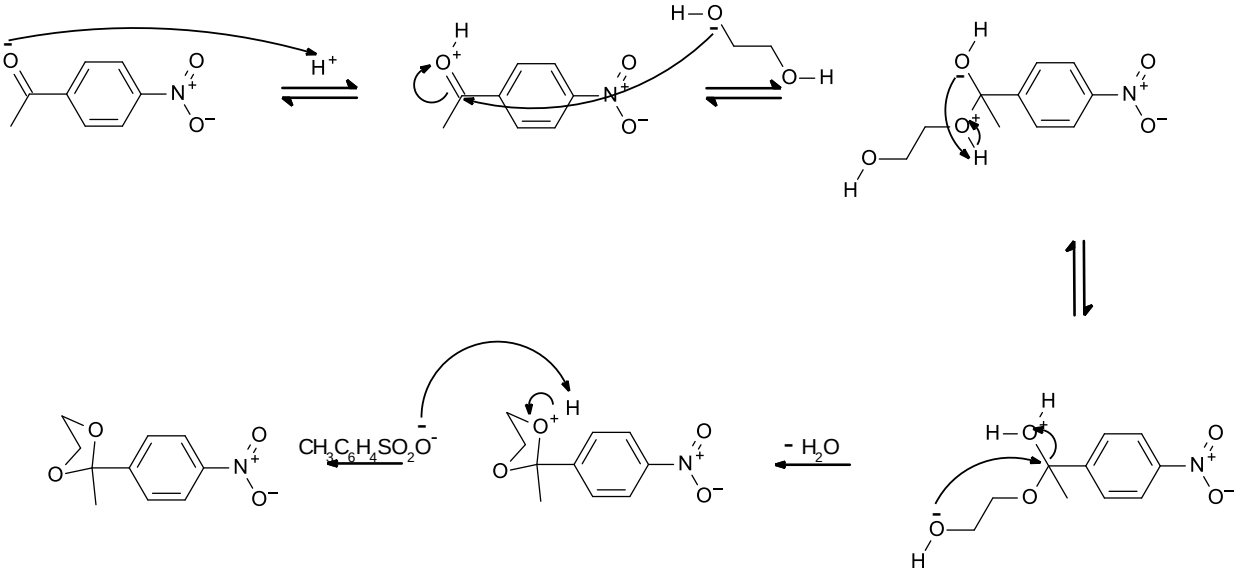
The experiment was repeated, at rt for 24.5 h and at 50 ^{o}C for 11 h. This time, both starting materials were observed and no reaction products were formed. The explanation for this is similar to that of compound **20**, namely the presence of an electron withdrawing acetyl substituent on the aromatic ring. It pulls the electrons away from the ring and from NH_2, which reduces its nucleophilicity[9].

The total synthesis of compound **24** is depicted in scheme 18.
The procedure used for the synthesis of compound **21** consisted of an acetal formation of 1-(4-nitrophenyl)ethanone. 1-(4-Nitrophenyl)ethanone was used instead of the amino-form, since the nitro-form resulted in a higher yield of compound **21**. This is probably due to the nitro-group, being an electron withdrawing group decreasing the electron density. This makes the carbonyl carbon more positive and more susceptible to nucleophilic attack.



*Scheme 18: 4 steps reaction obtaining compound **24**. Reagents and conditions: (a) ethylene glycol, P-toluenesulfonic acid, benzene, 120 °C Dean-Stark, 4 h, 93%; (b) iron powder, ammonium chloride, EtOH/H_2O, 80 °C, 30 min, 80%; (c) compound **2**, EDCI, HOBt, Et_3N, DMF, rt, 20 h, 44%; (d) 1M HCl, THF, rt, 1.5 h, 29%.*

The mechanism of the formation of compound **21** is depicted in scheme 19. First, the carbonyl group is protonated, which makes the carbon more susceptible to be attacked by the nucleophile. The alcohol acts as a nucleophile and attacks the carbonyl carbon. Protonation of the OH group of the hemiacetal occurs, which results in the loss of H_2O. This leaves a species that is susceptible to be deprotonated by the P-toluenesulfonic acid anion, ultimately leading to the acetal formation.



*Scheme 19: Acetal formation mechanism obtaining compound **24**.*

After the workup 93% yield of compound **21** was obtained. ^{1}H-NMR confirmed the identity of compound **21**. The procedure used for the synthesis of compound **22** consisted of an Bechamp reduction of compound **21**. After the workup 80% yield of compound **22** was obtained. ^{1}H-NMR indicated byproducts.

The spectrum is depicted in appendix 7.3.1. At 7.82, 6.73 and 2.50 ppm signals are present that identify 1-(4-aminophenyl)ethanone.
However, this procedure was repeated a couple of times. This ^{1}H-NMR spectrum showed that only 20% 1-(4-aminophenyl)ethanone was formed. Spectra from other experiments showed around 50-60%.

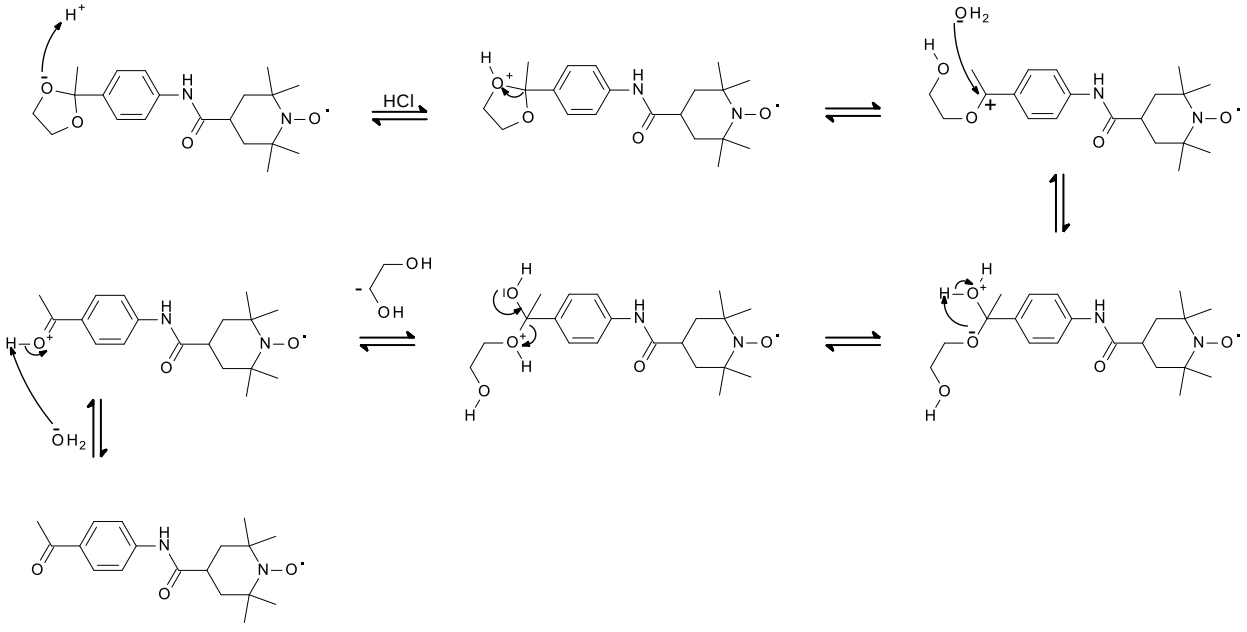
An explanation for these ^{1}H-NMR signals is that acetal hydrolysis formed 1-(4-aminophenyl)ethanone. Ammonium chloride solutions are mildly acidic, which (in conjunction with H_2O) can result in an acetal hydrolysis. The procedure used for the synthesis of compound **23** consisted of an amide formation between compound **22** and **2**. The general mechanism is depicted in scheme 6 on page 7.

The experimental was continued without purification of compound **22**, because:

- The viable solvent system for column chromatography to separate compound **22** from 1-(4-aminophenyl)ethanone was unable to dissolve the compound mixture.
- The 1-(4-aminophenyl)ethenone should not interfere with the experiment because of the presence of the electron withdrawing acetyl group.

The reaction mixture was stirred at rt for 20 h and purified using column chromatography to give compound **23** in 44% yield. LC-MS indicated a purity of 100%.

The mechanism of the formation of compound **24** is depicted in scheme 20[9]. First, one of the oxygens is protonated, which makes it a good leaving group. Leading to the formation of a carbocation, which is attacked by H_2O, forming a hemiacetal after deprotonation. The ethylene glycol leaves while H_2O deprotonates the carbonyl group to form compound **24**.



*Scheme 20: Acetal hydrolysis obtaining compound **24**.*

The procedure used for the synthesis of compound **24** consisted of an acetal hydrolysis of compound **23.** After purifying, there were two fractions collected. LC-MS indicated that one fraction had mass +1. (See appendix 7.3.2. mass 317 to 318). On TLC this fraction showed a lower retention factor. HCl probably acted

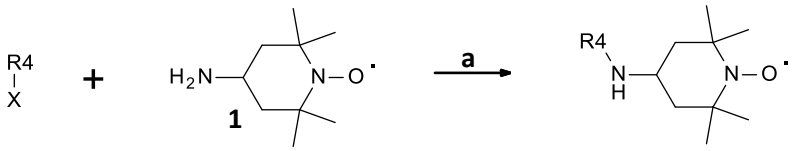
as a radical scavenger, as is seen in scheme 8 on page 10. LC-MS of the other fraction showed a purity of 97% with a yield of 29%.

Compound 25:
The synthesis of compound **25** was performed twice and consisted of an amide formation between pyridin-3-amine and **2**. In the first procedure, the reaction mixture in DMF was stirred at rt for 2 h. After work-up, the crude product was purified using column chromatography to give compound **25** in 19% yield. LC-MS indicated a purity of 100%. In the second procedure, the reaction mixture in THF was stirred at rt for 24 h. The crude product was purified using column chromatography to give compound **25** in 32% yield. LC-MS indicated a purity of 96%. It seemed that in both procedures the reaction was not fully completed, as both starting materials were still present. In the first procedure TLC at the end of the reaction using 10% i-PrOH in DCM showed that **2** was absent. However, using a different solvent system (5% MeOH in DCM) **2** was present, therefore indicating an incomplete reaction. In the second procedure on TLC with 5% MeOH in DCM the starting material was almost invisible. However, using column chromatography **2** could be detected.

An explanation for the low yields, is the presence of an electron withdrawing N heteroatom in the aromatic ring. This pulls electrons away from the amine, which reduces its nucleophilicity[9].

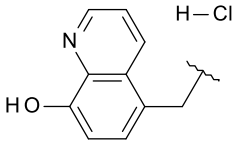
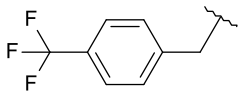
2.2.4. TEMPO labelling results of various other aliphatic monohalide compounds

The results of the reaction of different starting compounds with **1** are summarized in table 3. The reaction is depicted in scheme 21.



*Scheme 21: S_N2 reaction obtaining compound **26** and **27**. Reagents and conditions: (a) Et_3N, DCM or K_2CO_3, DMF.*

*Table 3: R4-X groups in a nucleophilic substitution with **1**.*

Compound nr.	R4 group	X
26		Cl
27		Br

Compound 26:

The mechanism of the formation of compound **26** and **27** is depicted in appendix 7.2.4. The procedure used for the synthesis of compound **26** consisted of a nucleophilic substitution between 5-(chloromethyl)quinolin-8-ol hydrochloride and **1**. The reaction mixture was stirred at 40 °C for 4 h and overnight at rt dissolved in DCM, Et_3N was used as base. The crude product was purified using column chromatography to give compound **26** in 7% yield. LC-MS indicated 72% of a different mass. LC-MS confirmed the presence of compound **26** (mass 328.45). However, compound **26** was not the main mass found. Therefore, the purity of compound **26** could not be confirmed.

Because of the low yield and purity, the experiment was repeated under different conditions. The crude product was purified using column chromatography to give compound **26** in 12% yield. However, LC-MS indicated 70% purity of a compound with a different mass, also 15% starting material **1** was present.

Because of the low yield and purity this labeled product was not submitted for testing in the COVID-19-NMR project.

Compound 27:

The procedure used for the synthesis of compound **27** consisted of a nucleophilic substitution between 1-(bromomethyl)-4-(trifluoromethyl)benzene and **2**. The reaction mixture in DMF was stirred at rt for 46 h. The crude product was purified using column chromatography to give compound **27** in 49% yield. LC-MS indicated purity of 79%. Attempts to further purify the end product by column chromatography and recrystallization were not successful. ^{1}H-NMR indicated compound **27** with few impurities. Because of this unsuccessful attempt, reductive amination reaction was tried. However, this did not result in an improved purity either.

Because of the low yield and purity this labeled product was not submitted for testing in the COVID-19-NMR project.

2.3. Synthesis of linked fragments

The TEMPO labeled active first-site fragments were submitted for testing in the COVID-19-NMR project. The original purpose was that on the basis of the "second hit", outcomes to chemically link those fragments that bind to the RNA and in the vicinity of each other by means of a spacer. However, since the second hit screening results were not provided timely, two fragments were selected on the basis of other criteria. One criterium was the observed binding of fragment **II** to the protease enzyme of the SARS-CoV-2 virus (see figure 4). The two fragments were linked to each other in order to provide a proof of principle for the present linker design.

On the basis of molecular modeling (see figure 5), the binding site distance was determined by a collaborating party in the COVID-19-NMR project (Saverna Therapeutics AG) to be 3, which means that the number of atoms between the two fragments is 9. On the basis of this information, the synthesis of the two fragments linked by a spacer of 9 atoms in length was performed by an 8-step reaction sequence. The synthesis pathway of the spacer is depicted in scheme 22. The synthesis of the spacer was started on a small scale. Later on, it appeared there was not enough compound left to continue, so the focus was directed at the scale-up of the synthesis. However, in this thesis mainly the small-scale procedures are described. If some steps in the scale-up differed from the small scale, they will be discussed. The overall yield per step is depicted in table 4.

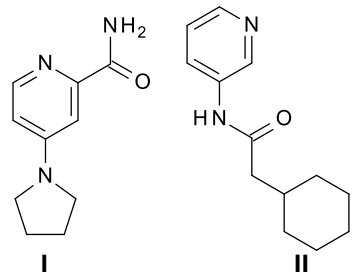


Figure 4: The first-site hit fragments.

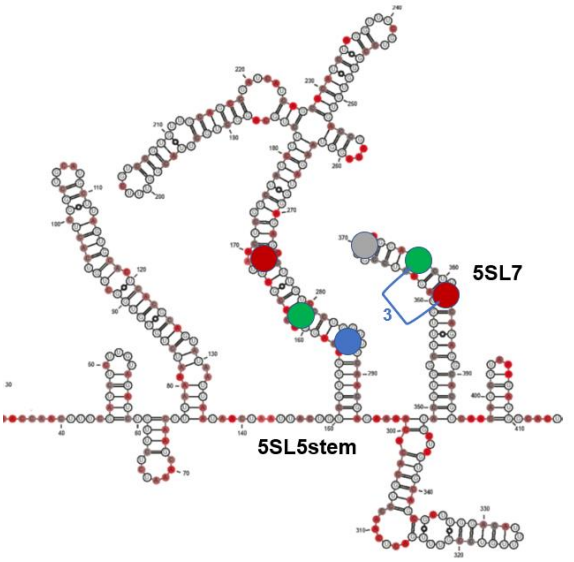
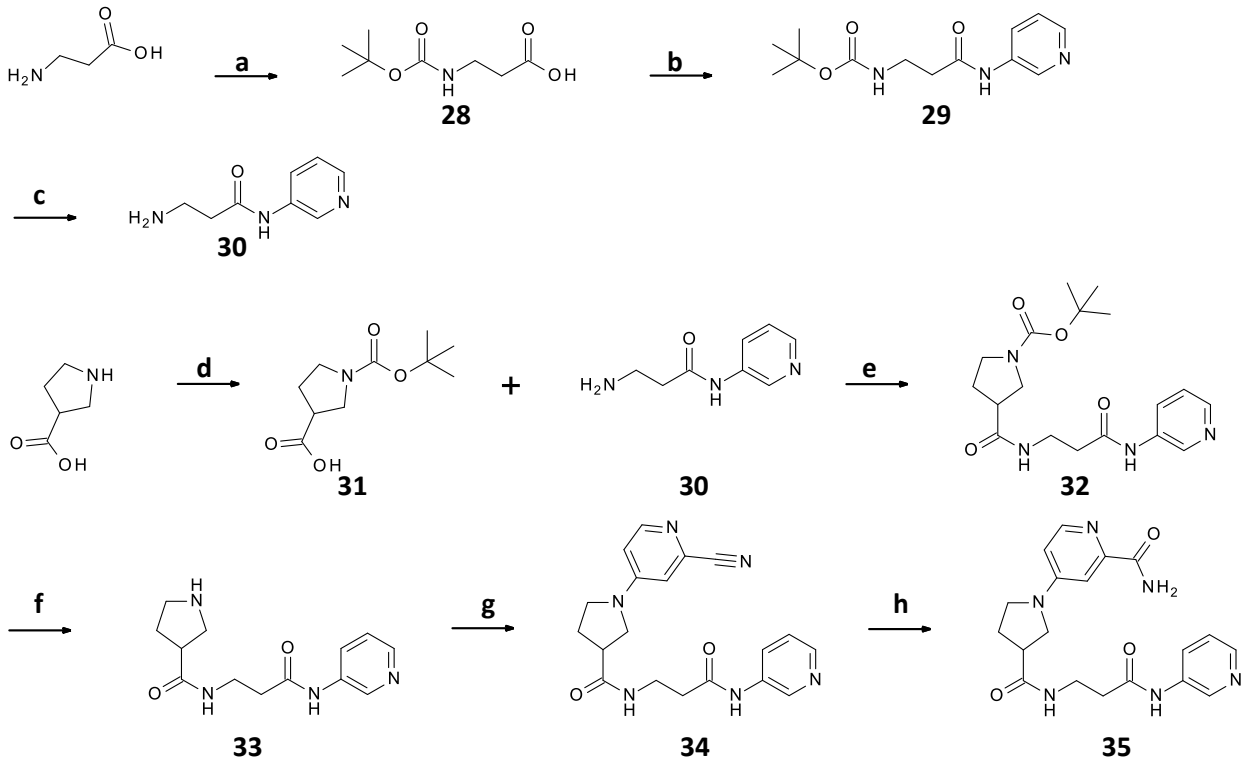


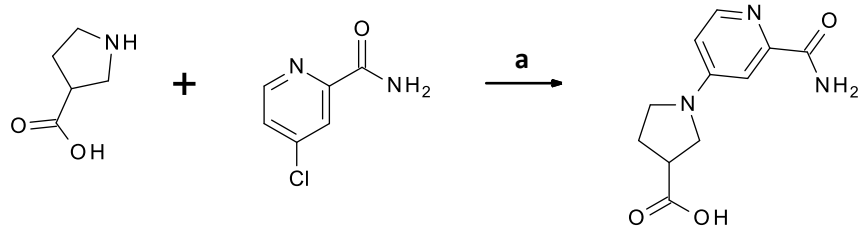
Figure 5: Molecular modeling SARS-CoV-2 RNA.

Table 4: The overall yield, small scale and scale-up.

Spacer	Yield small scale
Step 1	Quantitative
Step 2	56%
Step 3	75%
Step 4	Quantitative
Step 5	55%
Step 6	76%
Step 7	57%
Step 8	43%



*Scheme 22: Synthesis pathway of compound **35**. Reagents and conditions: (a) Boc_2O, NaOH, THF, 0°C-rt , 23 h, quantitative yield; (b) pyridine-3-amine, HATU, Et_3N, DCM, rt; (c) TFA, DCM, rt; (d) Boc_2O, 1:3 water: acetone, 0°C-rt, 22 h, quantitative yield; (e) DIPEA, PyBOP, DMF, rt; (f) TFA, DCM, rt; (g) 4-bromopyridine-2-carbonitrile, Et_3N, DMSO, 130 °C, 2 h, 57%; (h) K2CO3, H2O2 35%, DMSO, rt, 22 h, 43%.*



Scheme 23: Original synthesis. Reagents and conditions: (a) Et_3N, DMSO, 120 °C-150 °C, 12.5 h. second reaction: Et_3N, BuOH, 120 °C, 4 days.

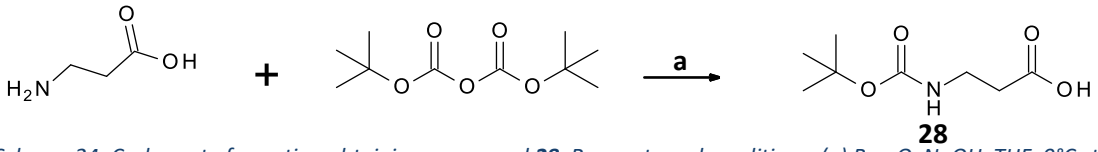
The original plan was to synthesize the molecule depicted in scheme 23 at the right. This molecule would then be used to be attached to the spacer.

The procedure used for this synthesis consisted of an amide formation between pyrrolidine-3-carboxylic acid and 4-chloropyridine-2-carboxamide. The reaction was performed twice. In the first procedure, DMSO was used as solvent. After 3 h stirring at 150 °C the reaction was complete. While taking TLC the product was washed with water. The product was water soluble.

In the second procedure, 1-butanol (BuOH) was used as solvent. After stirring at 120 °C for almost 4 days, there was still starting material left according to TLC. LC-MS indicated a purity of 37% product. Clearly, BuOH was not suited for this reaction. BuOH (polar protic solvent) forms hydrogen bonds with the nucleophile and hinders the attack on the halogenoalkane substrate[11].

Below, the reactions are described to form the intermediate products in scheme 22.

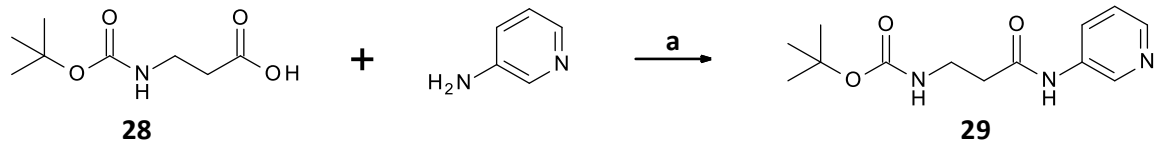
Compound 28: carbamate formation



*Scheme 24: Carbamate formation obtaining compound **28**. Reagents and conditions: (a) Boc_2O, NaOH, THF, 0°C-rt, 23 h, quantitative yield.*

The mechanism of the carbamate hydrolysis is depicted in appendix 7.2.5. To simplify the mechanism, R_2NH is used as a primary or secondary amine what is depicted in scheme 24 and 27. The procedure used for the synthesis of compound **28** consisted of a carbamate formation between 3-aminopropanoic acid and Boc_2O**.** The product was not purified and afforded compound **28** in quantitative yield. ^{1}H-NMR confirmed the identity of compound **28**.

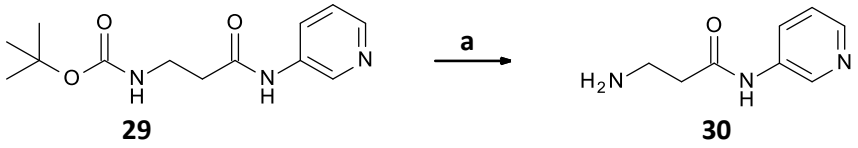
Compound 29: amide formation



*Scheme 25: Amide formation obtaining compound **29**. Reagents and conditions : (a) pyridine-3-amine, HATU, Et_3N, DCM, rt, 70 h, 56%.*

The mechanism of the carbamate hydrolysis is depicted in appendix 7.2.6. The procedure used for the synthesis of compound **29** consisted of an amide formation between pyridin-3-amine and **28** (see scheme 25). The crude product was purified using column chromatography, resulting in different fractions with purities of 100 and 89% as determined by LC-MS. The yield of the collected fractions of compound **29** was 56%. ^{1}H-NMR confirmed the identity of compound **29**.

Compound 30: carbamate hydrolysis



*Scheme 26: Carbamate hydrolysis obtaining compound **30**. Reagents and conditions: (a) TFA, DCM, rt, 1 h, 75%.*

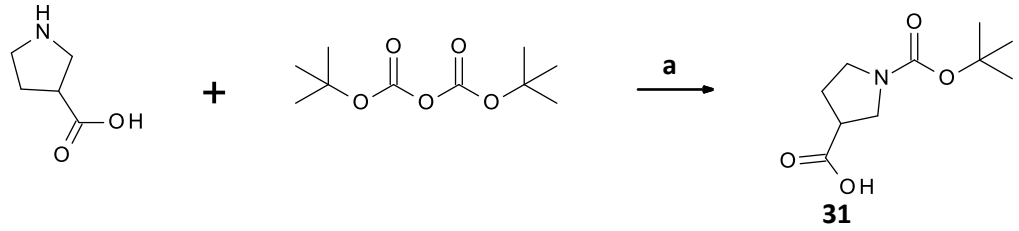
The mechanism of the carbamate hydrolysis is depicted in appendix 7.2.7. To simplify the mechanism, R_2NH is used as a primary or secondary amine what is depicted in scheme 26 and 29.

The synthesis of compound **30** consisted of a carbamate hydrolysis of compound **29**. Two experiments were performed. In the first experiment, the yield could not be calculated since the excess TFA salt that was formed in the reaction, was not removed. LC-MS indicated a purity of 72%. ^{1}H-NMR indicated that compound **30** formed, but that starting material **29** was left and some byproducts formed.

In the second experiment, the crude product was purified by column chromatography with ammonia/MeOH as solvent system after completion. The ammonia deprotonates the TFA salt to obtain compound **30** in quantitative yield. LC-MS indicated a purity of 59%. LC-MS indicated also that there was still 4% starting compound present. ^{1}H-NMR confirmed the identity of compound **30** with a few impurities.

For the scale-up reaction, the same procedure as the small scale was used. The crude product was purified using column chromatography to give compound **30** in 75% yield. LC-MS indicated a purity of 100%. ^{1}H-NMR confirmed the identity of pure compound **30**. The yield for the scale up (75% yield) is quite low in comparison with the small scale (quantitative yield). As seen from LC-MS, the scale-up purity is 100%. In the small scale the product is not as pure, so it makes sense that the yield seems much higher.

Compound 31: carbamate formation

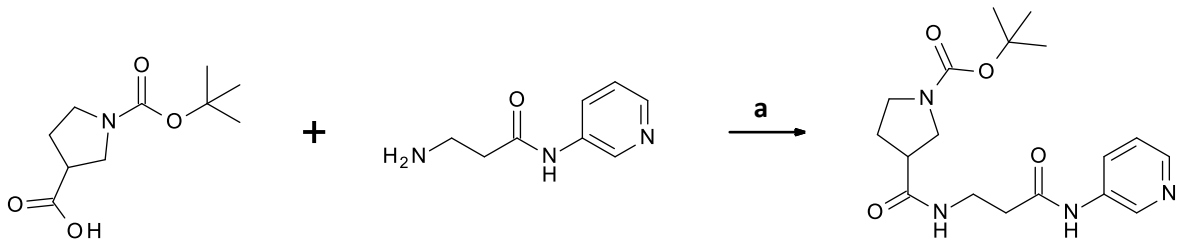


31

*Scheme 27: Carbamate formation obtaining compound **31**. Reagents and conditions: (a) Boc_2O, 1:3 water: acetone, 0°C-rt, 22 h, quantitative yield.*

The mechanism of the carbamate formation is depicted in appendix 7.2.5. The procedure used for the synthesis of compound **31** consisted of a carbamate formation between pyrrolidine-3-carboxylic and Boc_2O (see scheme 27). The product was not purified and afforded compound **31** in quantitative yield. ^{1}H-NMR confirmed the identity of compound **31**.

Compound 32: amide formation



31 **30** **32**

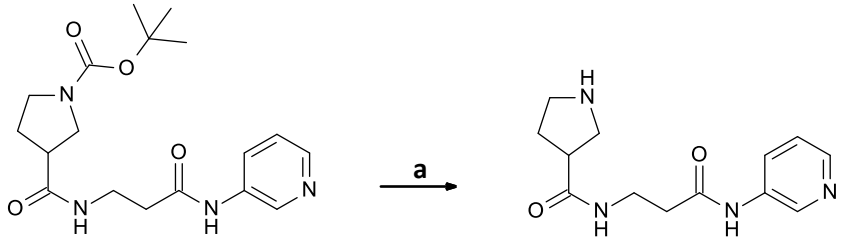
*Scheme 28: Amide formation obtaining compound **32**. Reagents and conditions: (a) DIPEA, PyBOP, DMF, rt, 45 min, 55%.*

The mechanism of the amide formation is depicted in appendix 7.2.8. The reaction to form compound **32** consisted of an amide formation between compound **30** and **31** (see scheme 28). As had been mentioned in the synthesis of compound **30**, two experiments were performed. In the first experiment, there was TFA salt still present in compound **30**. Moreover, this did not result in compound **32**, as seen by LC-MS. For the reaction, 7 equivalents of DIPEA were added to neutralize TFA, but this was apparently not sufficient. Another factor which may cause the absence of any product is that DCM was used as solvent instead of DMF.

In the second experiment used for the synthesis of compound **30**, the TFA salt was removed. After reaction with compound **31** and purification by column chromatography a yield of 55% of compound **32** was achieved. LC-MS indicated a purity of 92%. ^{1}H-NMR confirmed the identity of compound **32**, some impurities in the aromatic region were detected, probably some starting material **30** left.

For the scale-up reaction, the same procedure as the small scale (were the TFA salt was removed) was used. The crude product was purified using column chromatography to give compound **32** in 78% yield. LC-MS indicated a purity of 100%. However, ^{1}H-NMR indicated two big signals at 3.02 and 1.73 ppm that indicate the formation of a byproduct. In order to remove it, diethyl ether was added and a part of the byproduct was filtered off. By this procedure 500 mg of byproduct was removed. However, ^{1}H-NMR still indicated a lot of byproduct. An explanation for the seemingly high yield in the scale-up is because there is much byproduct present.

Compound 33: carbamate hydrolysis



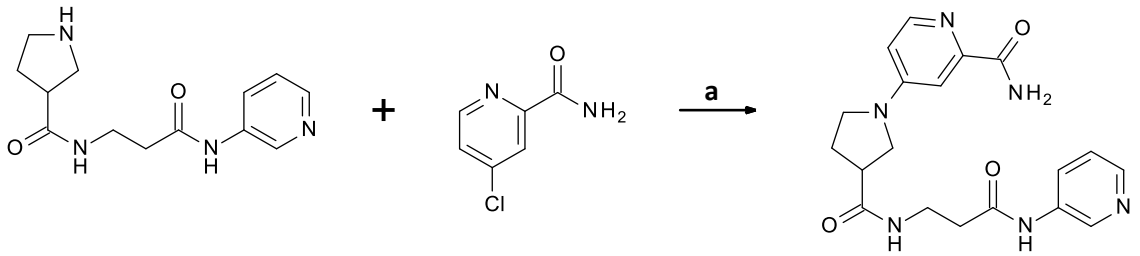
32 **33**

*Scheme 29: Carbamate hydrolysis obtaining compound **33**. Reagents and conditions: (a) TFA, DCM, rt, 1 h, 76%.*

The procedure used for the synthesis of compound **33** consisted of carbamate hydrolysis of compound **32** (see scheme 29). The crude product was purified using column chromatography to give compound **33** in

76% yield. LC-MS indicated a purity of 87%. ^{1}H-NMR confirmed the identity of compound **33** with few impurities.

Compound 35: aromatic nucleophilic substitution

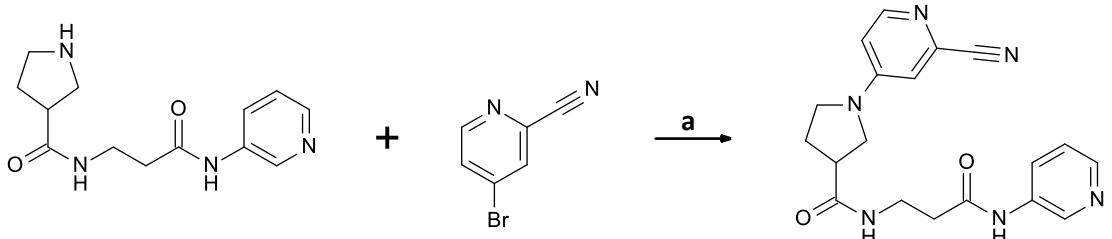


33 **35**

*Scheme 30: Aromatic nucleophilic substitution obtaining compound **35**. Reagents and conditions: (a) Et_3N, DMSO, 110 ^{o}C, 17.5 h, 0%.*

The procedure used for the synthesis of compound **35** consisted of an aromatic nucleophilic substitution between compound **33** and 4-chloropyridine-2-carboxamide (see scheme 30). LC-MS indicated 85% of 4-chloropyridine-2-carboxamide and no compound 35. Therefore, the following alternative reactions were tried (see scheme 31 and 32).

Compound 34: aromatic nucleophilic substitution

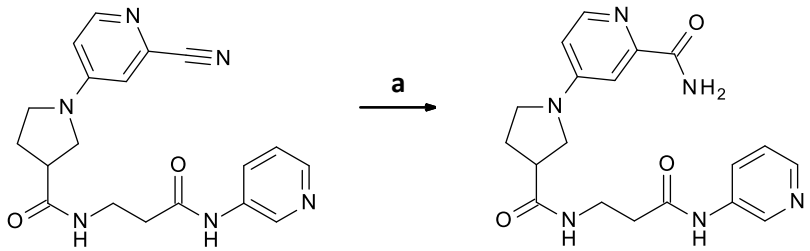


33 **34**

*Scheme 31: Aromatic nucleophilic substitution obtaining compound **34**. Reagents and conditions: (a) Et_3N, DMSO, 130 ^{o}C, 2 h, 57%.*

Scheme 29 shows the aromatic nucleophilic substitution reaction to obtain compound **34**, which was consequently oxidized to obtain compound **35** (see scheme 31). The procedure used for the synthesis of compound **34** consisted of a reaction between 4-bromopyridine-2-carbonitrile and compound **33**. The crude product was purified using column chromatography to give compound **34** in 57% yield. LC-MS indicated a purity of 100%. ^{1}H-NMR confirmed the identity of compound **34**. Even after multiple extractions of the water layer, TLC showed the presence of compound **34** in the water layer, which may explain the moderate yield.

Compound 35: nitrile oxidation



*Scheme 32: Nitrile oxidation obtaining compound **35**. Reagents and conditions: (a) K_2CO_3, H_2O_2 35%, DMSO, rt, 22 h, 43%.*

The general mechanism obtaining compound **35** is depicted in scheme 11 on page 13. The procedure used for the synthesis of compound **35** consisted of a nitrile oxidation of compound **34** (see scheme 32). The unpurified product yielded 43% of compound **35**. LC-MS indicated a purity of 100%. ^{1}H-NMR confirmed the identity of compound **35**. Even after multiple extractions of the water layer, TLC showed the presence of compound **35** in the water layer, which may explain the moderate yield.

A possible explanation for the fact that the second procedure did work (scheme 31 and 32). From the two different functional groups, cyano and amide, cyano is more electron withdrawing than amide. In case of a cyano group, the ring becomes more electron deficient, which makes a nucleophilic attack easier.

In summary, compound **35** was successfully synthesized in an 8-step synthesis, with an overall yield of 5%. If compound **35** proves to be a potential canidate for targeting SARS-CoV-2 RNA, optimization is probably needed (described in 5.4. Recommendations).

3. Second-site screening results

Below is a summary given of an example of a second-site hit. This screening is not done against the SARS-CoV-2 RNA, but against the A-site of the ribosome (supplied by Saverna Therapeutics AG).

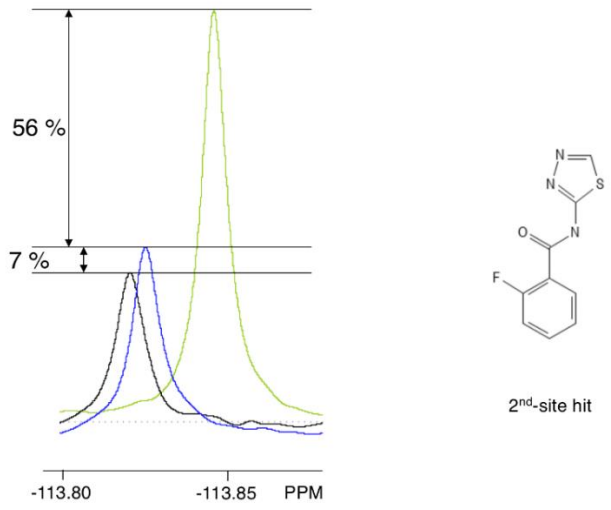


Figure 6: Detection of a second hit by ^{19}F-NMR.

Detection of a second-site hit by ^{19}F-NMR. A library of 100 fluorine-containing compounds was screened in the presence of spin labeled molecule compound **19**. On the left, figure 6 shows the superposition of three spectra at the resonance of the second site hit, for which the structure is shown on the right. The green signal is the resonance of the hit in the absence of the target RNA. The signal in black is the resonance of the same compound in the presence of the target RNA and in the presence of compound **19**. Comparison with the green signal shows that the signal has reduced intensity caused by ligand binding to the RNA. The blue signal is the signal after addition of ascorbic acid. In this sample, the unpaired electron that causes the paramagnetic relaxation is deactivated and the effect of the unpaired electron of compound **19** is no longer observed. As a result, the additional relaxation effect of the spin label is not observed anymore. The difference in intensity of the blue and black signal shows that the hit is binding simultaneously and in the vicinity to compound **19**.

Screening of the library of 16 spin labeled compounds in the presence of ascorbic acid using ^{1}H NMR. The chemical shift perturbation (CSP) of the signals indicates the binding of the compounds to the target RNA (see table 5). The value of CSP = 0 Hz can be indicated for the other compounds that are not present in table 5. Interestingly, the spin labeled compounds that are closest to a previously found fragment hit (compound **11** and compound **10**) show no binding or weak binding, whereas, compound **19** shows the best binding. This result shows the power of the library approach to find the best spin label.

Table 5: Compound spin labeled and the chemical shift perturbation (CSP).

Compound	CSP (Hz)
3	1
19	8.8
10	0.7
24	1.1
9	0.7

4. Conclusion

Selected fragments which have been shown to bind to the SARS-CoV-2 RNA, have been successfully coupled with TEMPO spin label. Three different organic reactions (Buchwald-Hartwig amination, aromatic nucleophilic substitution and amide formation) were used. Buchwald-Hartwig amination and amide formation could be applied successfully, showing good or moderate yields. Aromatic nucleophilic substitution, however, showed low yield or no products at all. Aromatic nucleophilic substitution was not successful, specifically with pyridines, probably because TEMPO is easily degraded by the necessary high temperatures. The labeled compounds were submitted for second site NMR screening. The preliminary second site screening results are promising because a major second site hit was found in the presence of labeled compound **19**, as seen by quenching of the NMR signals.

Proof of principle for the synthesis of a 9 atom length spacer between two fragments, was provided. Since the second hit screening results were not provided timely, two other fragments were selected. The resulting product of the linked fragments (compound **35**) was successfully synthesized in an 8-step synthesis, with an overall yield of 5%.

The present approach by FBDD using NMR first and second-site screening may be a promising way for efficient development of inhibitor drugs targeting the SARS-CoV-2 RNA.

5. Recommendations

5.1. Recommendation Buchwald-Hartwig amination

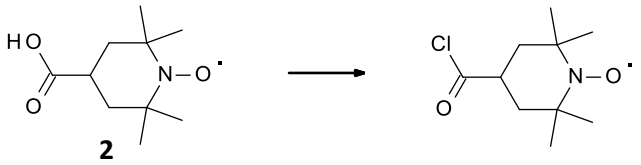
The presently applied Buchwald-Hartwig amination showed low to moderate yields. Optimization may be performed by the use of different ligands and catalysts.

In this thesis two different ligands were used, that is, Davephos and Xphos. However, DavePhos was still present in the product after purification. Therefore, Xphos was tried and the solids were filtered off before work-up and purification, which resulted in pure product. The recommendation is therefore to filter the ligand off before the work-up and purification.

5.2. Recommendation amide formation

In the synthesis of spin labeled compounds by amide formation, EDCI and HOBt were used as coupling reagents. However, instead of using both EDCI and HOBt, only an access of EDCI may also be used as a coupling reagent. HATU or PyBOP were used in the synthesis of the spacer as coupling reagents. These two coupling reagents may also be tried for the synthesis of the different spin labels.

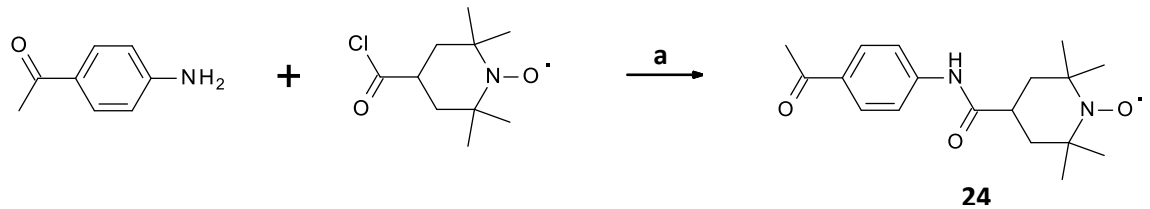
5.3. Recommendation obtaining compound 24:



Scheme 33: Conversion of carboxylic acid to acid chloride

An recommendation for obtaining compound **24**, is to make an acid chloride-TEMPO (see scheme 33). An acid chloride is more reactive, that the reaction can take place. For this reaction thionyl chloride and Et_3N in DMF is used to obtain the acid chloride-TEMPO. The sequence of the addition of the reagents is important, because pure thionyl chloride destroys compound **2**. It is necessary to treat thionyl chloride with DMF to obtain the amide chloride complex, which reacts with compound **2** to form the corresponding chloride[20].

This will results next in a plausible Schotten-Baumann reaction (see scheme 34) to obtain compound **24**.



*Scheme 34: Plausible Schotten-Baumann reaction obtaining compound **24**. Reagents and conditions: (a): Et_3N in DCM or DMF.*

5.4. Recommedation linked fragments (obtaining compound 35):

A recommendation for the 8-step synthesis obtaining compound **35**. The overall yield in the 8-step synthesis obtaining compound **35** is only 5%. If compound **35** proves to be a potential candidate for targeting SARS-CoV-2 RNA, optimization is probably needed. Since the individual steps in the synthesis show reasonable yields, no major improvement can be expected by optimizing the individual steps. In stead, by aiming to decrease the number of steps the overall yield may be substantially increased.

6. References

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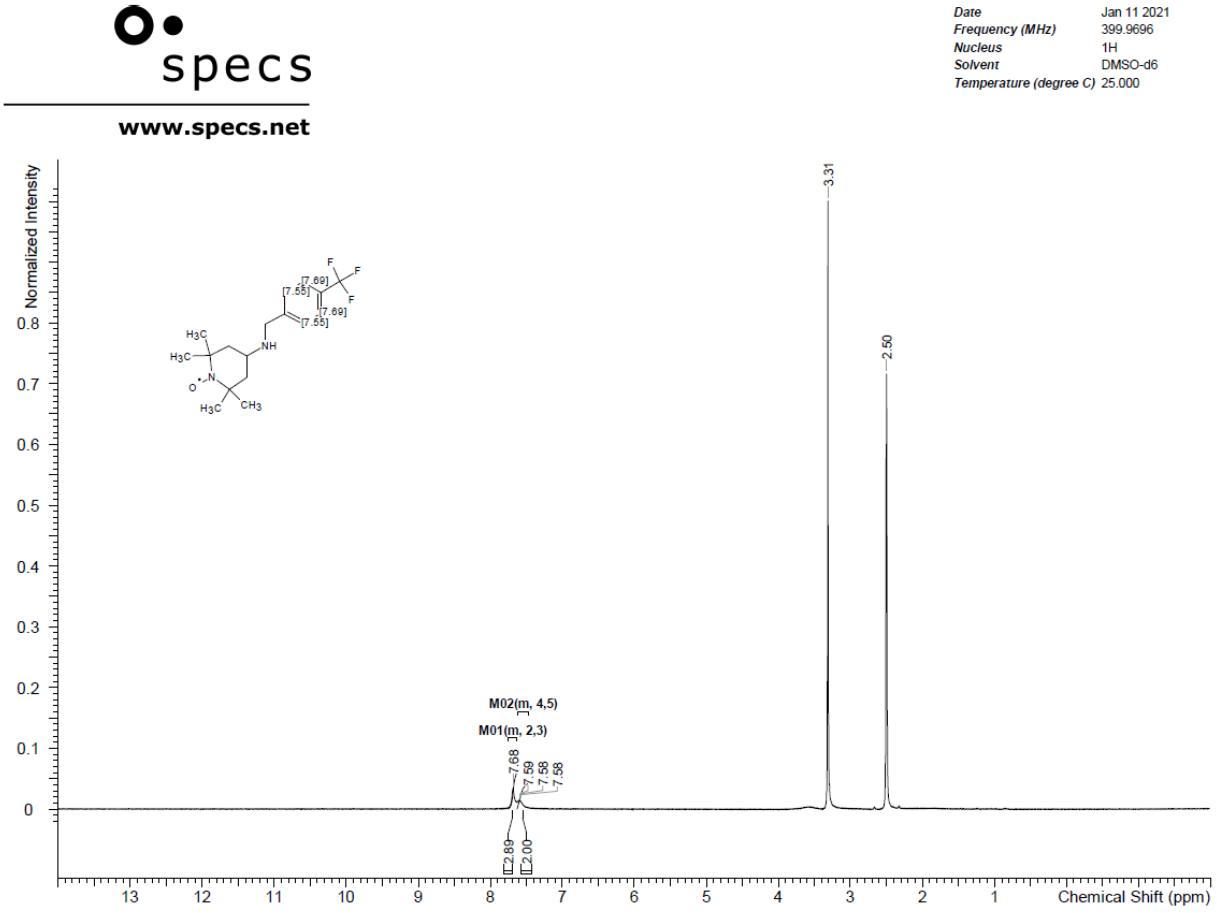
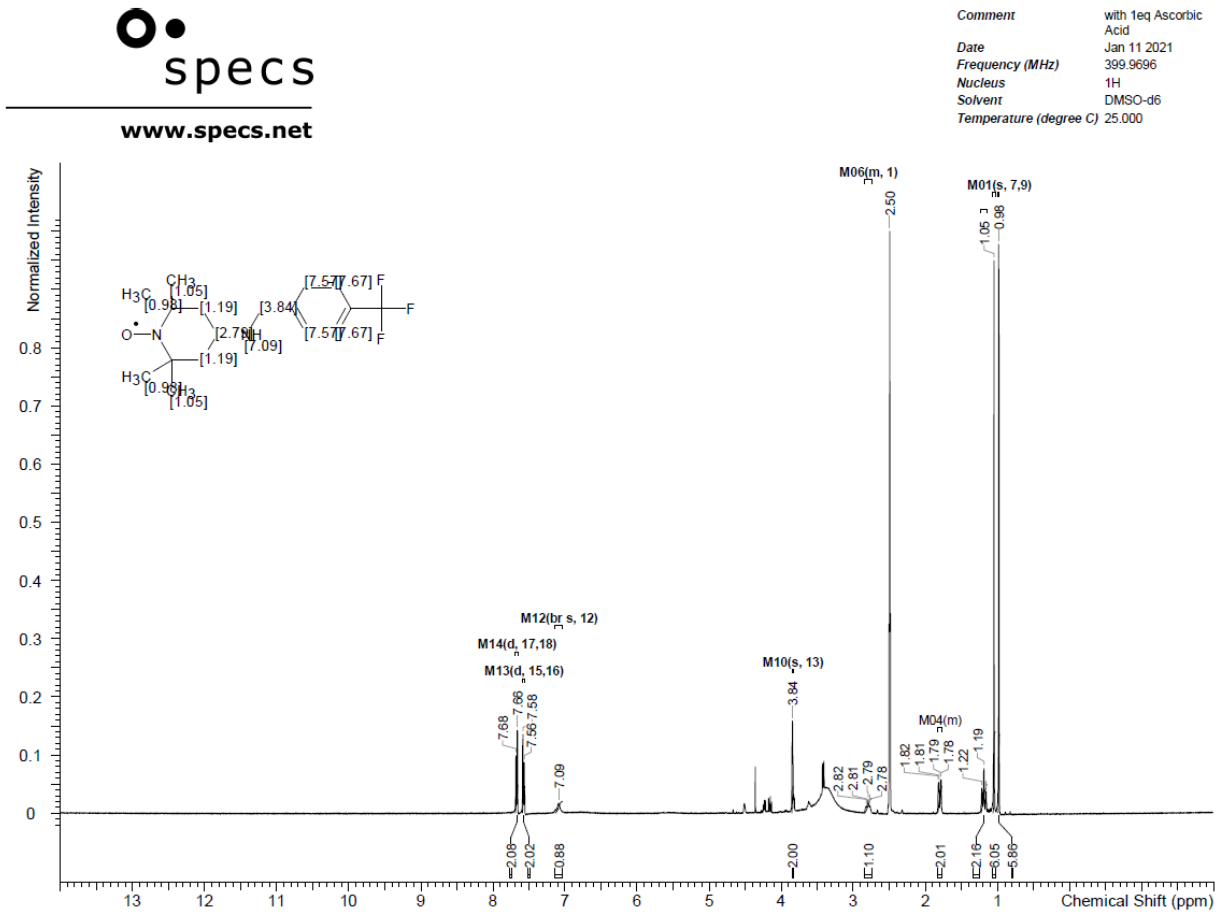
7. Appendix

7.1. Appendix 1: Radical-scavenger ^{1}H-NMR

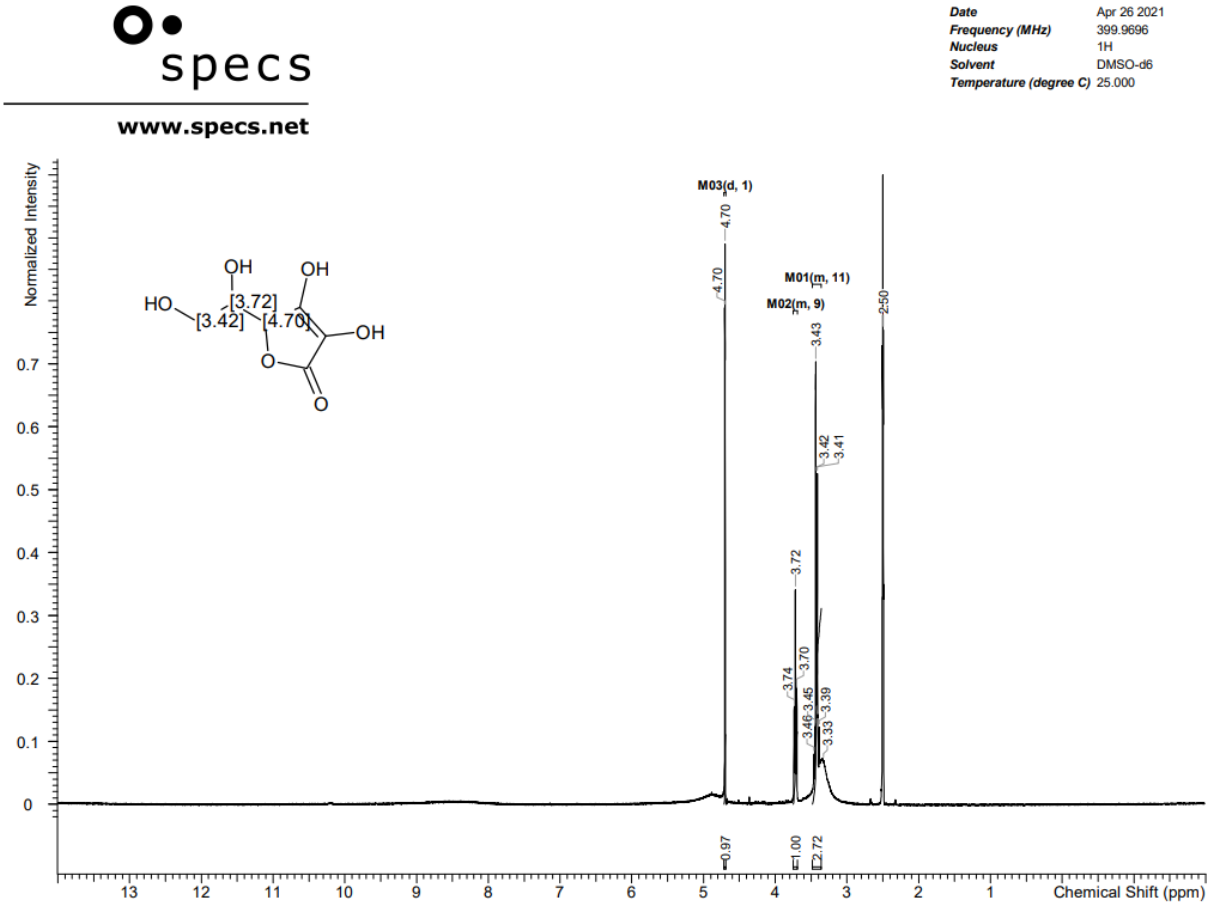
7.1.1 ^{1}H-NMR compound **27** in DMSO without ascobic acid



7.1.2. ^{1}H-NMR compound **27** in DMSO with ascobic acid



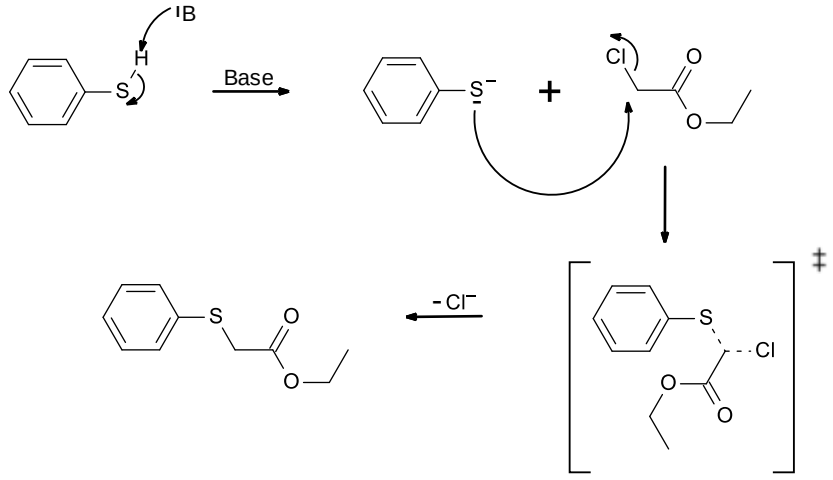
7.1.3. ^{1}H-NMR ascorbic acid in DMSO



7.2. Appendix 1: reaction mechanisms

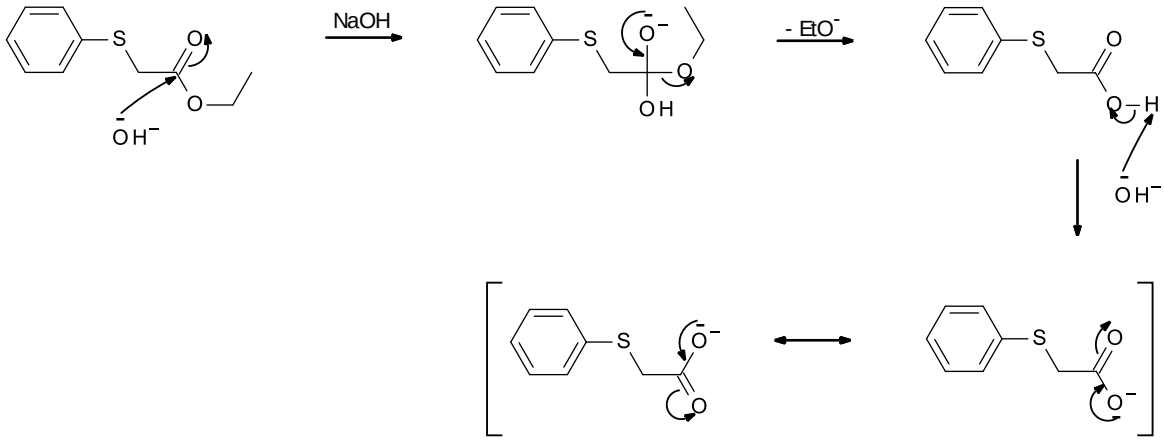
7.2.1. S_N2 reaction mechanism to obtain compound **14**

Since thiols are weakly acidic, the thiol is first deprotonated, which results in a thiol anion. A thiol anion group is a good nucleophile to perform a S_N2 reaction. A nucleophilic attack at the δ positive carbon atom takes place and results in the direct loss of Cl^- [9].



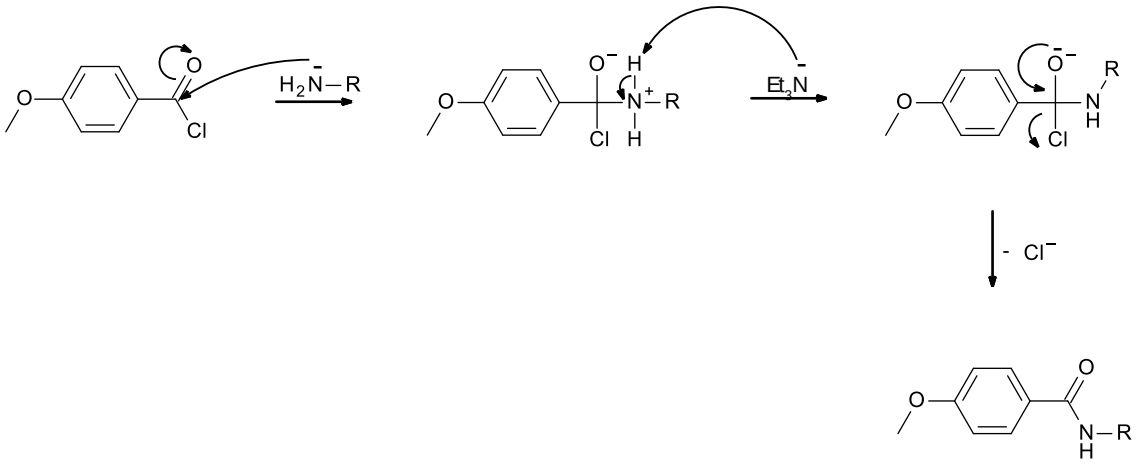
7.2.2. Ester hydrolysis to obtain compound 15

First, a nucleophilic attack at the carbonyl carbon takes place. This causes the carbonyl bond to open up, followed by the loss of alkoxide (-EtO^-). The carboxylic acid is deprotonated by the base, which results in a carboxylate anion. The carboxylate anion is less electrophilic than carboxylic acid and therefore cannot be attacked by the alkoxide (-EtO^-) anymore[11].



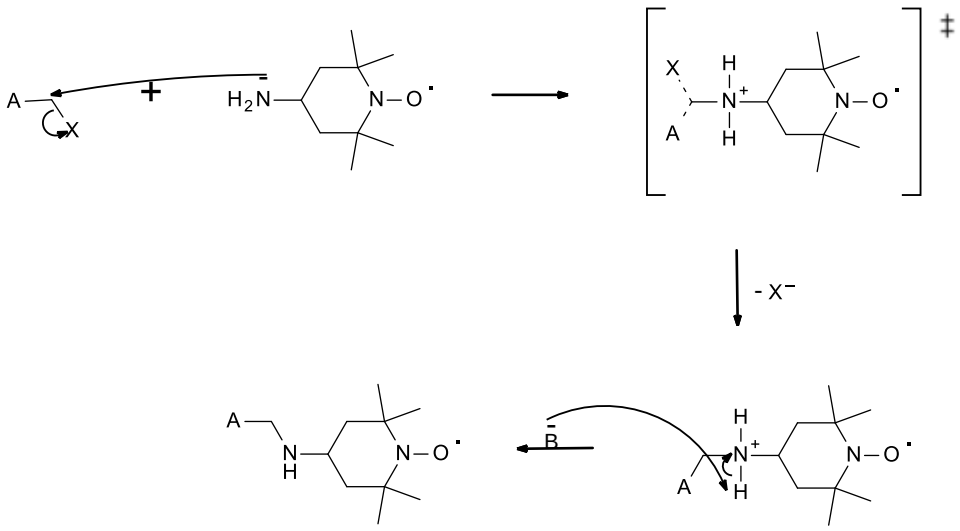
7.2.3. Schotten-Baumann to obtain compound 18

First a nucleophilic attack at the carbonyl carbon atom takes place. This attack does not result in the direct loss of Cl^-, but causes the carbonyl bond to open up. The amine is deprotonated by the base, which results in the formation of a carbonyl carbon, this results in the loss of Cl^- [11].



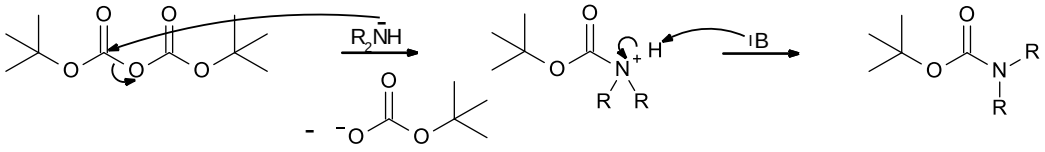
7.2.4. Nucleophilic substitution to obtain compound 26 and 27

A nucleophilic attack at the δ positive carbon atom takes place. This attack results in the direct loss of Cl^- [11]. A= Aromatic compound.

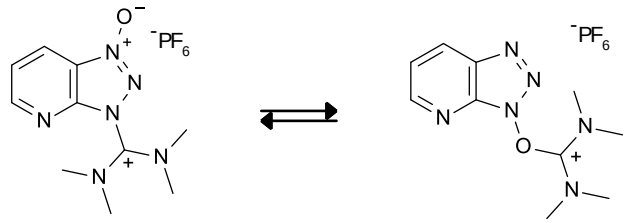


7.2.5. Carbamate formation to obtain compound 28 and 31

First a nucleophilic attack at the carbonyl carbon atom takes place. This result in the direct loss of $BocO^-$. The amine gets deprotonated by the base, which forms compound **28** and **31**[21].

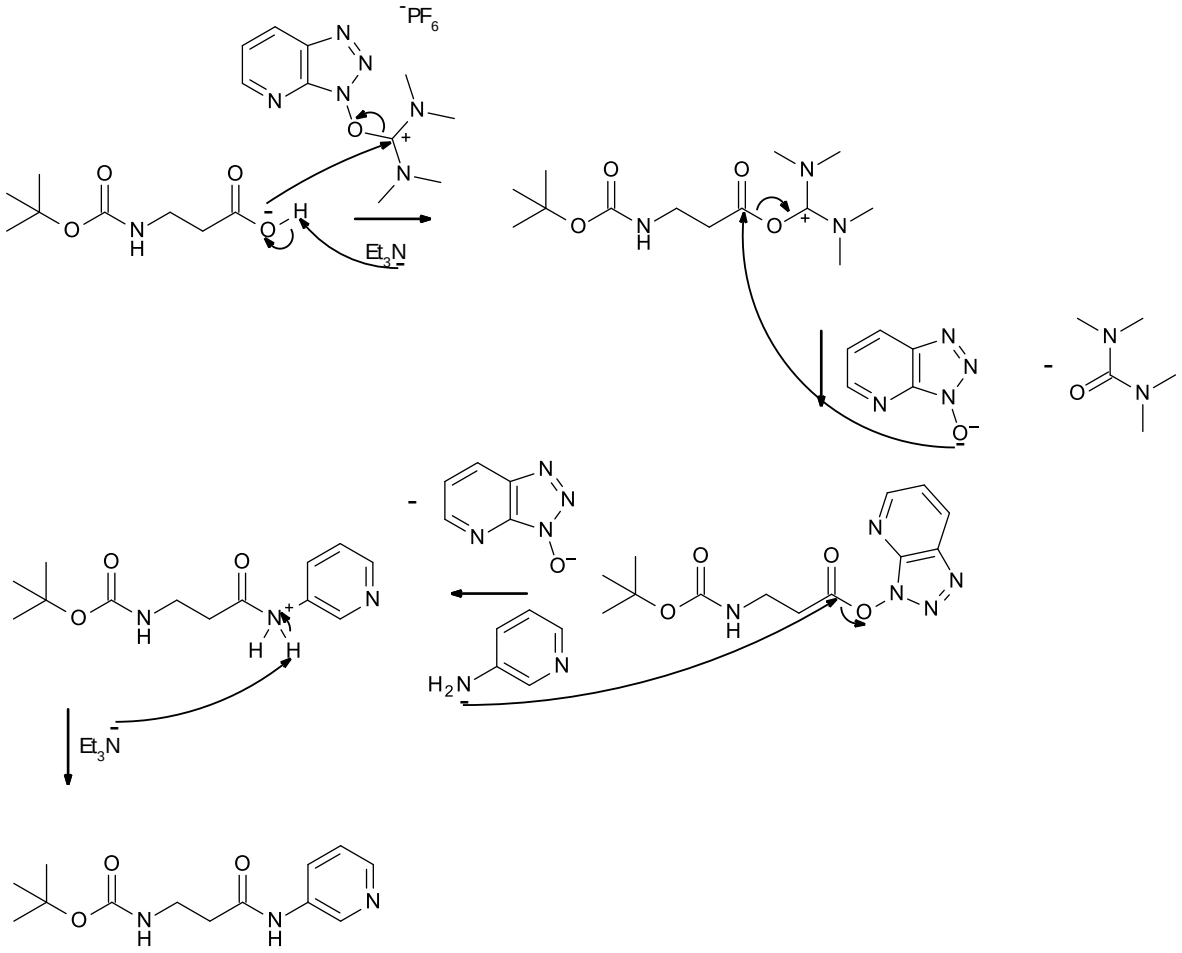


7.2.6. Amide formation obtaining compound 29 (HATU)



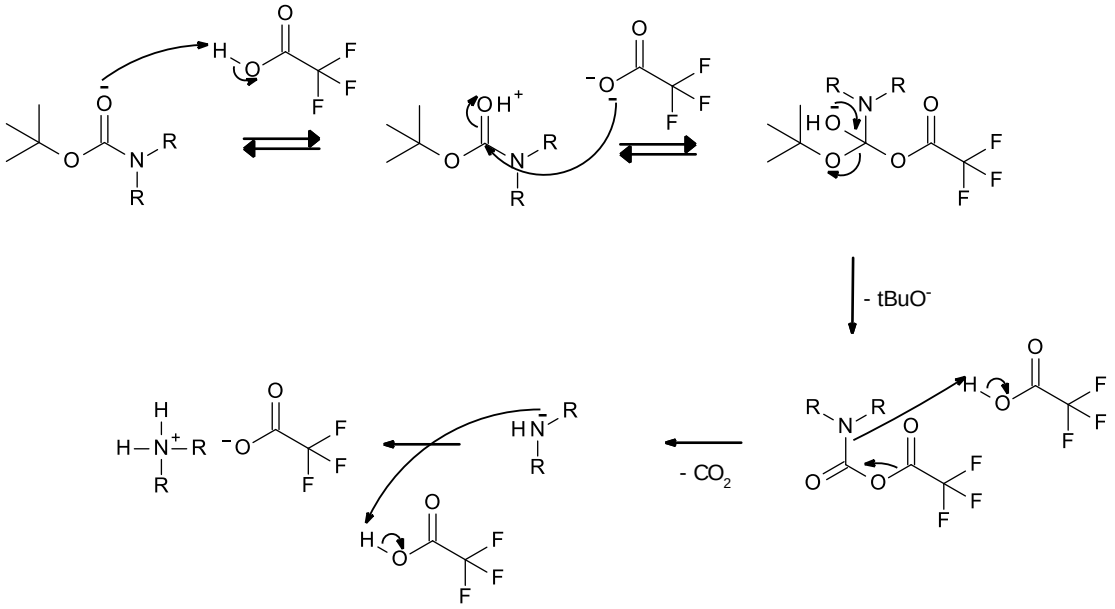
N-form O-form

For the first step, the carboxylic acid is deprotonated Et_3N, which allows a nucleophilic attack of the anion of the carboxylic acid at the positive carbon from HATU. In the mechanism the O-form is used, because that form is more reactive. Next, the negative oxygen resulted from HOBt attacks the carbonyl carbon, which removes the corresponding urea. The R-NH_2 attacks the carbonyl carbon, which removes the resulting HOBt anion. Finally, the base (Et_3N) deprotonates the amine, to form compound **29**[22].



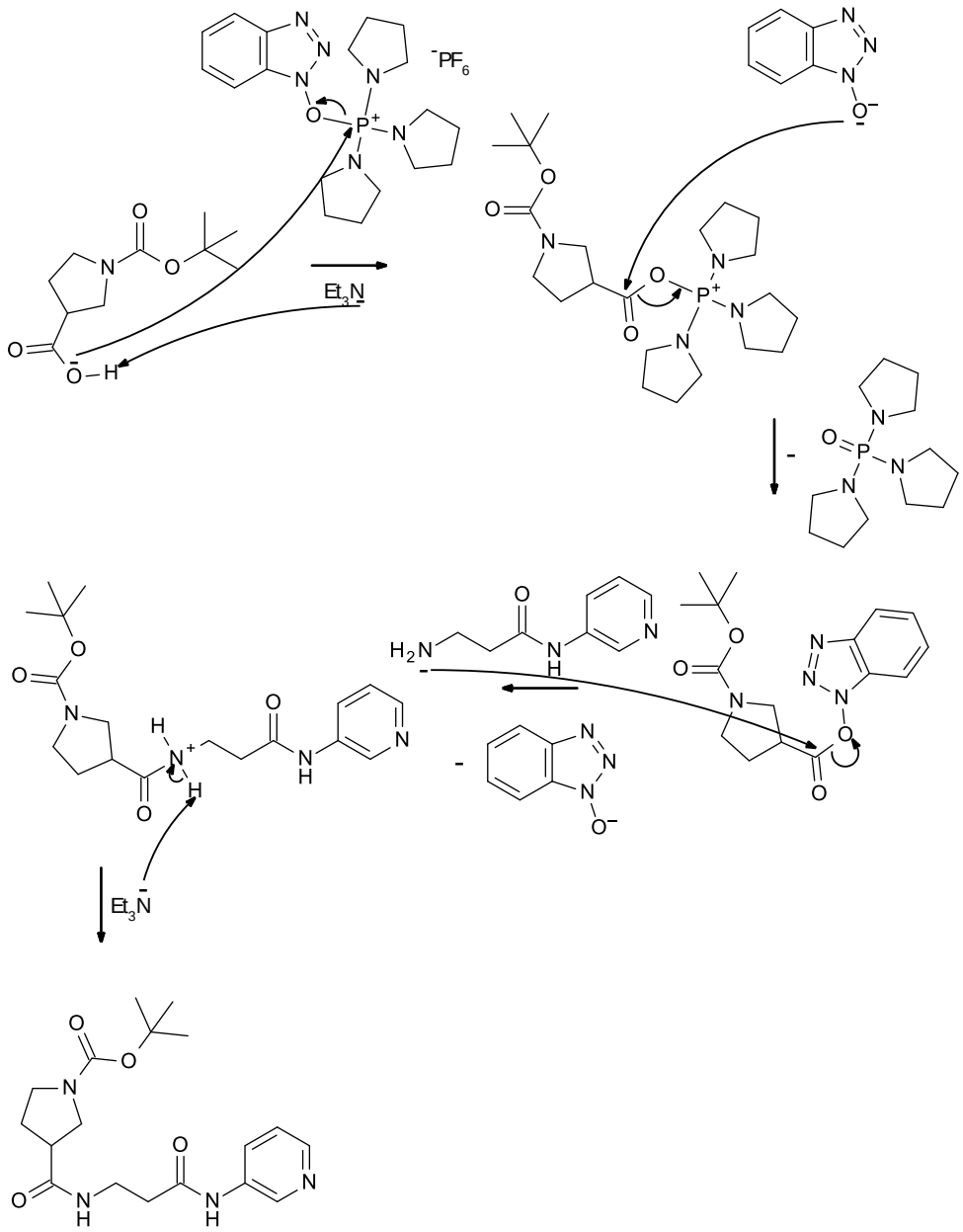
7.2.7. Carbamate hydrolysis to obtain compound 30 and 33

First, the carbonyl carbon is protonated by TFA, a nucleophilic attack from the TFA anion at the carbon takes place. Carbonyl bond is formed, which results in the loss of $tBuO^-$. CO_2 is expelled and the amine is protonated to form the TFA salt[23].



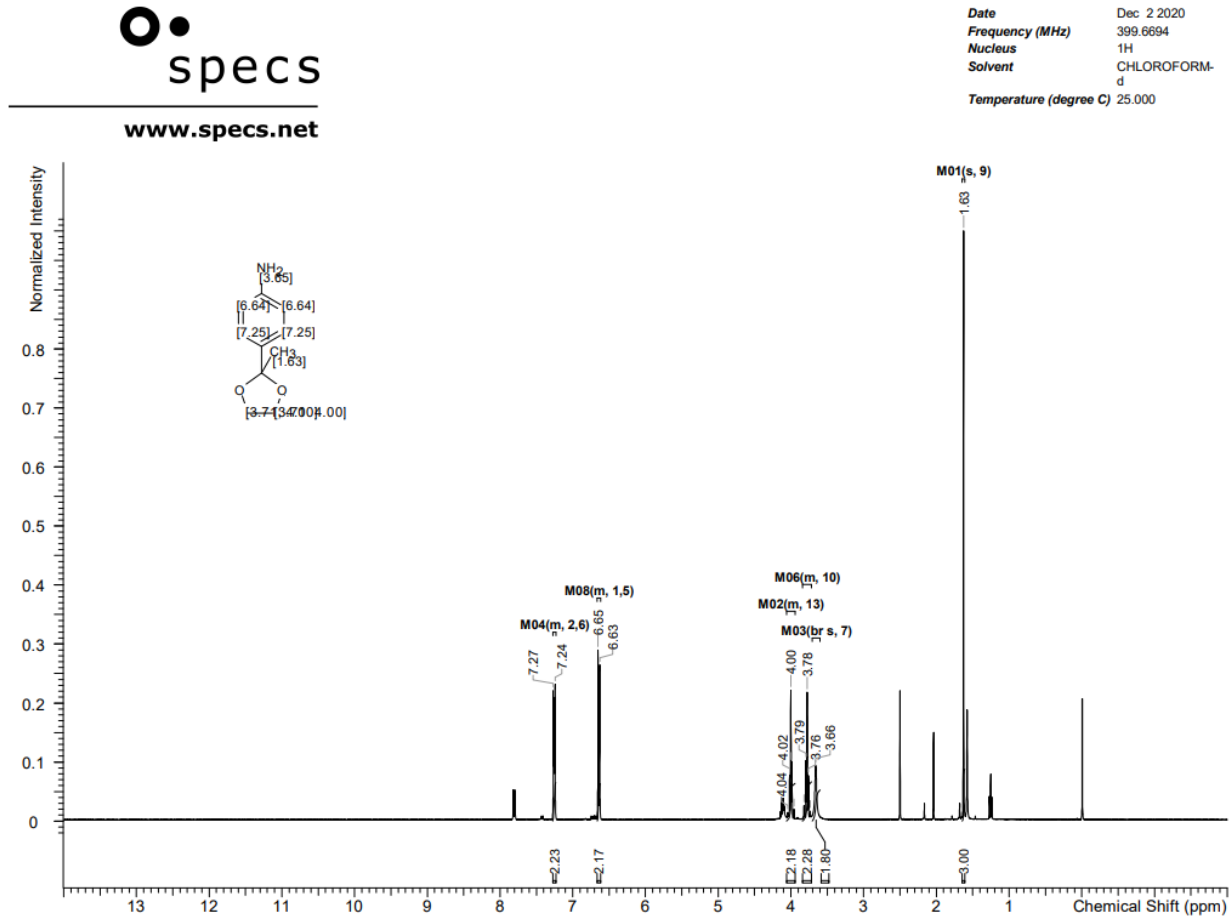
7.2.8. Amide formation obtaining compound **32** (PyBOP)

For the first step, the carboxylic acid is deprotonated Et_3N, which allows a nucleophilic attack of the anion of the carboxylic acid at the positive phosphine from PyBOP. Next, the negative oxygen (resulted from PyBOP) attacks the carbonyl carbon, which removes the corresponding urea. The R-NH_2 now attacks the carbonyl carbon, which removes the resulting PyBOP anion. The base (Et_3N) deprotonates the amine to form the product **32**[24,25].



7.3. Appendix 3: ^{1}H-NMR and LC-MS spectra

7.3.1. ^{1}H-NMR Bechamp reduction obtaining compound **22**

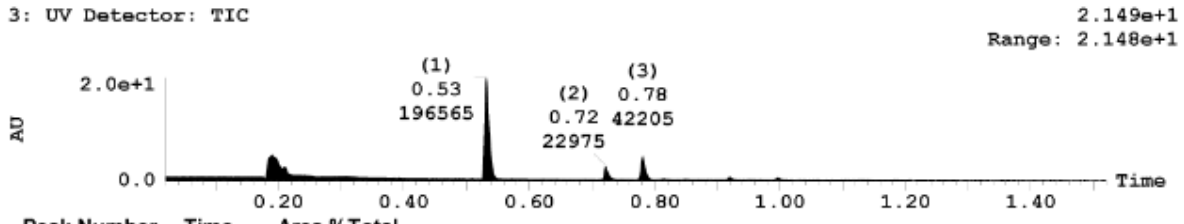


7.3.2. Acetal hydrolysis, radical scavenger of compound **24**

Specs info@specs.net, www.specs.net Page 1

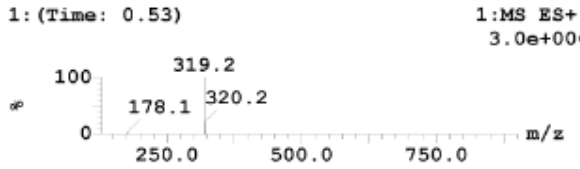
File:SRL_AS_0034C ID:SRL_AS_0034C Description:C18H26N2O3

Vial:1:6 Date:11-Dec-2020 Time:10:27:46

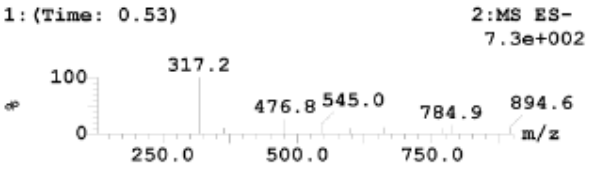


Peak Number	Time	Area %Total
1	0.53	72.50
2	0.72	8.47
3	0.78	15.57
4	0.92	1.81
5	1.00	1.64

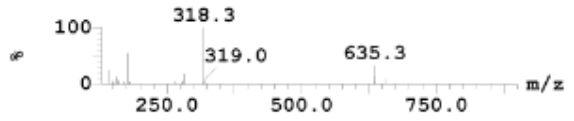
Peak ID	Time	Mass Found	BPM
1	0.53	318.19	319.17



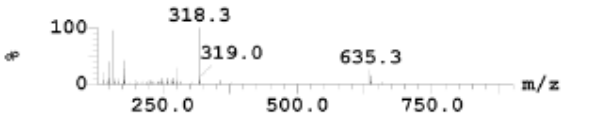
Peak ID	Time	Mass Found	BPM
1	0.53	318.19	317.24



Peak ID	Time	Mass Found	BPM
2	0.72	318.19	318.31



Peak ID	Time	Mass Found	BPM
3	0.78	318.19	318.29



Peak ID	Time	Mass Found	BPM
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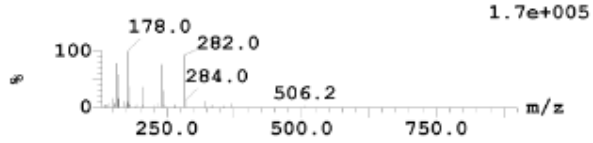

Peak ID	Time	Mass Found	BPM
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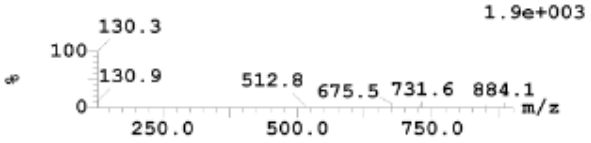

Peak ID	Time	Mass Found	BPM
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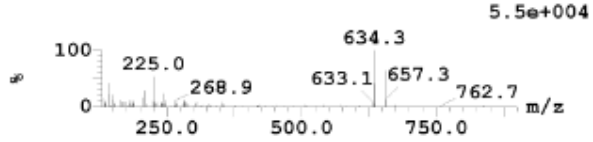
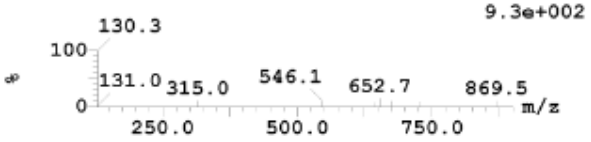
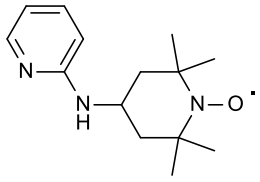


Peak ID	Time	Mass Found	BPM
5	1.00		130.27



7.4. Appendix 4: Experimental

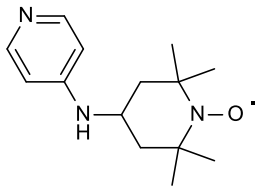
All the achieved compounds are identified by use of ^{1}1H-NMR-analysis. The ^{1}1H-NMR of the spin labels are measured with 1 equivalent ascorbic acid added. To determine the purity of the final analogues LC-MS analysis is also executed alongside ^{1}1H-NMR- analysis. All the ^{1}1H-NMR spectra were recorded at Agilent 400 MHz. The LC-MS were taken on an Acquity UPLC-SQD system from Waters. Specs' general LC-MS method involves a gradient elution of Water (Formic acid 0.1%)/Acetonitrile on an HSS-T3 column (2,1 x 50 mm, Waters), 1.8 µm, at 30°C, PDA detection between 240-320 nm, and MS detection by simultaneous ES+/ES- ionization in a mass range of 150-800. The flow is set to 0.9 ml/min, and the gradient time is 1.5 min.



N-(TEMPO)pyridin-2-amine (3):

2-bromopyridine (371.2 mg, 2.349 mmol), NaOtBu (311.5 mg, 3.241 mmol), DavePhos (54.1 mg, 0.137 mmol) and $Pd_2(dba)_3$ (61.1 mg, 0.0667 mmol) where added to a mixture of **1** (218.3 mg, 1.275 mmol) which was dissolved in 6 mL dioxane. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and stirred at 91 °C for 3 h. TLC (20% EtOAc in PE (40-60)) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with water. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 30% EtOAc in PE (40-60) to give compound **3** (210.0 mg, 0.8462 mmol, 66%) as an orange solid. LC-MS: 100%.

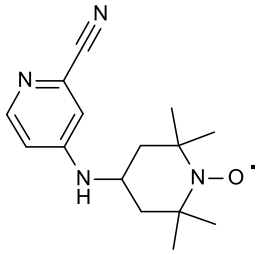
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 7.94 (dd, *J* = 5.1, 2.0 Hz, 1H), 7.29-7.35 (m, 1H), 7.10 (br s, 1H), 6.37-6.44 (m, 2H), 3.99-4.11 (m, 1H), 1.78-1.82 (m, 2H), 1.25-1.31 (m, 2H), 1.10 (s, 6H), 1.06 (s, 6H).



N-(TEMPO)pyridin-4-amine (4):

4-chloropyridine hydrochloride (357.8 mg, 2.385 mmol), NaOtBu (314.1 mg, 3.268 mmol), DavePhos (59.5 mg, 0.151 mmol), Et_3N (0.20 mL, 1.40 mmol) and $Pd_2(dba)_3$ (0) (68.0 mg, 0.0743 mmol) where added to a mixture of **1** (225.1 mg, 1.314 mmol) which was dissolved in 6 ml dioxane. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and stirred at 91 °C for 10 h. TLC (10% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM, washed with water and saturated solution of $NaHCO_3$. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 10% MeOH /DCM) to give compound **4** (70.0 mg, 0.282 mmol, 21%) as an orange solid. LC-MS: 96%.

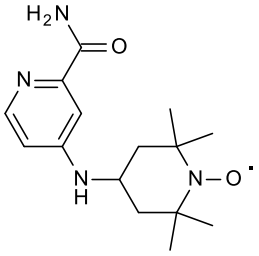
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm= 8.05 (d, *J* = 6.6 Hz, 2H), 7.16-7.25 (m, 1H), 6.62 (d, *J* = 7.0 Hz, 2H), 4.13-4.28 (m, 1H), 1.81 (dd, *J* = 12.5, 3.5 Hz, 2H), 1.33 (t, *J* = 12.1 Hz, 2H), 1.14 (s, 6H), 1.08 ppm (s, 6H).



4-[(TEMPO)amino]pyridine-2-carbonitrile (5):

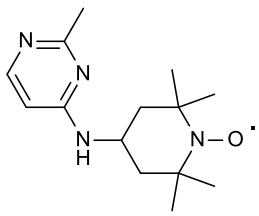
4-bromopyridine-2-carbonitrile (287.4 mg, 1.570 mmol), XPhos (70.5 mg, 0.148 mmol), K_3PO_4 (754.9 mg, 3.556 mmol) where added to a mixture of **1** (225.1 mg, 1.314 mmol) which was dissolved in 8 mL dioxane. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and $pd_2(dba)_3$ (89.1 mg, 0.0973 mmol) was added. The reaction mixture was stirred at 90°C for 10 h. Color of the reaction mixture turned from yellow-orange to yellow-grey. TLC (10% MeOH in DCM) indicated almost complete consumption of the starting material. The solids were filtered off over celite. The filtrate mixture was diluted with EtOAc and washed with water.

The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 20% PE (40-60) in EtOAc) to give compound **5** (26.9 mg, 0.0984 mmol, 7%) as a light-orange solid. LC-MS: 97%.



4-[(TEMPO)amino]pyridine-2-carboxamide (6):

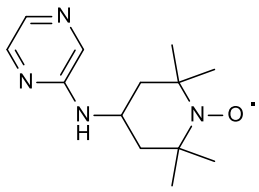
Compound **25** (48.1 mg, 0.176 mmol), K_2CO_3 (11.3 mg, 0.0818 mmol) and hydrogen peroxide 35% (7.90 mg, 0.232 mmol) where dissolved in 3 mL DMSO. The reaction mixture was stirred at room temperature for 48 h. Color of the reaction mixture changed from orange to brown-orange after 22 h. TLC (EtOAc) indicated half consumption of the starting material. The reaction mixture was diluted with 10 mL water while stirring, extracted with DCM and the organic layers were washed with brine. The combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, EtOAc) to give compound **6** (12.1 mg, 0.0415 mmol, 24%) as a light-orange solid. LC-MS: 94%.



N-(TEMPO)-2-methyl-pyrimidin-4-amine: (7):

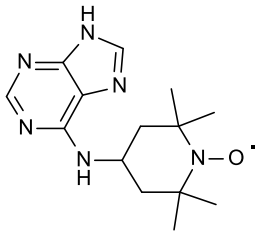
4-chloro-2-methyl-pyrimidine (205.9 mg, 1.202 mmol), NaOtBu (318.6 mg, 3.315 mmol), DavePhos (55.8 mg, 0.142 mmol) and $Pd_2(dba)_3$ (65.2 mg, 0.0712 mmol) where added to a mixture of **1** (205.9 mg, 1.202 mmol) which was dissolved in 6 mL dioxane. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and stirred at 91°C for 6.5 h. TLC (5% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with water. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 5% MeOH in DCM) to give compound **7** (90.0 mg, 0.342 mmol, 28%) as an orange solid. LC-MS: 100%.

^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ = 7.85-7.96 (m, 1H), 7.06 (br d, *J* = 6.6 Hz, 1H), 6.17-6.25 (m, 1H), 4.11-4.29 (m, 1H), 2.30 (s, 3H), 1.77 (br d, *J* = 9.4 Hz, 2H), 1.25-1.38 (m, 2H), 1.12 (s, 6H), 1.06 ppm (s, 6H).



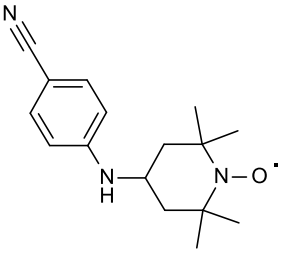
N-(TEMPO)pyrazin-2-amine (8):

2-bromopyrazine (0.15 mL, 1.69 mmol), XPhos (91.9 mg, 0.193 mmol), K_3PO_4 (875.4 mg, 4.124 mmol), $pd_2(dba)_3$ (83.9 mg, 0.0916 mmol) where added to a mixture of **1** (223.9 mg, 1.307 mmol) which was dissolved in 6 mL toluene. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and stirred at 110°C for 6.5 h and at room temperature for 63 h. TLC (10% MeOH in DCM) indicated complete consumption of the starting material. The solids were filtered off over celite. The filtrate mixture was diluted with EtOAc and washed with water. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 5% MeOH in DCM) to give compound **8** (80.7 mg, 0.324 mmol, 25%) as an orange solid. LC-MS: 100%.



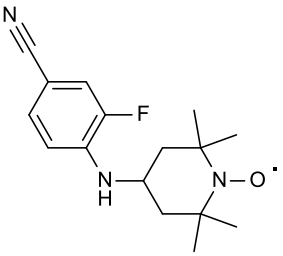
N-(TEMPO)-9H-purin-6-amine (9):

6-bromo-9H-purine (240.9 mg, 1.210 mmol) and Et_3N (0.30 mL, 2.16 mmol) where added to a mixture of **1** (193.7 mg, 1.131 mmol) which was dissolved in 5 mL DMF. The reaction mixture was stirred at 125 °C for 4.5 h and 18 h at room temperature. Color of the reaction mixture changed from red-brown to dark red-brown. TLC (20% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc and washed with water. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over MgSO4, filtered and evaporated. The crude product did not dissolve in the solvent system (15% MeOH in EtOAc), the product was filtered off to give compound **9** (37.4 mg, 0.129 mmol, 11%) as an orange solid. LC-MS: 92%.



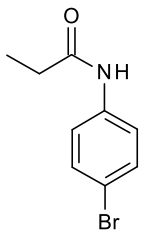
4-[(TEMPO)amino]benzonitrile (10):

4-bromobenzonitrile (416.1 mg, 2.286 mmol), XPhos (85.4 mg, 0.179 mmol), K_3PO_4 (863.0 mg, 4.066 mmol), $pd_2(dba)_3$ (83.8 mg, 0.0915 mmol) where added to a mixture of **1** (251.4 mg, 1.468 mmol) which was dissolved in 6 mL toluene. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and stirred at 110°C for 2 h. TLC (5% MeOH in DCM) indicated complete consumption of the starting material. The solids were filtered off over celite. The filtrate mixture was diluted with EtOAc and washed with water. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 40% EtOAc in PE (40-60)) to give compound **10** (160.0 mg, 0.5874mmol, 40%) as an (pink-)orange solid. LC-MS: 100%.



3-fluoro-4-[(TEMPO)amino]benzonitrile (11):

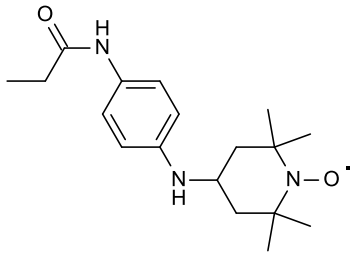
4-bromo-3-fluoro-benzonitrile (447.5 mg, 2.237 mmol), XPhos (88.0 mg, 0.185 mmol), K_3PO_4 (887.2 mg, 4.18 mmol), $pd_2(dba)_3$ (94.2 mg, 0.103 mmol) where added to a mixture of **1** (268.1 mg, 1.565mmol) which was dissolved in 6 mL toluene. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and stirred at 110°C for 5 h and at room temperature for 17 h. TLC (40% EtOAc in PE (40-60)) indicated complete consumption of the starting material. The solids were filtered off over celite. The filtrate mixture was diluted with EtOAc and washed with water. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 20%-30% EtOAc in PE (40-60) to give compound **11** (190.0 mg, 0.6543 mmol, 42%) as an orange solid. LC-MS: 100%.



N-(4-bromophenyl)propanamide (12):

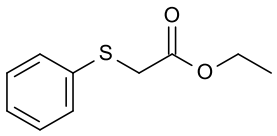
Propanoyl chloride (745.5 mg, 8.057 mmol) dissolved in 5 mL DCM and Et_3N (1.80 mL, 12.98 mmol) where added dropwise to a mixture of 4-bromoaniline (1.14 g, 6.62 mmol) which was dissolved in 10 mL DCM while stirring at 0 °C. The reaction mixture was stirred at room temperature for 3 h. TLC (10% PE (40-60) in EtOAc) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with water. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated to give compound **12** (1.48 g, 6.48 mmol, 98%) as an off-white solid.

^{1}H NMR (400 MHz, CHLOROFORM-d, 25ºC): δ ppm = 7.40-7.44 (m, 4H), 7.23 (br s, 1H), 2.39 (q, *J* = 7.6 Hz, 2H), 1.25 (t, *J* = 7.4 Hz, 3H).



N-[4-[(TEMPO)amino]phenyl]propanamide (13):

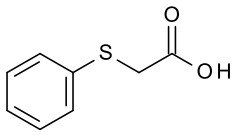
1 (233.3 mg, 1.362 mmol), XPhos (80.0 mg, 0.168 mmol), K_3PO_4 (773.0 mg, 3.642 mmol) where added to a mixture of compound **22** (345.6 mg, 1.515 mmol) which was dissolved in 10 mL dioxane. The reaction mixture was evacuated *in vacuo* and flushed three times with N_2 and $pd_2(dba)_3$ (88.9 mg, 0.0971 mmol) was added. The reaction mixture was stirred at 91°C for 3.5 h. Color of the reaction mixture turned from yellow-orange to yellow-grey. TLC (5% MeOH in DCM) indicated complete consumption of the starting material. The solids were filtered off over celite. The filtrate mixture was diluted with EtOAc and washed with water. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 15% PE (40-60) in EtOAc) to give compound **13** (65.7 mg, 0.206 mmol, 15%) as an light-orange solid. LC-MS: 95%.



Ethyl 2-phenylsulfanylacetate (14):

A mixture of benzenethiol (2.20 mL, 21.6 mmol), ethyl 2-chloroacetate (2.30 mL, 21.9 mmol) and K_2CO_3 (10.83 g, 78.34 mmol) in 100 mL acetone was stirred at 70 °C for 2.5 h. TLC (5% EtOAc in PE (40-60)) indicated complete consumption of the starting material. The reaction mixture was filtered and rinsed with acetone. The filtrate was evaporated, dissolved in EtOAc and washed with 1M HCl. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 5% EtOAc in PE (40-60)) to give compound **14** (3.42 g, 17.4 mmol, 81%) as an off-white solid.

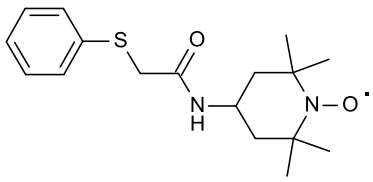
^{1}H NMR (400 MHz, CHLOROFORM-d, 25ºC): δ ppm = 7.36-7.43 (m, 2H), 7.23-7.31 (m, 2H), 7.16-7.23 (m, 1H), 4.09-4.17 (m, 2H), 3.61 (d, *J* = 2.3 Hz, 2H), 1.16-1.23 ppm (m, 3H).



2-phenylsulfanylacetic acid (15):

NaOH (1.10 g, 27.5 mmol) dissolved in 5 mL water, was added to a mixture of compound **14** (3.13 g, 15.9 mmol) which was dissolved in 40 mL EtOH. The reaction mixture was stirred at room temperature for 2 h. More NaOH (1.017 g, 25.42 mmol) was added, stirred at 68 °C for 20 h. TLC (5% EtOAc in PE (40-60)) indicated complete consumption of the starting material. White solid formed, EtOH was evaporated and 1M HCl was added to Ph= 1-2. The water layer was extracted with EtOAc and the organic layer was washed with brine, dried over $MgSO_4$, filtered and evaporated to give compound **4** (2.70 g, 16.0 mmol, 101%) as an off-white solid.

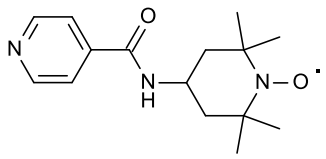
^{1}H NMR (400 MHz, CHLOROFORM-d, 25ºC): δ ppm = 7.41-7.45 (m, 2H), 7.29-7.35 (m, 2H), 7.23-7.28 (m, 1H), 3.68 ppm (s, 2H).



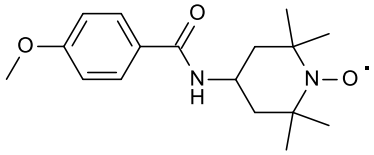
N-(TEMPO)-2-phenylsulfanyl-acetamide (16):

Compound **15** (226.3 mg, 1.345 mmol), HOBt Hydate (33.8 mg, 0.250 mmol), EDCI (252.9 mg, 1.319 mmol) and Et3N (0.50 mL, 3.50 mmol) were added to a mixture of **1** (203.2 mg, 1.187 mmol) which was dissolved in 5 mL DCM. The reaction mixture was stirred at room temperature for 3 h. TLC (20% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc and washed with a

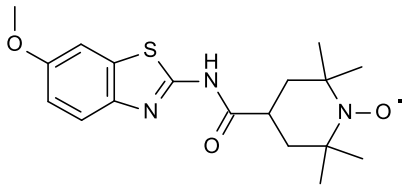
saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 5% MeOH in DCM) to give compound **16** (180.0 mg, 0.5599 mmol, 47%) as an orange solid. LC-MS: 100%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 7.28-7.38 (m, 4H), 7.17-7.21 (m, 1H), 7.12 (br s, 1H), 3.81-3.97 (m, 1H), 3.57 (s, 2H), 1.59 (dd, *J* = 12.9, 3.5 Hz, 2H), 1.20-1.29 (m, 2H), 1.03 (d, *J* = 1.6 Hz, 12H).



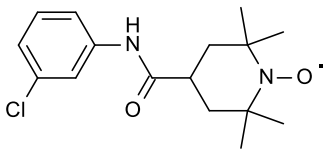
N-(TEMPO)pyridine-4-carboxamide (17):
Isonicotinic acid (215.5 mg, 1.750 mmol), HOBt Hydrate (53.3 mg, 0.394 mmol), EDCI (303.3 mg, 1.582 mmol) and Et_3N (0.50 mL, 3.61 mmol) where added to a mixture of **1** (207.9 mg, 1.214 mmol) which was dissolved in 5 mL EtOH. The reaction mixture was stirred at 50 ºC for 30 min and at room temperature for 70 h. TLC (20% MeOH in DCM) indicated complete consumption of the starting material. The solvent was evaporated and EtOAc was added, the organic layer was washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 10% MeOH in DCM) to give compound **17** (230.0 mg, 0.8322 mmol, 69%) as an orange solid. LC-MS: 97%.



N-(TEMPO)-4-methoxy-benzamide (18):
4-methoxybenzoyl chloride (308.3 mg, 1.807 mmol) dissolved in 3 mL DCM and Et_3N (0.40 mL, 2.89 mmol) where added dropwise to a mixture of **1** (216.1 mg, 1.262 mmol) which was dissolved in 3 mL DCM while stirring at 0 ºC. The reaction mixture was stirred at 0 ºC for 15 min and at room temperature for 1.5 h. TLC (5% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with water. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 3% MeOH in DCM) to give compound **18** (133.6 mg, 0.4375 mmol, 35%) as a pink-orange solid. LC-MS: 93%.

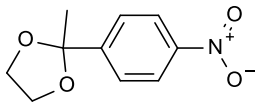


N-(6-methoxy-1,3-benzothiazol-2-yl)-TEMPO-4-carboxamide (19):
6-methoxy-1,3-benzathiazol-2-amine (182.9 mg, 1.015 mmol), HOBt Hydrate (32.4 mg, 0.240 mmol), EDCI (237.1 mg, 1.237 mmol) and Et3N (0.50 mL, 3.71 mmol) where added to a mixture of **1** (253.6 mg, 1.254 mmol) which was dissolved in 5 mL THF. The reaction mixture was stirred at room temperature for 24 h. TLC (5% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 5% MeOH in DCM) to give compound **19** (190.0 mg, 0.5242 mmol, 52%) as an orange solid. LC-MS: 97%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ = 7.62 (d, *J* = 9.0 Hz, 1H), 7.55 (d, *J* = 2.3 Hz, 1H), 7.20 (br s, 1H), 7.02 (dd, *J* = 9.0, 2.7 Hz, 1H), 3.80 (s, 3H), 2.95 (br t, *J* = 12.3 Hz, 1H), 1.66-1.73 (m, 2H), 1.51-1.60 (m, 2H), 1.09 (s, 6H), 1.07 ppm (s, 6H).



N-(3-chlorophenyl)-TEMPO-4-carboxamide (20):

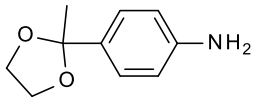
3-chloroaniline (145.8 mg, 1.143 mmol), HOBt Hydrate (29.0 mg, 0.215 mmol), EDCI (206.6 mg, 1.078 mmol) and Et_3N (0.45 mL, 3.25 mmol) where added to a mixture of **1** (222.6 mg, 1.100 mmol) which was dissolved in 5 mL THF. The reaction mixture was stirred at room temperature for 22 h. TLC (5% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 5% MeOH in DCM) to give compound **20** (50.0 mg, 0.162 mmol, 15%) as an orange solid. LC-MS: 100%.



2-methyl-2-(4-nitrophenyl)-1,3-dioxolane (21):

Ethylene glycol (3.04 g, 48.9 mmol) and P-Toluenesulfonic acid 978.1 mg, 5.680 mmol) where added to a mixture of 1-(4-nitrophenyl)ethanone (4.14 g, 25.1 mmol) which was dissolved in 100 mL benzene. The reaction mixture was stirred at 120 °C, dean-stark for 4 h. TLC (20% EtOAc in PE (40-60)) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated to give compound **21** (4.88 g, 23.3 mmol, 93%) as an white solid.

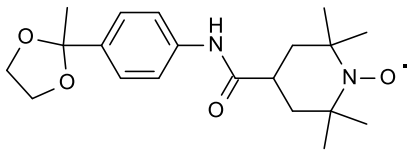
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 8.22 (d, *J* = 9.0 Hz, 2H), 7.69 (d, *J* = 9.0 Hz, 2H), 3.97-4.08 (m, 2H), 3.65-3.76 (m, 2H), 1.58 ppm (s, 3H).



4-(2-methyl-1,3-dioxolan-2-yl)aniline (22):

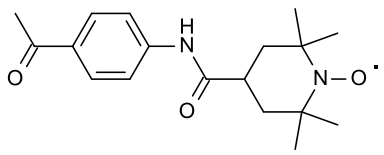
Ammonium chloride (218.2 mg, 4.079 mmol) and iron powder (1.43 g, 25.6 mmol) where added to a mixture of compound **21** (994.9 mg, 4.756 mmol) which was dissolved in 10 mL EtOH and 5 mL water. Refluxed at 80 °C for 30 min, the color from the reaction mixture changed from light yellow to red-brown to black. TLC (50% EtOAc in PE (40-60)) indicated complete consumption of the starting material. A saturated solution of $NaHCO_3$ was added and the solids were filtered off over celite. The filtrate mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated to give compound **22** (680.0 mg, 3.794 mmol, 80%) as an yellow solid.

^{1}H NMR (400 MHz, CHLOROFORM-d, 25ºC): δ ppm = 7.22-7.29 (m, 2H), 6.64 (m, *J* = 8.6 Hz, 2H), 3.94-4.06 (m, 2H), 3.72-3.84 (m, 2H), 3.66 (br s, 2H), 1.63 (s, 3H).

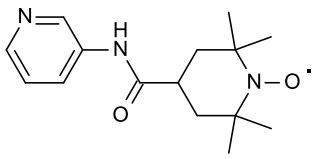


N-[4-(2-methyl-1,3-dioxolan-2-yl)phenyl-TEMPO-4-carboxamide (23):

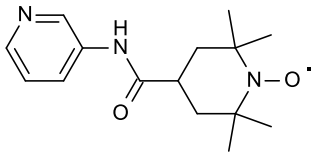
Compound **22** (261.6 mg, 1.460 mmol), HOBt Hydrate (44.4 mg, 0.329 mmol), EDCI (235.4 mg, 1.228 mmol) and Et_3N (0.45 mL, 3.25 mmol) where added to a mixture of **2** (213.8 mg, 1.068 mmol) which was dissolved in 5 mL DMF. The reaction mixture was stirred at room temperature for 20 h. TLC (10% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 20% PE (40-60) in ether) to give compound **23** (170.0 mg, 0.4703 mmol, 44%) as a yellow solid. LC-MS: 100%.



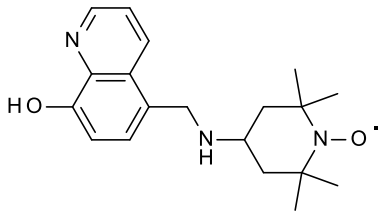
N-(4-acetylphenyl)-TEMPO-4-carboxamide (24):
Compound **23** (160.0 mg, 0.4426 mmol) was dissolved in 3 mL THF, 1M HCL (3.0 mL, 88.5 mmol) was added dropwise to the mixture. The reaction mixture was stirred at 50 °C for 15 min and at room temperature for 1.5 h. Color turned yellow. TLC (10% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 3,5% isopropanol in DCM) to give compound **24** (40.5 mg, 0.128 mmol, 29%) as a light orange solid. LC-MS: 97%.



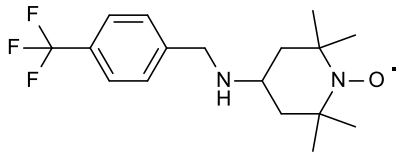
N-(3-pyridyl)-TEMPO-4-carboxamide (25):
pyridin-3-amine (126.5 mg, 1.344 mmol), HOBt Hydrate (32.8 mg, 0.243 mmol), EDCI (207.5 mg, 1.082 mmol) and Et_3N (0.50 mL, 3.61 mmol) where added to a mixture of **2** (199.1 mg, 0.9942 mmol) which was dissolved in 5 mL DMF. The reaction mixture was stirred room temperature for 2 h. TLC (15% isopropanol in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 10% isopropanol in DCM) to give compound **25** (53.2 mg, 0.193 mmol, 19%) as an orange solid. LC-MS: 100%.



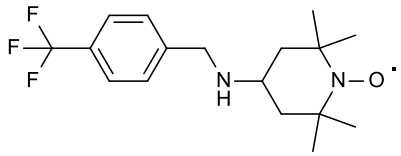
N-(3-pyridyl)-TEMPO-4-carboxamide (25):
pyridin-3-amine (128.1 mg, 1.361 mmol), HOBt Hydrate (52.0 mg, 0.385 mmol), EDCI (217.7 mg, 1.136 mmol) and Et_3N (0.50 mL, 3.61 mmol) where added to a mixture of **2** (198.8 mg, 0.9927mmol) which was dissolved in 5 mL THF. The reaction mixture was stirred room temperature for 24 h. TLC (5% MeOH in DCM) indicated complete consumption of the starting material. The solvent was evaporated and the crude reaction mixture was purified by column chromatography (silica, 10% isopropanol in DCM) to give compound **19** (87.5 mg, 0.317 mmol, 32%) as an orange solid. LC-MS: 96%.



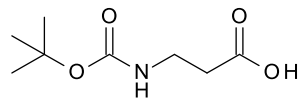
5-[[(TEMPO)amino]methyl]quinolin-8-ol (26):
1 (128.9 mg, 0.7527 mmol) and K_2CO_3 (509.6 mg, 3.687 mmol) where added to a mixture of 5-(chloromethyl)quinolin-8-ol hydrochloride (200.9 mg, 0.8731 mmol) which was dissolved in 5 mL DMF. The reaction mixture was stirred at 62°C for 3 h and 20 h overnight at room temperature. After 2 h the color of the reaction mixture changed from yellow-grey to darkgreen. TLC (20% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with EtOAc, washed with water and a saturated solution of $NaHCO_3$. The water layer was extracted with EtOAc and the combined organic layers were collected, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 10% MeOH in DCM) to give compound **26** (30.0 mg, 0.0913 mmol, 12%) as an orange solid.



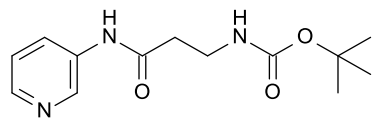
N-[[4-(trifluoromethyl)phenyl]methyl]-TEMPO-4-amine (27):
1 (225.1 mg, 1.314 mmol) and K_2CO_3 (755.6 mg, 5.467 mmol) where added to a mixture of 4-bromomethyl-trifluormethylbenzene (360.8 mg, 1.509 mmol) which was dissolved in 5 ml DMF. The reaction mixture was stirred at room temperature for 46 h. TLC (10% MeOH in DCM) indicated complete consumption of the starting material. The solids were filtered off over celite. The reaction mixture was diluted with EtOAc and washed with water. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 5% MeOH in DCM) and recrystallized (1:1 PE/Ether) to give compound **27** (53,7 mg, 0.163 mmol, 12%) as a red-orange solid. LC-MS: 67%. The mother liquor was recrystallized to give compound **27** (22.2 mg, 0.0674 mmol, 5%) as a red-orange solid. LC-MS: 75%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 7.67 (d, *J* = 7.8 Hz, 2H), 7.57 (d, *J* = 8.2 Hz, 2H), 7.09 (br s, 1H), 3.84 (s, 2H), 2.74-2.85 (m, 1H), 1.77-1.83 (m, *J* = 9.4 Hz, 2H), 1.19 (br t, *J* = 12.1 Hz, 2H), 1.05 (s, 6H), 0.98 (s, 6H).



N-[[4-(trifluoromethyl)phenyl]methyl]-TEMPO-4-amine (27):
Sodium cyanoborohydride (110.6 mg, 1.706 mmol) dissolved in 6 mL MeOH and 4-(trifluoromethyl)benzaldehyde (0.20 mL, 1.46 mmol) where added dropwise to a mixture of **1** (225.5 mg, 1.317 mmol) which was dissolved in 6 mL MeOH. The reaction mixture was stirred at room temperature for 2 h. TLC (10% MeOH in DCM) indicated complete consumption of the starting material. The solvent was evaporated, the crude was diluted with EtOAc and washed with water. The water layer was washed with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 8% MeOH in EtOAc) to give compound **27** (96.6 mg, 0.294 mmol, 22%) as a red-orange solid. LC-MS: 75%.

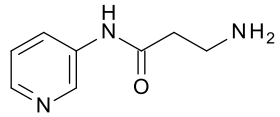


3-(tert-butoxycarbonylamino)propanoic acid (28):
Boc_2O (4.16 g, 19.1 mmol) dissolved in 2 mL THF was added to a solution of 3-aminopropanoic acid (1.41 g, 15.9 mmol) which was dissolved in 21 mL of 1:2 1M NaOH:THF, while stirring at 0 ºC. The reaction mixture was stirred at room temperature for 23 h. TLC (MeOH) indicated almost complete consumption of the starting material. The solvent was evaporated, the water layer was acidified to Ph=1. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated to give compound **28** (3.29 g, 17.4 mmol, 109%) as an see-through oil what changed to a white solid.
^{1}H NMR (400 MHz, CHLOROFORM-d, 25ºC): δ ppm = 5.04 (br s, 1H), 3.32-3.51 (m, 2H), 2.47-2.69 (m, 2H), 1.45 (br s, 9H).

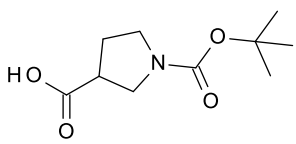


Tert-butyl N-[3-oxo-3-(3-pyridylamino)propyl]carbamate (29):
Pyridin-3-amine (246.3 mg, 2.617 mmol), HATU (1.00 g, 2.64 mmol) and Et_3N (0.70 mL, 5.05 mmol) were added to a mixture of compound **28** (446.2 mg, 2.358 mmol) in 8 mL DCM. The reaction mixture was stirred at room temperature for 70 h. TLC (5% MeOH in EtOAc) indicated complete consumption of the starting material. The reaction mixture was diluted with 50 mL water while stirring, extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. EtOAc was added and the product was filtered off. The filtrate was purified by

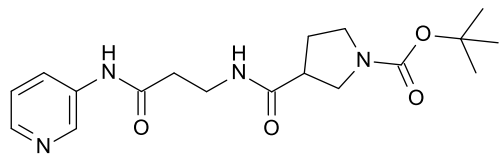
column chromatography (silica, 10% MeOH in EtOAc) to give compound **29** (350.3 mg, 1.320 mmol, 56%) as a light-pink solid (filtered product, LC-MS: 100%) and white solid (column product, LC-MS: 89%).
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 10.13 (s, 1H), 8.72 (s, 1H), 8.24 (d, *J* = 4.7 Hz, 1H), 8.02 (br d, *J* = 8.6 Hz, 1H), 7.32 (dd, *J* = 8.2, 4.7 Hz, 1H), 6.84-6.92 (m, 1H), 3.22 (q, *J* = 6.7 Hz, 2H), 2.46-2.49 (m, 2H), 1.37 (s, 9H).



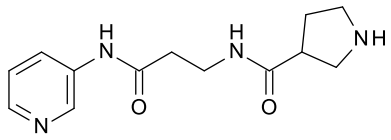
3-amino-N-(3-pyridyl)propenamide (30):
Added 10 mL DCM to compound **29** (1.86 g, 7.02 mmol). TFA (4.80 mL, 64.6 mmol) in 20 mL DCM was added dropwise to the reaction mixture while stirring in a cold water bath. While adding the TFA, compound **29** started to dissolve. The reaction mixture was stirred at room temperature for 1 h. TLC (MeOH) indicated complete consumption of the starting material. DCM was evaporated and the crude product was purified by column chromatography (silica, 10% ammonia (25% in water) in MeOH)) to give compound **30** (865.0 mg, 5.236 mmol, 75%) as a yellow oil. LC-MS: 100%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 8.72 (d, *J* = 2.3 Hz, 1H), 8.23 (dd, *J* = 4.7, 1.6 Hz, 1H), 8.03 (dt, *J* = 8.3, 1.9 Hz, 1H), 7.32 (dd, *J* = 8.2, 4.7 Hz, 1H), 2.85 (t, *J* = 6.6 Hz, 2H), 2.42 (t, *J* = 6.6 Hz, 2H).



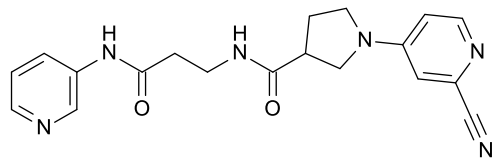
1-tert-butoxycarbonylpyrrolidine-3-carboxylic acid (31):
Dissolved Boc_2O (3.36 g, 15.4 mmol) and pyrrolidine-3-carboxylic acid (1.41 g, 12.2 mmol) in 2 mL water: 6m L acetone. Cooled the reaction mixture till 0 °C in an ice-bath and added sodium carbonate (795.8 mg, 7.508 mmol). The reaction mixture was stirred at 0 °C for 1 h and gradually to room temperature for 21 h. The color of the reaction mixture turned trouble off-white after 30 min. TLC (50% MeOH in EtOAc) indicated complete consumption of the starting material. Acetone was evaporated and citric acid monohydrate was added to pH=4. The water layer was extracted with EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated to give compound **31** (2.77 g, 12.9mmol, 105%) as an white solid.
^{1}H NMR (400 MHz, CHLOROFORM-d, 25ºC): δ ppm = 3.31-3.71 (m, 4H), 3.03-3.17 (m, 1H), 2.09-2.25 (m, 2H), 1.47 (s, 9H).



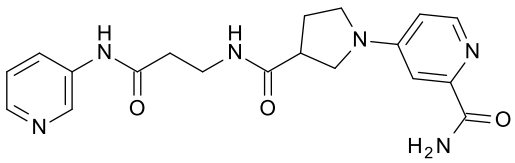
tert-butyl 3-[[3-oxo-3-(3-pyridylamino)propyl]carbamoyl] pyrrolidine-1-carboxylate (32):
Added compound **31** (224.1 mg, 1.041 mmol), DIPEA (0.30 mL, in 3 mL DMF, 1.89 mmol), when everything dissolved added PyBOP (596.0 mg, 1.145 mmol). The color from the reaction mixture turned from see-through to yellow to brown. Stirred at room temperature for 15 min before adding compound **30** (125.5 mg, 0.7597 mmol) dissolved in 4 mL DMF dropwise. The reaction mixture was stirred at room temperature for 45 min. TLC (MeOH) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with a saturated solution of $NaHCO_3$. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 20% MeOH in 1:1 EtOAc:DCM) to give compound **32** (152.4 mg, 0.4205 mmol, 55%) as an off white/light yellow oil, which turned to a solid while scraping. LC-MS: 92%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ = 10.15 (s, 1H), 8.71-8.73 (m, 1H), 8.23-8.25 (m, 1H), 8.10-8.12 (m, 1H), 8.02 (br d, 1H), 7.33 (dd, *J* = 8.2, 4.7, 3.5 Hz, 1H), 4.03 (q, 1H), 3.35-3.42 (m, 2H), 3.10-3.25 (m, 2H), 2.82-2.94 (m, 2H), 2.49-2.53 (m, 2H), 1.82-2.00 (m, 2H), 1.38 ppm (s, 9H).



N-[3-oxo-3-(3-pyridylamino)propyl]pyrrolidine-3-carboxamide (33):
Dissolved compound **32** (144.2 mg, 0.3979 mmol) in 6 mL DCM, added TFA (0.40 mL, 5.38 mmol) in 4 mL DCM dropwise to the reaction mixture while stirring. The reaction mixture was stirred at room temperature for 1 h. TLC (20% MeOH in DCM) indicated complete consumption of the starting material. DCM was evaporated and the crude product was purified by column chromatography (silica, 20% ammonia (25% in water) in MeOH)) to give compound **33** (79.9 mg, 0.305 mmol, 77%) as an off-white oil which changed to a solid while scraping. LC -MS: 87%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 10.16 (br s, 1H), 8.73 (s, 1H), 8.24 (br d, *J* = 3.9 Hz, 1H), 8.02 (br d, *J* = 8.6 Hz, 1H), 7.99 (br s, 1H), 7.33 (dd, *J* = 8.0, 4.5 Hz, 1H), 2.82-2.92 (m, 2H), 2.60-2.81 (m, 5H), 2.51-2.54 (m, 2H), 1.68-1.79 (m, 2H).

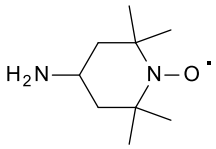
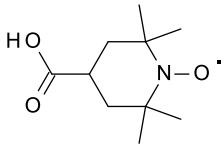
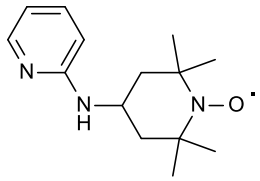
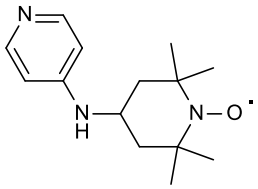
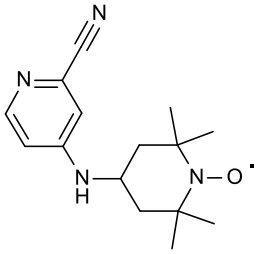


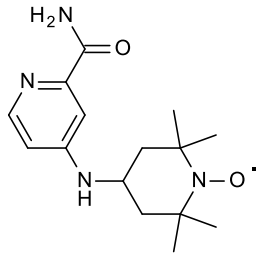
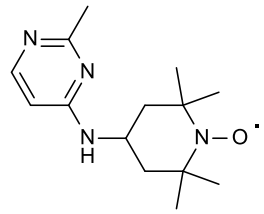
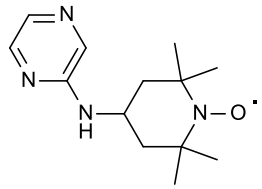
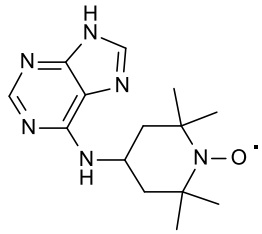
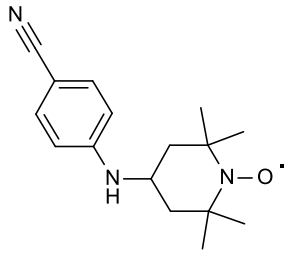
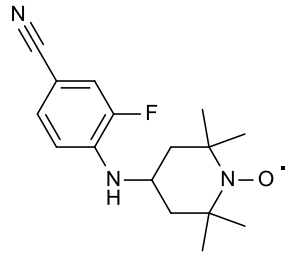
1-(2-cyano-4-pyridyl)-N-[3-oxo-3-(3-pyridylamino)propyl] pyrrolidine-3-carboxamide (34):
4-bromopyridine-2-carbonitrile (152.0 mg, 0.8306 mmol) dissolved in 3 mL DMSO and Et_3N (0.38 mL, 2.75 mmol) dissolved in 2 mL DMSO were added to compound **33** (248.0 mg, 0.9455 mmol). The reaction mixture was stirred at 130 ^{o}C for 2 h. The color of the reaction mixture changed from light-orange to dark orange after 1.5 h. TLC (20% MeOH in EtOAc) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with water. The water layer was extracted with DCM and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (silica, 10-20% MeOH in DCM) to give compound **34** (173.3 mg, 0.4756 mmol, 57%) as an off-white oil which turned to a solid. LC-MS: 100%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 1.17 (s, 1H), 8.74 (d, *J* = 2.3 Hz, 1H), 8.20-8.26 (m, 2H), 8.16 (d, *J* = 5.9 Hz, 1H), 8.00-8.05 (m, 1H), 7.33 (dd, *J* = 8.2, 4.7 Hz, 1H), 7.04 (d, *J* = 2.3 Hz, 1H), 6.64 (dd, *J* = 5.9, 2.3 Hz, 1H), 3.35-3.50 (m, 4H), 3.26-3.32 (m, 2H), 3.08 (quin, *J* = 7.4 Hz, 1H), 2.52-2.56 (m, 2H), 2.00-2.20 (m, 2H).

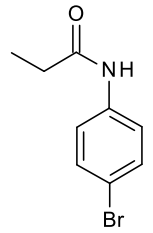
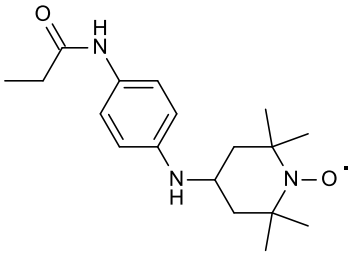
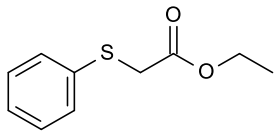
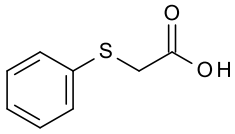
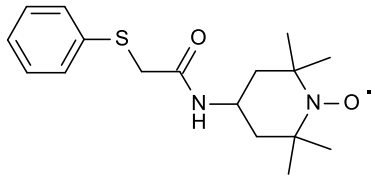
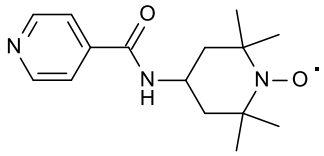
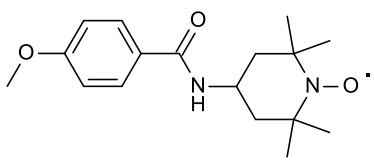


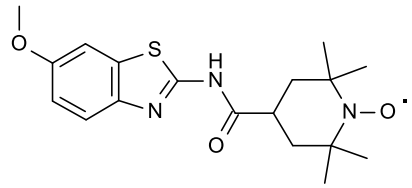
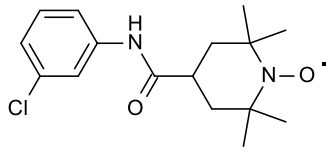
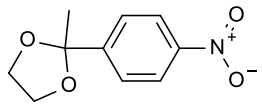
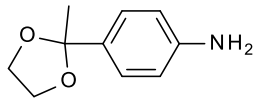
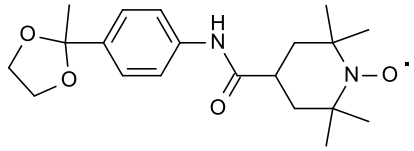
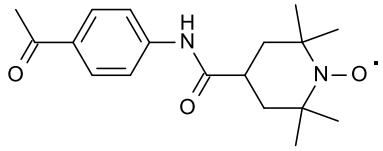
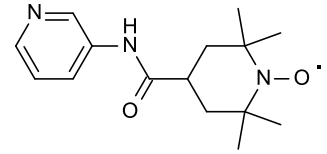
4-[3-[[3-oxo-3-(3-pyridylamino)propyl]carbamoyl] pyrrolidin-1-yl]pyridine-2-carboxamide (35):
Compound **34** (141.6 mg, 0.3886 mmol), K_2CO_3 (15.9 mg, 0.115 mmol) and hydrogen peroxide 35% (36.1 mg, 1.06 mmol) where dissolved in 4 mL DMSO. The reaction mixture was stirred at room temperature for 22 h. Color of the reaction mixture changed from off-white to lighter off-white. TLC (20% MeOH in DCM) indicated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with water. The water layer was extracted with DCM, warm EtOAc and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated to give compound **35** (63.5 mg, 0.166 mmol, 43%) as an off-white solid. LC-MS: 100%.
^{1}H NMR (400 MHz, DMSO-d_6, 25ºC): δ ppm = 10.19 (br s, 1H), 8.71-8.79 (m, 1H), 8.17-8.29 (m, 2H), 8.07-8.15 (m, *J* = 5.1 Hz, 1H), 7.99-8.06 (m, 1H), 7.96 (br s, 1H), 7.43-7.51 (m, 1H), 7.30-7.36 (m, 1H), 7.10 (br s, 1H), 6.51-6.58 (m, *J* = 4.3 Hz, 1H), 3.35-3.54 (m, 6H), 3.05-3.14 (m, 1H), 2.52-2.58 (m, 2H), 2.01-2.22 (m, 2H).

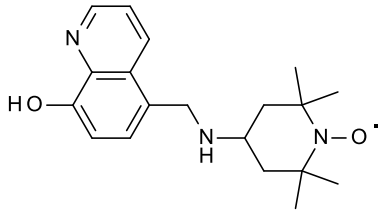
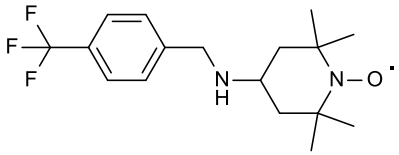
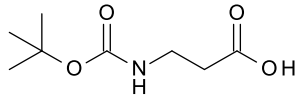
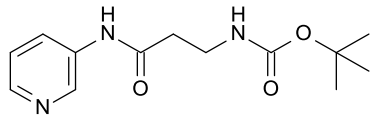
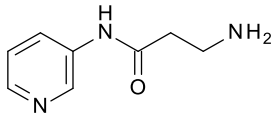
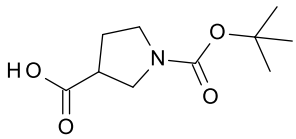
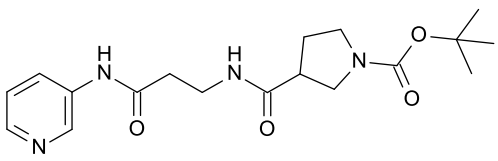
7.5. Appendix 5: Compound numbers and yield

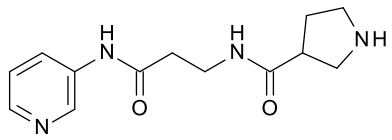
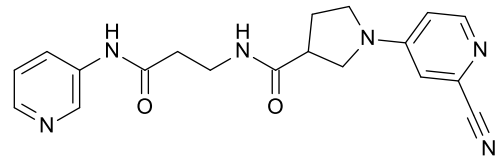
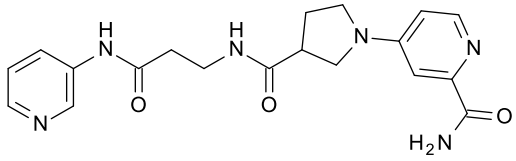
Compound structure	Compound nr	Yield
	1	-
	2	-
	3	66%
	4	21%
	5	7 and 10%

	6	24%
	7	28%
	8	25%
	9	11%
	10	40%
	11	42%

	12	98%
	13	15%
	14	81%
	15	Quantitative
	16	47%
	17	69%
	18	35%

	19	52%
	20	15%
	21	93%
	22	80%
	23	44%
	24	29%
	25	32%

	26	7 and 12% yield (not pure)
	27	22% yield (not pure)
	28	Quantitative
	29	56%
	30	75%
	31	Quantitative
	32	55%

	33	76%
	34	57%
	35	43%