Universitat de Girona

Facultat de Ciències

Memòria del Treball de Final de Grau

**Títol del treball:**

# SYNTHESIS AND EVALUATION OF IMAGING PROBES FOR PROLYL OLIGOPEPTIDASE (PREP)

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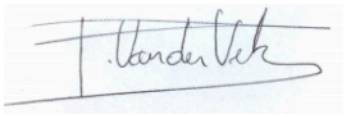

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**Abstract**

PREP is an endopeptidase member of the serine peptidase family that has a selective mechanism: it cleaves peptides with no more than 30 amino acid residues at the carboxyl side of proline residues. It has a high expression in brain and central nervous system (CNS) and has been found to be related to some neurodegenerative pathologies like Alzheimer's disease, Parkinson, Schizophrenia, mania, depression and epilepsy.

The main goal for this project was to synthesise molecular imaging probes to follow the evolution of PREP expression during epileptogenesis. This imaging probes consist of three main parts: a PREP inhibitor as a targeting moiety, biotin and rhodamine B piperazine anaolgues as signal agents and a linker that connects both parts. Two different signal agents have been synthesised in order to know which is the best option.

The generic structure for PREP inhibitor has been divided in three building blocks: (*S*)-pyrrolidine-2-carboxamide hydrochloride**,** (*2S,4S*)-4-azido-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid and 4-phenylbutanoyl chloride, which were synthesised separately and coupled in the end following the synthesis pathway reported before in 2012 by Van der Veken et al. Finally, a dehydration of the primary amide group was done in order to obtain a carbonitrile warhead that reduces the proximity between the imaging probe and the target, PREP.

**RESUM**

PREP és una endopeptidasa membre de la família de serin-proteases. Aquest enzim té un mecanisme de proteòlisi selectiu: talla pèptids de menys de 30 aminoàcids al grup carboxil de prolines. PREP té un alt nivell d'expressió al cervell i al sistema nerviós central (SNC) i s'ha vist que està relacionada amb algunes malalties neurodegeneratives com l'Alzheimer, el Parkinson, l'esquizofrènia, la depressió i l'epilèpsia.

El principal objectiu d'aquest projecte és sintetitzar sondes moleculars (molecular imaging probes) per seguir l'evolució de l'expressió de PREP durant l'epileptogènesi. L'estructura d'aquestes sondes consta de tres parts: un inhibidor per PREP com a "targeting moiety", anàlegs de biotina i rodamina B piperazina com a reporters i un enllaç per connectar les dues parts anteriors. S'han utilitzat dos reporters diferents per tal de trobar quina és la millor opció en aquest cas.

Pel que fa a l'inhibidor, l'estructura general s'ha dividit en tres blocs: hidroclorur de (*S*)-pirrolidina-2-carboxamida**,** àcid (2*S*,4*S*)-4-azido-1-(*tert*-butoxicarbonil)pirrolidina-2-carboxílic i clorur de 4-fenilbutanoïl*,* els quals s'han sintetitzat per separat i unit al final, seguint els passos descrits al 2012 per Van der Veken et al. Un cop obtinguda la molècula amb els tres blocs, s'ha fet una deshidratació de l'amida primària per tal d'obtenir un grup nitril que redueix la proximitat entre l'inhibidor, PREP i la sonda.

**RESUMEN**

PREP es una endopeptidasa que pertenece a la familia de las serin-proteasas. Tiene un mecanismo de proteólisis selectivo: corta péptidos de menos de 30 aminoácidos y lo hace en el grupo carboxilo de las prolinas. PREP tiene una elevada expresión en el cerebro y el sistema nervioso central (SNC) y se ha relacionado con algunas enfermedades neurodegenerativas como el Alzheimer, el Parkinson, la esquizofrenia, la depresión y la epilepsia.

El principal objetivo de este proyecto es sintetizar sondas moleculares (molecular imaging probes) para seguir la evolución de la expresión de PREP durante la epileptogenesis. La estructura de estas sondas está formada por tres partes: un inhibidor de PREP como "targeting moiety", análogos de la biotina y rodamina B piperazina como etiquetas "reporters", y un conector para juntar las dos partes anteriores. Se han usado dos "reporters" diferentes para encontrar cuál es mejor para visualizar PREP.

Por otro lado, la estructura general del inhibidor ha sido dividida en tres bloques: hidrocloruro de (*S*)-pirrolidina-2-carboxamida**,** ácido (2*S*,4*S*)-4-azido-1-(*tert*-butoxicarbonil)pirrolidina-2-carboxílico y cloruro de 4-fenilbutanoil, los cuales han sido sintetizados por separado y enlazados al final, siguiendo los pasos descritos en 2012 por Van der Veken et al. Una vez juntados los tres bloques, se ha hecho una deshidratación de la amida primaria para obtener un grupo nitrilo que reduce la proximidad entre la enzima, PREP, y la sonda.

# Index

- **Abbreviations**

| | |
|---|---|
| CNS | Central nervous system |
| CuAAC | Copper Catalysed Azide-Alkyne Cycloaddition |
| DCC | *N*,*N*'-Dicyclohexylcarbodiimide |
| DCM | Dicloromethan |
| DMF | Dimethylformamide |
| DMSO | Dimethyl sulfoxide |
| DPP | Dipeptidyl peptidase |
| ESI | Electrospray ionisation |
| EtOAc | Ethyl acetate |
| FAP | Fibroblast activation protein |
| HATU | 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate |
| HOSu | N-Hydroxysuccinimide |
| HPLC | High performance liquid chromatography |
| LC-MS | Liquid chromatography–mass spectrometry |
| MeOH | Methanol |
| NIR | Near infrared |
| NMR | Nuclear magnetic resonance |
| NP | Normal phase |
| PEG- | Polyethylene glycol |
| PREP | Prolyl oligopeptidase |
| RP | Reverse phase |
| rt | Room temperature |
| TEA | Triethylamine |
| TMA | Trimethylaluminum |
| TBTU | *O*-(Benzotriazol-1-yl)-*N,N,N',N*'-tetramethyluronium tetrafluoroborate |
| TFA | Trifluoroacetic acid |
| TLC | Thin-layer chromatography |
| TLE | Temporal Lobe Epilepsy |
| UPLC | Ultra performance liquid chromatography |
| UV | Ultraviolet |

## • Introduction

Serine proteases are one of the five recognised groups of proteolytic enzymes, which can be divided in aspartic-, cysteine-, metallo-, threonine and serine peptidases. The name of this group has been chosen since all the enzymes in this family have a Ser amino acid residue in the active site which is able to attack the carbonyl group of the peptide forming an acyl-enzyme intermediate. First, the Asp group interact with the His ring to orient and activate it and then the His act as a base to attack the OH group of Ser which act as a nucleophile and attack the carbonyl group of the substrate. In this mechanism, some intermediates are formed in order to avoid a high transition state (Patrick, 2009).

Serine proteases families are further classified into subfamilies according to their catalytic triad. The main focus of this project is Prolyl oligopeptidase (PREP), a peptidase that belongs to the clan SC of serine proteases, more specifically to the S9A family (Page & Di Cera, 2008) (Rosenblum & Kozarich, 2003).

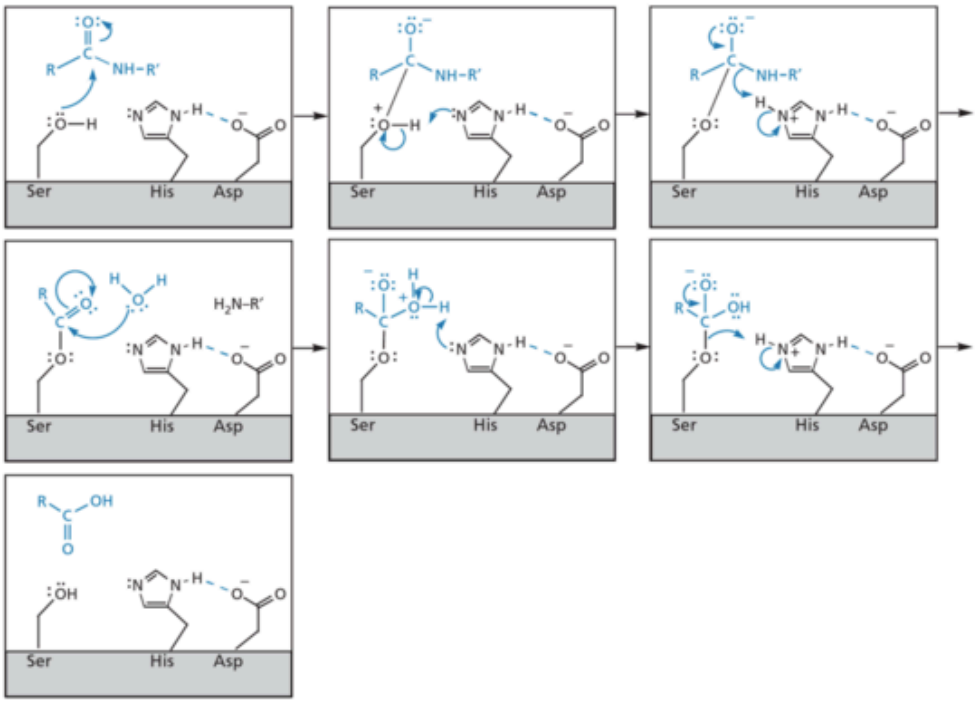



**Fig. 1 Hydrolysis mechanism of serine proteases active site** (Patrick, 2009)

PREP is an endopeptidase member of the serine peptidase family that cleaves peptides at the carboxyl side of proline residues. The peptidases of this group, like dipeptidyl peptidase IV, oligopeptidase B and acylaminoacyl peptidase, have a higher molecular mass than the other serine peptidases. Mainly their molecular mass will be approximately around 80KDa. PREP has a 710 amino acid residue-sequence in a single-chain structure and the catalytic residues, Ser554, His680 and Asp641, are found at the carboxyl terminus of the protease. Just like other enzymes of clan SC proteases, PREP

has a very interesting property, its selectivity; this serine protease is able to cleave peptides after a proline residue in the P1-position. Furthermore, this peptidase preferentially cleaves peptides with no more than 30 amino acids (Szeltner & Polgár, 2008) (Polgár, 2002).

The three-dimensional structure for PREP was determined by X-ray crystallography with the inhibitor Z-Pro-prolinal (PDB codes 1qfm and 1qfs) (Fülöp, Böcskei, & Polgár, 1998). PREP has a cylindrical structure with a height of 60 Å and a diameter of 50 Å. It is a single-chain protein divided into two domains, a peptidase or catalytic (residues 1-72 and 428-710) and a seven-bladed β-propeller (residues 73-427) (García-Horsman, Männistö, & Venäläinen, 2007). The peptidase domain contains the catalytic triad formed by Ser554, His680 and Asp641. This domain has a characteristic α/β hydrolase fold with a central eight-stranded β-sheet all distributed in a parallel manner except for the second one. The β-sheets are flanked with 2 helices in one side and 6 in the other one. The β-propeller domain is held to the catalytic domain by hydrophobic forces. This domain is formed by a seven-fold repeat of four-stranded antiparallel twisted β-sheets, which are arranged radially around the central tunnel. The first and seventh blades of the β-propeller are connected with weak hydrophobic interactions (Szeltner & Polgár, 2008) (Polgár, 2002).

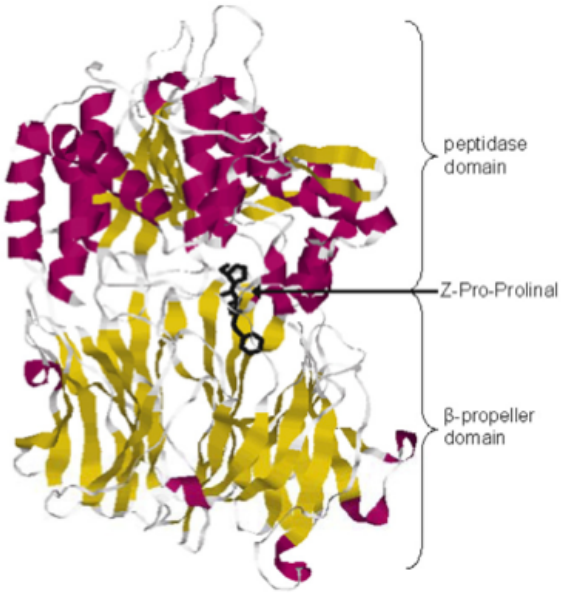



**Fig. 2 Structure of prolyl oligopeptidase with the inhibitor Z-Pro- Prolinal bound at the active site** (García-Horsman et al., 2007)

The active side (Ser554, Asp641 and His680) is located in a large cavity at the interface of the 2 domains. The Ser554 is found on the tip of a sharp turn, which is the typical nucleophile elbow from the α/β hydrolase structure. For this reason, the OH group of this residue is well exposed and accessible for the substrate and the imidazole group of His680, which is found in the middle of a loop. One of the oxygen of Asp641 is located in the plane of the imidazole group of His680, which is placed in the ideal position to form a hydrogen bond. This oxygen is also forming a hydrogen bond with a well-structured water molecule. Two main-chain NH- groups coordinate the other oxygen of the Asp641. The binding mode of PREP is different from the other serine peptidases. Instead of a regular S1-P1 bond formation, the bond is formed between the S2

(guanadinium group of Arg643) and the carbonyl oxygen of the P2 residue (Szeltner & Polgár, 2008) (Polgár, 2002) (García-Horsman et al., 2007).

As mentioned before PREP preferably cleaves short peptides. This selectivity of short peptides can be explained by studying the three-dimensional structure of the protein. The diameter of the entrance of the propeller domain is narrower (about 4 Å) than in the average peptides (6-10 Å). Although during catalysis the diameter enlarges, because blades 1 and 7 are opened, forming a gating filter. The hypothesis has been confirmed by altering the enzymatic structure through the placement of disulphide bonds between blades 1 and 7; which reduces the enzymatic activity (Szeltner & Polgár, 2008).

PREP expression is elevated in particular brain regions like substantia nigra, hippocampus, cerebellum and caudate putamen. The distribution of this expression changes between a healthy brain and a diseased brain. In healthy brains, PREP was found in the neurons' cytoplasm while in diseased brains it was found next to amyloid deposits and α-synuclein aggregates. These differences in distribution depend on the integrity of the microtubules-network. Previous reports predict that PREP interacts with some proteins like GAP43, β-tubulin and α-synuclein, so it is thought that PREP takes part of a larger molecular network (Schulz et al., 2005) (Di Daniel et al., 2009). It seems like this molecular network, that modulates the microtubules-dynamics has an important role in the maintenance of the neurons.

Although PREP is related to some processes like neurodegeneration and synaptic plasticity; nowadays, the role and the localization of PREP in cells is not completely clear. PREP has been found in the cytosol but it is also present in the extracellular space. Previous studies show that the PREP expression level after epilepsy induction is reduced in the hippocampus. For this reason it is believed that PREP may be released from the cell. Furthermore, some studies show that PREP is involved in some metabolic pathways like the inositol phosphate signalling pathway (IP3) (Szeltner & Polgár, 2008) (Myöhänen, Kääriäinen, Jalkanen, Piltonen, & Männistö, 2009).

It is known that PREP is involved in processing and degrading of neuropeptides because of its high activity level in brain and central nervous system (CNS). Therefor, PREP has been related to neurodegenerative disorders like Alzheimer's disease and Parkinson, and psychiatric disorders like Schizophrenia, mania and depression. Several studies demonstrate that PREP inhibitors have a positive effect on memory, learning and cognition in model animals with brain injury by prolonging the action of some specific neuropeptides related to memory, learning and cognition (Van Der Veken et al., 2012a).

**PREP inhibitors**

Neurodegenerative diseases affect hundreds of millions people all over the world and are currently not curable. For this reason, over the last years, neurodegenerative processes have been widely studied as well as new targets for therapeutics interventions and treatments. One of the most important targets that have been studied is PREP, which has been found to be related to neurodegeneration and therefore, to some neurodegenerative and psychiatric pathologies. Many PREP inhibitors have been designed and synthesised in order to improve the knowledge about localization and function of the protein (Szeltner & Polgár, 2008) (The EU Joint Programme – Neurodegenerative Disease Research, 2015). In this case, PREP inhibitors have been used as targeting moieties in several imaging and activity-based probes for PREP.

The first effective PREP inhibitor was Z-Pro-prolinal and it was shown to reverse scopolamine-induced amnesia in rats in some studies with other inhibitors like Ono-1603, JTP-4819 and S-17092. A great amount of derivatives of this lead compounds have been synthesised and it is shown that the modifications influence the binding mechanism, the association rate constant and the duration of the inhibition (Szeltner & Polgár, 2008).

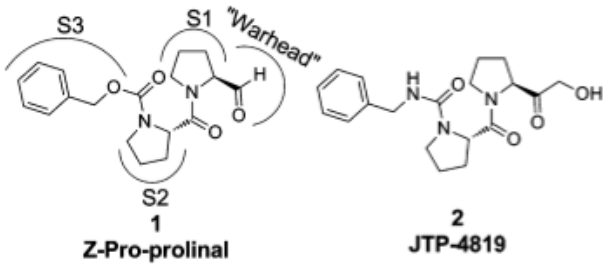
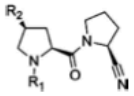


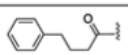
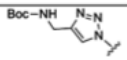

**Fig. 3 Structure of PREP inhibitors** (Van Der Veken et al., 2012a)

Most of the reported PREP inhibitors have a similar structure: a proline or a proline analogue residue in the P1 and P2 sites and an hydrophobic structure in P3 site (Szeltner & Polgár, 2008). These three positions usually interact with three binding sites of PREP. The S1 pocket binds to the P1-proline residue, the S2 pocket, which is less known, is supposed to bind to some other residues and the S3 pocket is filled with an aliphatic spacer attached to an aromatic group. Moreover, most of these reported inhibitors have a "warhead" in P1 site. This is an electrophilic functional group that can interact with the catalytically active serine hydroxyl group of the enzyme. It is known that this warhead can potentially increase the affinity for the target without changing the druglikeness but it is thought that it can also decrease the stability and modify the selectivity of the inhibitor (Van Der Veken et al., 2012a).

Some of these inhibitors have been reviewed previously by Van der Veken et al. in 2012 and it is known that most of them have space to insert substituents in the P2 site. The P2 substitution maximizes the selectivity of the inhibitor and allows fine-tuning of the physicochemical characters: the activity, the selectivity and the biopharmaceutical profile.

**Table 1. Results for inhibitory potency for PREP, FAP, DPP IV, DPP II and DPP9 of inhibitors synthesised before in this group.** (Van Der Veken et al., 2012b)



| | | | $IC_{50}$ ($\mu$M) | | | | |
|---|---|---|---|---|---|---|---|
| Cmpd. | $R_1$ | $R_2$ | PREP | FAP | DPP IV | DPP II | DPP9 |
| **17** |  | -H | 0.006 ± 0.004 | >10 | >100 | >100 | >100 |
| **13** |  | $-N_3$ | 0.003 ± 0.0003 | >100 | >>100 | >>100 | >100 |
| **14** |  |  | 0.009 ± 0.0001 | >>100 | >>100 | >>100 | >100 |

The $IC_{50}$ values for inhibitors previously synthesised by Van der Veken et al. were determined and some of the results are shown in the Table 1. The inhibitory potency for PREP is really high for these inhibitors that carry a carbonitrile warhead in P1 compared with the inhibitory potency for other serine proteases like FAP, DPP IV, DPP II and DPP9. The P1-carbonitrile warhead stabilized by hydrogen bonds with the NH of Asn555 and the phenolic OH of Tyr473 and it's very important for the localization of the enzyme because it reduces the proximity between the inhibitor and the target.

**Imaging probes for PREP**

The molecular imaging probes are a useful alternative for the traditional diagnostic imaging techniques since these allow to detect the diseases in a non-invasively way and provide biochemical information about these pathologies by specifically reaching the target of interest *in vivo.* For this reason, a desirable molecular imaging probe needs some specific and unique characteristics like high binding affinity and high specificity to the target, high sensitivity, high contrast ratio, high stability *in vivo*, low immunogenicity and toxicity and production and economical feasibility (Chen & Chen, 2013).

Molecular imaging probes are agents to visualize, characterize and quantify a biological process in living systems (Chen & Chen, 2013), in this case, PREP expression in fresh slices of rat brains. Most of molecular imaging and activity-based probes have a general structure that consists of a signal agent, a targeting moiety and a linker, which connects both parts.

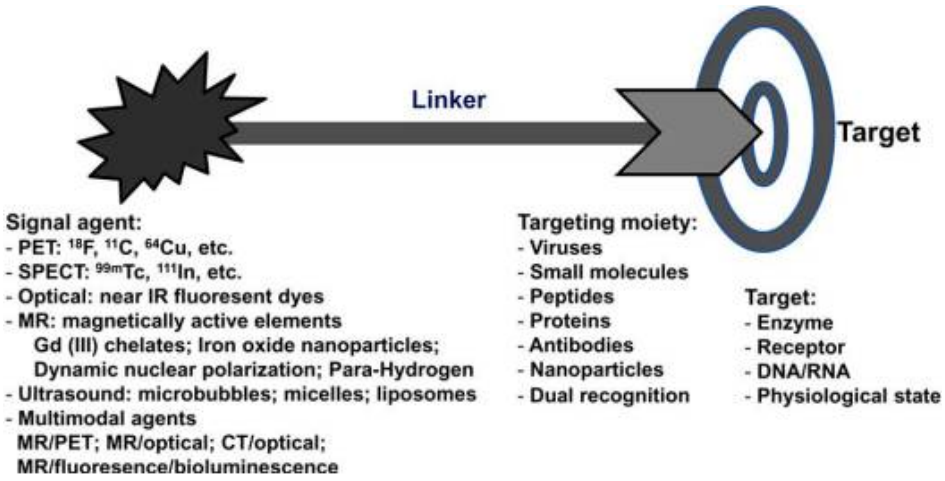



**Fig. 4 General structure for an imaging probe.** (Chen & Chen, 2013)

There is a broad variety of signal agents and targeting moieties available for molecular imaging. Depending on the preferred imaging technique a specific type of signal agent will be used. Over the last years a great variety of imaging probes have been developed and used for clinical settings. Some of them allow to perceive anatomical changes related to a disease (like ultrasound and computed tomography) meanwhile others can provide functional and biochemical information (like positron emission tomography, single photon emission computed tomography and optical fluorescence imaging) (Chen & Chen, 2013).

- **Objectives**

The main goal of this project is to synthesise and characterise molecular imaging probes to follow the evolution of the PREP expression levels during the epileptogenesis. In order to reach this final goal, the following objectives need to be accomplished:

1) Synthesis of the PREP inhibitor and optimization of the synthesis pathway.
    a. Synthesis of the first building block: (*S*)-pyrrolidine-2-carboxamide hydrochloride.
    b. Synthesis of the second building block: (*2S,4S*)-4-azido-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid.
    c. Synthesis of the third building block: 4-phenylbutanoyl chloride.
    d. Coupling of the three building blocks.
2) Synthesis of biotin and rhodamine B analogues, which will be used as signal agents.
3) Coupling of the inhibitor with the signal agents by using “click chemistry”.

- **Methodology**

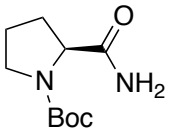
## 1) Equipment

Characterisation of all compound was done with $^{1}$H-NMR, $^{13}$C-NMR, and LC-MS. $^{1}$H-NMR spectra were recorded on a 400 MHz Bruker Avance III nanobay spectrometer with ultrashield. Chemical shifts are in ppm and coupling constants are in Hertz (Hz). Purity of the compounds was verified by using an HPLC system. In this case: UPLC: Waters acquity H-class UPLC system coupled to a Waters TQD ESI mass spectrometer and a Waters TUV detector. A Waters acquitty UPLC BEH C18 1.7µm particle size, 2.1 x 50 mm column was used. Solvent A: water with 0.1% formic acid, solvent B: Acetonitrile (MeCN) with 0.1% formic acid. The wavelenght for UV detection was 254 nm.

Where necessary, flash purification was performed on a Biotage® ISOLERA One flash system equipped with an internal, variable dual-wavelength diode array detector (200-400 nm). For normal phase (NP) purification, SNAP cartridges (10-340 g; flow rate: 10 ml/min - 150 ml/min) were used. Reversed phase (RP) purifications were performed on KP-C18 cartridges (12-30 g; flow rate: 10ml/minute – 15 ml/min). Dry sample loading was done in Self Packing Samplet® cartridges using silica or Celite 545, for respectively NP- and RP- purifications. Used gradients are variable for every purification.

## 2) Synthesis

### (*S*)-*tert*-Butyl 2-carbamoylpyrrolidine-1-carboxylate (1)




1-Hydroxypyrrolidine-2,5-dione (2,95 g, 25,7 mmol) was added to a solution of (*S*)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (5,02 g, 23,34 mmol) in DCM (100 ml). Then, *N,N*'-dicyclohexanamine (5,30 g, 25,7 mmol) was added and the reaction mixture was left stirring for 30minutes at 0°C. After this time, ammonia (6,67 ml, 46,7 mmol) was added and reaction was brought to rt and left stirring another 30 minutes. After reaction completion the volatiles were evaporated under reduced pressure. The residue was redissolved in cold EtOAc (200 ml) and was filtered over celite. The filtrate was extracted with $NaHCO_3$ (3x150 ml). The organic phase was dried over anhydrous $Na_2SO_4$ and filtered off and further concentrated under reduced pressure, yielding the title compound (*S*)-*tert*-butyl 2-carbamoylpyrrolidine-1-carboxylate as a white crystaline solid (5,4 g, 25,2 mmol, 100 % yield).

**$t_R$:** 1.20 min; ESI ***m/z***: 237.2 $[M+Na]^+$ 115.1 $[M+H\text{-}Boc]^+$

**$^{1}$H NMR (400 MHz, DMSO-*$d_6$*) δ** 1.18 (t, J = 7.1 Hz, 1H), 1.37 (ss, J = 20.1 Hz, Boc), 1.68 - 1.87 (m, 3H, $CH_2$), 2.03 - 2.12 (m, 1H, $CH_2$), 3.19 - 3.30 (m, 1H, $CH_2$), 3.37 (dt, J = 7.9, 4.2 Hz, 1H, $CH_2$), 3.98 (dd, J = 8.4, 3.7 Hz, 1H, CH), 6.84 - 6.94 (m, 1H, $NH_2$), 7.24 - 7.33 (m, 1H, $NH_2$).

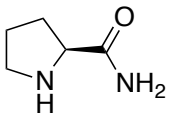
**$^{13}$C NMR (101 MHz, DMSO-*$d_6$*) δ** 23.15 ($CH_2$), 28.00 (Boc), 28.12 ($CH_2$), 46.32 ($CH_2$), 59.74 (CH), 78.28 (Boc), 153.29 (Boc), 174.57 (C).

The obtained spectra are in agreement with what was reported in literature (Tang, F, 2007).

## (*S*)-Pyrrolidine-2-carboxamide hydrochloride (2)

### Method A

Trifluoroacetic acid (38,5 ml, 503 mmol) was added to a solution (*S*)-*tert*-butyl 2-carbamoylpyrrolidine-1-carboxylate (5.39 g, 25,2 mmol) in DCM (100 ml). The reaction mixture was left stirring for 1h at rt. After reaction completion the volatiles were evaporated under reduced pressure. The excess TFA was evaporated with diethyl ether, yielding the title compound (*S*)-pyrrolidine-2-carboxamide hydrochloride as yellow oil (4,37 g, 29,0 mmol, 100 % yield).



TFA

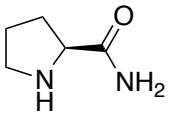
**$t_R$:** 0.20 min; ESI ***m/z***: 112.9 $[M-H]^+$

**$^{1}$H NMR (400 MHz, Methanol-*$d_4$*) δ** 1.98 - 2.18 (m, 3H, 1, $CH_2$), 2.39 - 2.58 (m, 1H, $CH_2$), 3.34 - 3.48 (m, 2H, $CH_2$), 4.26 - 4.37 (m, 1H, CH).

**$^{13}$C NMR (101 MHz, Methanol-*$d_4$*) δ** 25.13 ($CH_2$), 31.13 ($CH_2$), 47.35 ($CH_2$), 61.06 (CH), 171.84 (C).

### Method B

To a solution of hydrogen chloride (51,3 ml, 205 mmol) in dioxane, (S)-*tert*-butyl 2-carbamoylpyrrolidine-1-carboxylate (5.5 g, 25,7 mmol) was added. The reaction mixture was left stirring on at rt. After reaction completion, the reaction mixture was dried with ether and filtered, yielding the title compound (*S*)-pyrrolidine-2-carboxamide hydrochloride (3,278 g, 21,77 mmol, 65,6 % yield) as a white crystalline solid.



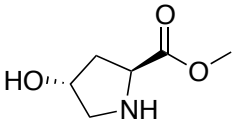
HCl

**$t_R$**: 0.19; ESI ***m/z***: 115$[M+H]^+$, 137$[M+Na]^+$

**$^{1}$H NMR (400 MHz, Methanol-*$d_4$*) δ** 1.98 - 2.18 (m, 3H, 1, $CH_2$), 2.50 (dtd, J = 11.3, 8.4, 7.4, 3.1 Hz, 1H, $CH_2$), 3.40 (dddd, J = 18.2, 13.5, 8.9, 5.4 Hz, 2H, $CH_2$), 4.27 - 4.38 (m, 1H, CH).

**$^{13}$C NMR (101 MHz, Methanol-*$d_4$*) δ** 25.15 ($CH_2$), 31.16 ($CH_2$), 47.37 ($CH_2$), 61.07 (CH), 171.87 (C).

## (*2S,4R*)-Methyl 4-hydroxypyrrolidine-2-carboxylate (3)



A round bottom flask containing MeOH (75 ml) was placed in an icebath. Sulfurchlorideoxide (2,51 ml, 34,4 mmol) was added followed by the addition of L-Hydroxyproline (4,52 g, 34,4 mmol). The reaction mixture was placed under reflux conditions and was left stirring for 2h at 65°C. After reaction completion the reaction mixture was cooled down to rt. The volatiles were evaporated under reduced pressure and the residue was washed with ether (100 ml), yielding the title compound (*2S,4R*)-methyl 4-hydroxypyrrolidine-2-carboxylate as a white crystalline solid (6,32 g, 43,5 mmol, 100 % yield).

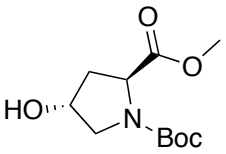
**$t_R$:** 1.44 min; ESI ***m/z***: 146.1 $[M+H]^+$ 168 $[M+Na]^+$

**$^1$H NMR (400 MHz, Methanol-*d*$_4$) δ** 2.23 (ddd, J = 13.7, 10.8, 4.2 Hz, 1H, $CH_2$), 2.39 - 2.50 (m, 1H, $CH_2$), 3.30 - 3.39 (m, 1H, $CH_2$), 3.48 (dd, J = 12.2, 3.7 Hz, 1H, $CH_2$), 3.88 (s, 3H, $CH_3$), 4.58 - 4.68 (m, 2H, 2, CH).

**$^{13}$C NMR (101 MHz, Methanol-*d*$_4$)** δ 38.58 ($CH_2$), 54.06 ($CH_3$), 55.04 ($CH_2$), 59.46 (CH), 70.66 (CH), 170.66 (C).

The obtained spectra are in agreement with what was reported in literature (Aldridge, & Steven, 2006).

## (*2S,4R*)-1-*tert*-Butyl 2-methyl 4-hydroxypyrrolidine-1,2-dicarboxylate (4)



Triethylamine (11,71 ml, 84 mmol) was added to a solution of (*2S,4R*)-methyl 4-hydroxypyrrolidine-2-carboxylate (6,1 g, 42,0 mmol) in DCM (120 ml). The reaction mixture was put in an icebath. A solution of di-*t*-butyldicarbonate (9,17 g, 42,0 mmol) in DCM was added. The reaction mixture was removed from the icebath and left stirring for 3h at rt. After reaction completion the volatiles were evaporated under reduced pressure. Residue was redissolved in EtOAc (150 ml). The organic phase was washed with a 2M solution of HCl (100 ml). The organic phase was dried over anhydrous $Na_2SO_4$, filtered off and further concentrated under reduced pressure, yielding de title compound (*2S,4R*)-1-*tert*-butyl 2-methyl 4-hydroxypyrrolidine-1,2-dicarboxylate as a white solid (9,7 g, 39,5 mmol, 94 % yield).

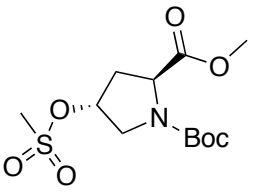
**$t_R$:** 1.27min; ESI ***m/z***: 146.1 $[M-Boc+H]^+$ 268.1 $[M+Na]^+$

**$^1$H NMR (400 MHz, Methanol-*d*$_4$)** δ 1.54 (s, 9H, Boc, $CH_3$), 2.22 - 2.31 (m, 2H, $CH_2$), 3.48 (dt, J = 4.8, 1.9 Hz, 1H, $CH_2$), 3.52 - 3.54 (m, 1H, $CH_2$), 3.54 - 3.58 (m, 1H, CH), 3.76 (s, 3H, $CH_3$), 4.34 - 4.38 (m, 2H, CH).

**$^{13}$C NMR (101 MHz, Methanol-*d*$_4$) δ** 27.62 (Boc), 40.00 ($CH_2$), 52.76 ($CH_3$), 55.60 ($CH_2$), 59.40 (C), 70.03 (CH), 81.72 (Boc), 155.81 (Boc), 175.33 (C).

The obtained spectra are in agreement with what was reported in literature (Mollica & Adriano, 2006).

## (*2S,4R*)-1-*tert*-Butyl 2-methyl 4-((methylsulfonyl)oxy)pyrrolidine-1,2-dicarboxylate (5)



Triethylamine (5,51 ml, 39,5 mmol) was added to a solution of (*2S,4R*)-1-*tert*-butyl 2-methyl 4-hydroxypyrrolidine-1,2-dicarboxylate (9,70 g, 39,5 mmol) in DCM (50 ml). A solution of methanesulfonyl chloride (3,06 ml, 39,5 mmol) in DCM (50 ml) was added to the reaction mixture in a dropwise manner over 20 minutes. The reaction mixture was left stirring for 30 minutes at rt. After reaction completion the volatiles were evaporated under reduced pressure. The residue was redissolved in EtOAc (100 ml) and was washed with brine (75 ml). The organic phase was dried over anhydrous $Na_2SO_4$, filtered off and further concentrated under reduced pressure, yielding the title compound (*2S,4R*)-1-*tert*-butyl 2-methyl 4-((methylsulfonyl)oxy)pyrrolidine-1,2-dicarboxylate as a white crystalline solid (11,14 g, 34,5 mmol, 87 % yield).

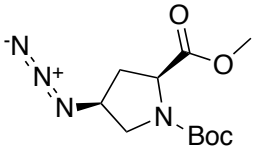
**$t_R$**: 1.48 min; ESI ***m/z***: 224 $[M\text{-}Boc+H]^+$; 346 $[M+Na]^+$

**$^1$H NMR (400 MHz, Methanol-*$d_4$*) δ** 1.42 (s, 9H, Boc, $CH_3$), 2.11 - 2.25 (m, 1H, $CH_2$), 2.46 - 2.60 (m, 1H, $CH_2$), 3.04 (s, 3H, $CH_3$), 3.56 - 3.63 (m, 1H, $CH_2$), 3.66 (s, 3H, $CH_3$), 3.68 - 3.73 (m, 1H, $CH_2$), 4.24 - 4.34 (m, 1H, CH), 5.15 - 5.23 (m, 1H, CH).

**$^{13}$C NMR (101 MHz, MeOD-*$d_4$*) δ** 27.61 (Boc), 28.50 ($CH_2$), 38.31 ($CH_3$), 52.96 ($CH_3$), 53.67 ($CH_2$), 58.92 (CH), 82.26 (Boc), 86.44 (CH), 155.31 (Boc), 174.42 (C).

The obtained spectra are in agreement with what was reported in literature (Greenwood, 2003).

## (*2S,4S*)-1-*tert*-Butyl 2-methyl 4-azidopyrrolidine-1,2-dicarboxylate (6)



Sodium azide (4,62 g, 71,1 mmol) was added to a solution of (*2S,4R*)-1-*tert*-butyl 2-methyl 4-((methylsulfonyl)oxy)pyrrolidine-1,2-dicarboxylate (11,5 g, 35,6 mmol) in DMF (45 ml). The reaction mixture was left stirring overnight at rt. The reaction mixture was diluted with EtOAc (100 ml). The organic phase was washed with brine (3x50 ml), dried over anhydrous $Na_2SO_4$ and filtered off. The volatiles were evaporated under reduced pressure, yielding the title compound (*2S,4S*)-1-*tert*-butyl 2-methyl 4-azidopyrrolidine-1,2-dicarboxylate as a yellow oil (12.8 g, 47,4 mmol, 100 % yield).

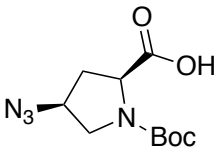
**$t_R$**: 1.60; ESI ***m/z***: 293.1 $[M+Na]^+$; 171.1 $[M\text{-}Boc]^+$; 573.4 $[2M+Na]^+$

**$^1$H NMR (400 MHz, Methanol-*$d_4$*) δ** 1.41 - 1.53 (m, 9H, Boc $CH_3$), 2.10 - 2.20 (m, 1H, $CH_2$), 2.49 - 2.61 (m, 1H, $CH_2$), 3.41 (dtd, J = 11.9, 3.5, 1.0 Hz, 1H, $CH_2$), 3.65 - 3.72 (m, 1H, $CH_2$), 3.76 (d, J = 4.8 Hz, 3H, $CH_3$), 4.26 - 4.37 (m, 1H, CH), 4.41 (ddd, J = 8.9, 5.1, 3.4 Hz, 1H, CH).

**$^{13}$C NMR (101 MHz, Methanol-*$d_4$*) δ** 28.52 (Boc), 28.67 ($CH_2$), 52.04 ($CH_2$), 52.55 ($CH_3$), 52.85 (CH), 60.72 (CH), 81.88 (Boc), 155.50 (Boc), 173.99 (C).

The obtained spectra are in agreement with what was reported in literature (Aldridge & Steven, 2006).

## (*2S,4S*)-4-Azido-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (7)




(*2S,4S*)-1-*tert*-Butyl 2-methyl 4-azidopyrrolidine-1,2-dicarboxylate (9.2 g, 34,0 mmol) was dissolved in a 1M potassium hydroxide (35,7 ml, 35,7 mmol) in MeOH (100 ml). The reaction was left stirring for an overnight at room temperature. After reaction completion the volatiles were evaporated. The product was redissolved in water (100 ml). The aqueous phase was washed with ether (3x10 ml). The combined organic phases were discarded. The aqueous phase was brought to pH=1 with a 2M HCl solution and extracted with EtOAc (3x150 ml). The combined organic phases were dried over sodium sulphate, filtered of and further concentrated under reduced pressure, yielding the title compound (*2S,4S*)-4-azido-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (8,069 g, 31,5 mmol, 93 % yield) as a white crystaline solid.

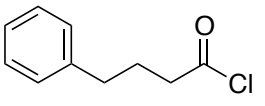
**$t_R$:** 1.37min; ESI ***m/z***: 255 $[M-H]^-$ 511 $[2M-H]^-$

**$^1$H NMR (400 MHz, Methanol-*d*$_4$) δ** 1.32 (d, J = 17.8 Hz, 9H, Boc $CH_3$), 1.95 - 2.08 (m, 1H, $CH_2$), 2.32 - 2.50 (m, 1H, $CH_2$), 3.20 - 3.30 (m, 1H, $CH_2$), 3.49 - 3.60 (m, 1H, $CH_2$), 4.09 - 4.26 (m, 2H, CH).

**$^{13}$C NMR (101 MHz, Methanol-*d*$_4$) δ** 28.54 (Boc), 37.00 ($CH_2$), 52.02 ($CH_2$), 52.59 (CH), 60.73 (CH), 81.84 (Boc), 155.70 (Boc), 175.34 (C).

The obtained spectra are in agreement with what was reported in literature (Le Quement, 2011).

## 4-Phenylbutanoyl chloride (8)




4-Phenylbutanoic acid (4,50 g, 27,4 mmol) was heated to 70°C. When it was melted, thionyl chloride (1,997 ml, 27,4 mmol) was added. The reaction mixture was left stirring for 1h at 70°C and then 10minutes at 90°C. After the reaction completion the reaction mixture was allowed to reach room temperature, yielding the title compound 4-phenylbutanoyl chloride (5 g, 27,4 mmol, 100 % yield).

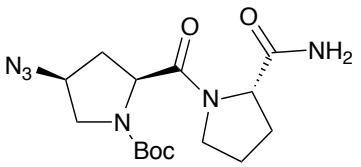
**$t_R$:** 1.78 min; ESI ***m/z***: 147.1 $[M-Cl]^+$ 179.1 $[M-Cl+O+C+3H]^+$

**$^1$H NMR (400 MHz, Chloroform-*d*) δ** 1.99 - 2.14 (m, 2H, $CH_2$), 2.72 (t, J = 7.5 Hz, 2H, 7), 2.93 (t, J = 7.2 Hz, 2H, $CH_2$), 7.17 - 7.43 (m, 5H, Ar-H).

**$^{13}$C NMR (101 MHz, Chloroform-*d*) δ** 26.59 ($CH_2$), 34.31 ($CH_2$), 46.27 ($CH_2$), 126.39 (CH), 128.46 (CH), 128.63 (CH), 140.35 (C), 173.71 (C).

The obtained spectra are in agreement with what was reported in literatura (Darvesh & Sultan, 2007).

**(*2S,4S*)-*tert*-Butyl 4-azido-2-((S)-2-carbamoylpyrrolidine-1-carbonyl)pyrrolidine-1-carboxylate (9)**

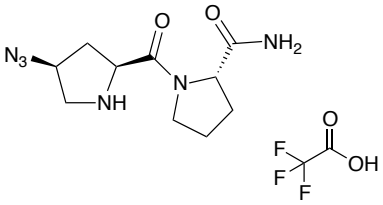


Triethylamine (7,18 ml, 51,5 mmol) and O-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (10,28 g, 27,0 mmol) were added to a solution of (*2S,4S*)-4-azido-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (6.6 g, 25,8 mmol) in DMF (50 ml). After 15 minutes (*S*)-pyrrolidine-2-carboxamide hydrochloride (3,88 g, 25,8 mmol) was added and the reaction mixture was left stirring at rt overnight. The reaction mixture was redissolved in EtOAc (200 ml) and washed with a 1M solution of HCl aq (2x75 ml). The organic phase was washed with brine (75 ml), sodium carbonate (75 ml) and brine (75 ml). The organic phase was dried over anhydrous $Na_2SO_4$, filtered off and further concentrated under reduced pressure. The residue was purified using a flash chromatography (EtOAc/MeOH 9:1 to EtOAc), yielding the title compound (*2S,4S*)-*tert*-butyl 4-azido-2-((S)-2-carbamoylpyrrolidine-1-carbonyl)pyrrolidine-1-carboxylate (7,96 g, 22,59 mmol, 88 % yield) as a yellow oil.

**$t_R$**: 1.15 min; ESI ***m/z***: 253 $[M\text{-Boc}+H]^+$; 353 $[M+H]^+$; 375 $[M+Na]^+$

**$^1$H NMR (400 MHz, DMSO-$d_6$) δ** 1.34 (2xs, 9H, Boc $CH_3$), 1.58 - 2.05 (m, 3H, $CH_2$), 2.69 (m, 1H, $CH_2$), 3.07 (m, 1H, $CH_2$), 3.35 (s, 2H, $CH_2$), 3.46 - 3.64 (m, 2H, $CH_2$), 3.77 (m, 1H, $CH_2$), 4.18 - 4.34 (m, 2H, CH), 4.43 (m, 1H, CH), 6.88 - 6.96 (m, 1H, $NH_2$), 7.18 - 7.28 (m, 1H, $NH_2$).

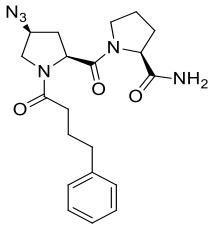
**(*S*)-1-((*2S,4S*)-4-Azidopyrrolidine-2-carbonyl)pyrrolidine-2-carboxamide trifluoroacetate (10)**



Trifluoroacetic acid (10 ml, 131 mmol) was added to a solution of (*2S,4S*)-*tert*-butyl 4-azido-2-((*S*)-2-carbamoylpyrrolidine-1-carbonyl)pyrrolidine-1-carboxylate (0.2 g, 0.568 mmol) in DCM (10 ml). The reaction mixture was left stirring for 45min at rt. After reaction completion, the volatiles were evaporated under reduced pressure. The excess TFA was co-evaporated with heptane (4x20 ml), yielding the title compound (*S*)-1-((*2S,4S*)-4-Azidopyrrolidine-2-carbonyl)pyrrolidine-2-carboxamide trifluoroacetate. The product was directly used in the next step without further purification.

**$t_R$**: 0.19 min; ESI ***m/z***: 253 $[M+H]^+$; 275 $[M+Na]^+$

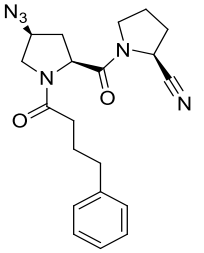
## (*S*)-1-((*2S,4S*)-4-Azido-1-(4-phenylbutanoyl)pyrrolidine-2-carbonyl)pyrrolidine-2-carboxamide (11)



To a solution of (*S*)-1-((*2S,4S*)-4-azidopyrrolidine-2-carbonyl)pyrrolidine-2-carboxamide 2,2,2-trifluoroacetate (0.208 g, 0,568 mmol) in dry DCM (10 ml), triethylamine (0,158 ml, 1,136 mmol) was added. The reaction mixture was put in an ice bath and a solution of 4-phenylbutanoyl chloride (0,104 g, 0,568 mmol) in DCM (10 ml) was added. The reaction mixture was left stirring for 2h in the ice bath. After reaction completion, the volatiles were evaporated under reduced pressure. The residue was redissolved in EtOAc (50ml) and washed with a 1M aq HCl (50 ml) solution and brine (2x25 ml). The organic phase was dried over sodium sulphate, filtered off and further concentrated under reduced pressure. The residue was purified using a reverse phase chromatography, yielding the title compound (*S*)-1-((*2S,4S*)-4-azido-1-(4-phenylbutanoyl)pyrrolidine-2-carbonyl)pyrrolidine-2-carboxamide (0,1636 g, 0,411 mmol, 72,3 % yield). The product was directly used in the next step without taking an NMR.

$\mathbf{t_R}$: 1.36 min; ESI ***m/z***: 399,3 $[M+H]^+$; 421,3 $[M+Na]^+$; 819,5 $[2M+Na]^+$; 397,3 $[M-H]^-$

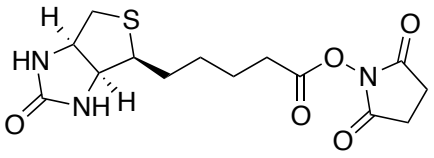
## (*S*)-1-((*2S,4S*)-4-Azido-1-(4-phenylbutanoyl)pyrrolidine-2-carbonyl)pyrrolidine-2-carbonitrile (12)



Pyridine (14,61 ml, 181 mmol) was added to a solution of (*S*)-1-((*2S,4S*)-4-azido-1-(4-phenylbutanoyl)pyrrolidine-2-carbonyl)pyrrolidine-2-carboxamide (2,88 g, 7,23 mmol) in DCM (50 ml). The reaction mixture was placed in an ice bath and trifluoroacetic anhydride (1,407 ml, 10,12 mmol) was added. The reaction mixture was left stirring for 1h at 0°C. After reaction completion, the volatiles were evaporated under reduced pressure. The resiude was purified using a flash chromatography (70% heptane - 30% EtOAc to 25% heptane - 75% EtOAc) yielding the title compound (*S*)-1-((*2S,4S*)-4-azido-1-(4-phenylbutanoyl)pyrrolidine-2-carbonyl)pyrrolidine-2-carbonitrile.

$\mathbf{t_R}$: 1.60 min; ESI ***m/z***: 403,2 $[M+Na]^+$

## 2,5-Dioxopyrrolidin-1-yl 5-((*3aS,4S,6aR*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanoate (13)

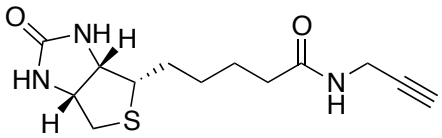


D-Biotin (0,501 g, 2,050 mmol) and *N*-hydroxysuccinimide (0,283 g, 2,461 mmol) were dissolved in hot anhydrous DMF (10 ml) (70°C). 1,3-Dicyclohexylcarbodiimide (0,508 g, 2,461 mmol) was added to the reaction mixture under vigorous stirring. The reaction mixture was left to reach room temperature and was left stirring for an overnight. After reaction completion the reaction mixture was filtered off. The filtrate was evaporated under reduced pressure and the resulting crude was redissolved in boiling isopropanol and cooled down to rt. Thereafter the resulting precipitate was collected by filtration over a glass filter yielding 2,5-dioxopyrrolidin-1-yl 5-((*3aS,4S,6aR*)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (0,709 g, 2,077 mmol, 101 % yield) as a white crystalline solid.

**$t_R$:** 1.39 min; ESI ***m/z*:** 342 $[M+H]^+$ (100%); 227 $[M-NHS]^+$ (5%)

**$^1$H NMR (400 MHz, Methanol-$d_4$) δ** 4.51 (ddd, J = 7.9, 5.0, 0.8 Hz, 1H), 4.33 (dd, J = 7.9, 4.4 Hz, 1H), 3.52 - 3.41 (m, 1H), 3.32 (dt, J = 3.3, 1.6 Hz, 1H), 2.85 (s, 1H), 2.76 - 2.59 (m, 1H), 1.83 - 1.76 (m, 1H), 1.62 - 1.55 (m, 1H), 1.40 - 1.32 (m, 1H).

## 5-((*3aS,4S,6aR*)-2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-*N*-(prop-2-yn-1-yl)pentanamide (14)

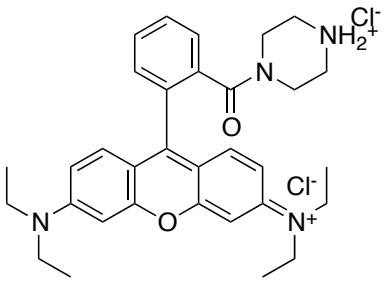


To a solution of 2-propyn-1-amine (0.038 ml, 0.586 mmol) in DMF (10 ml) *N,N*-diiso-propylethylamine (0.102 ml, 0.586 mmol) was added followed by the addition 2,5-dioxopyrrolidin-1-yl 5-((*3aS,4S,6aR*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanoate (0,200 g, 0.586 mmol) and the reaction was left stirring at room temperature overnight. After reaction completion the reaction mixture was diluted with water (40 ml) and extracted with EtOAc (3x150 ml). The combined organic fractions were dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure yielding the title compound, 5-((*3aS,4S,6aR)*-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-*N*-(prop-2-yn-1-yl)pentanamide (0.1393 g, 0.495 mmol, 85 % yield) as a white crystaline solid.

**$t_R$:** 1.02 min; ESI ***m/z*:** 282 $[M+H]^+$ (70%); 304.1 $[M+Na]^+$

**$^1$H NMR (400 MHz, DMSO)** δ 4.29 (dd, J = 7.7, 5.2 Hz, 1H), 4.14 - 4.08 (m, 1H), 4.01 (dd, J = 14.2, 7.1 Hz, 1H), 3.82 (dd, J = 5.5, 2.5 Hz, 1H), 3.10 - 3.04 (m, 1H), 2.81 (dd, J = 12.4, 5.1 Hz, 1H), 2.59 - 2.53 (m, 1H), 2.06 (t, J = 7.4 Hz, 1H), 1.66 - 1.38 (m, 4H), 1.34 - 1.14 (m, 2H).

## 4-(2-(6-(Diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzoyl)piperazin-1-ium chloride (15)



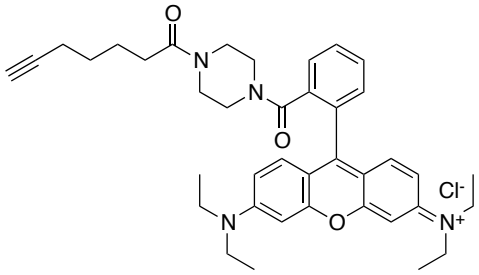
A solution of trimethylaluminum (20 ml, 40,0 mmol) in toluene was added dropwise to a solution of piperazine (7,66 g, 89 mmol) in dry DCM (30ml) at rt. After 2h, a solution of 3',6'-bis(diethylamino)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (10 g, 22,60 mmol) in DCM (20 ml) was added in a dropwise manner to the reaction mixture and the temperature was altered to 65°C. The reaction mixture was left stirring for 4 days. After reaction completion the reaction mixture was quenched with 1M HCl until gas evolution ceased. The heterogeneous solution was filtered (paper filter) and the retained solids were rinsed with DCM and a 4:1 DCM/MeOH solution. The combined filtrate was concentrated and the residue was dissolved in DCM, filtered to remove insoluble salts and concentrated again. The resulting glassy solid was then partitioned between dilute aqueous $NaHCO_3$ (400 ml) and EtOAc (200 ml). The retained aqueous layer was saturated with NaCl, acidified with 1M aqueous HCl, and then extracted with multiple portions of 2:1 isopropanol/DCM, until a faint pink color persisted. The combined organic layers were then dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The glassy purple solid was dissolved in a minimal amount of MeOH and precipitated by dropwise addition to a large volume of $Et_2O$. The precipitate was collected by filtration yielding 4-(2-(6-(diethylamino)-3-(diethyliminio)-3H-xanthen-9-yl) benzoyl) piperazin-1-ium chloride (9.653 g, 16,54 mmol, 74,4 % yield) as a dark purple crystalline solid.

**$t_R$:** 1.37; ESI ***m/z*:** 1021 $[2M+H]^+$, 539.3 $[M+Na]^+$, 511.3 $[M-H]^-$; 256.4 $[0.5M+H]^+$

**$^1$H NMR (400 MHz, DMSO) δ** 1.22 (t, J = 6.9 Hz, 12H), 2.84 - 3.06 (m, 4H), 3.53 (s, 2H), 3.66 (ddt, J = 22.0, 14.4, 7.2 Hz, 8H), 3.73 - 3.77 (m, 2H), 6.95 (s, 1H), 6.96 (s, 1H), 7.09 (d, J = 2.3 Hz, 1H), 7.11 (d, J = 2.3 Hz, 1H), 7.16 (d, J = 9.5 Hz, 2H), 7.52 - 7.56 (m, 1H), 7.72 - 7.79 (m, 2H), 7.81 - 7.85 (m, 1H), 10.07 - 10.26 (m, 2H).

**$^{13}$C NMR (101 MHz, DMSO) δ** 12.48, 38.86, 45.42, 95.91, 112.92, 114.24, 127.61, 129.86, 130.82, 131.63, 134.51, 155.09, 155.42, 157.00, 166.56.

### *N*-(6-(Diethylamino)-9-(2-(4-(hept-6-ynoyl)piperazine-1-carbonyl)phenyl)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium chloride (16)



To a solution of hept-6-ynoic acid (11.53 mg, 0.091 mmol) in DMF (3 ml) was added TBTU (32.3 mg, 0.101 mmol) and N-ethyl-N-isopropylpropan-2-amine (0.045 ml, 0.274 mmol). The solution was stirred for 15 minutes at room temperature. 4-(2-(6-(diethylamino)-3-(diethyliminio)-3H-xanthen-9-yl)benzoyl)piperazin-1-ium chloride (50 mg, 0.091 mmol) was added and the solution was allowed to stir overnight at rt. After reaction completion, water (100 ml) was added and was extracted with EtOAc (3x100 ml). The organic layer was washed with saturated $NaHCO_3$ solution (3x300 ml). The aqueous layer was extracted with EtOAc (3x300 ml). The organic layers were combined, washed with brine, dried over anhydrous $Na_2SO_4$ and filtered off. The solvent was evaporated and a solid was formed. The solid was washed 3 times with a small portion of EtOAc/Hexane 1:1, yielding the title compound *N*-(6-(diethylamino)-9-(2-(4-(hept-6-ynoyl)piperazine-1-carbonyl)phenyl)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium chloride as a dark purple solid.

## 3) Ethical and sustainability criteria

The lab is equipped with waste barrels for acids and bases separately. There are also waste barrels for non-halogenated and halogenated compounds. The lab also has containers for needles and another one for glass. Large amounts of solvents are used, mainly in the purification steps. However, all the residues are stored properly and nothing harmful for the environment is thrown away into the sink.

## • Results and discussion

In order to reach the objectives of this project, several imaging probes (general structure **17**) for PREP were synthesised. As discussed in the introduction and shown in Figure 4, the general structure of the imaging probes can be separated into three main parts: a signal agent, a linker and an inhibitor moiety (**12**) (vide supra).

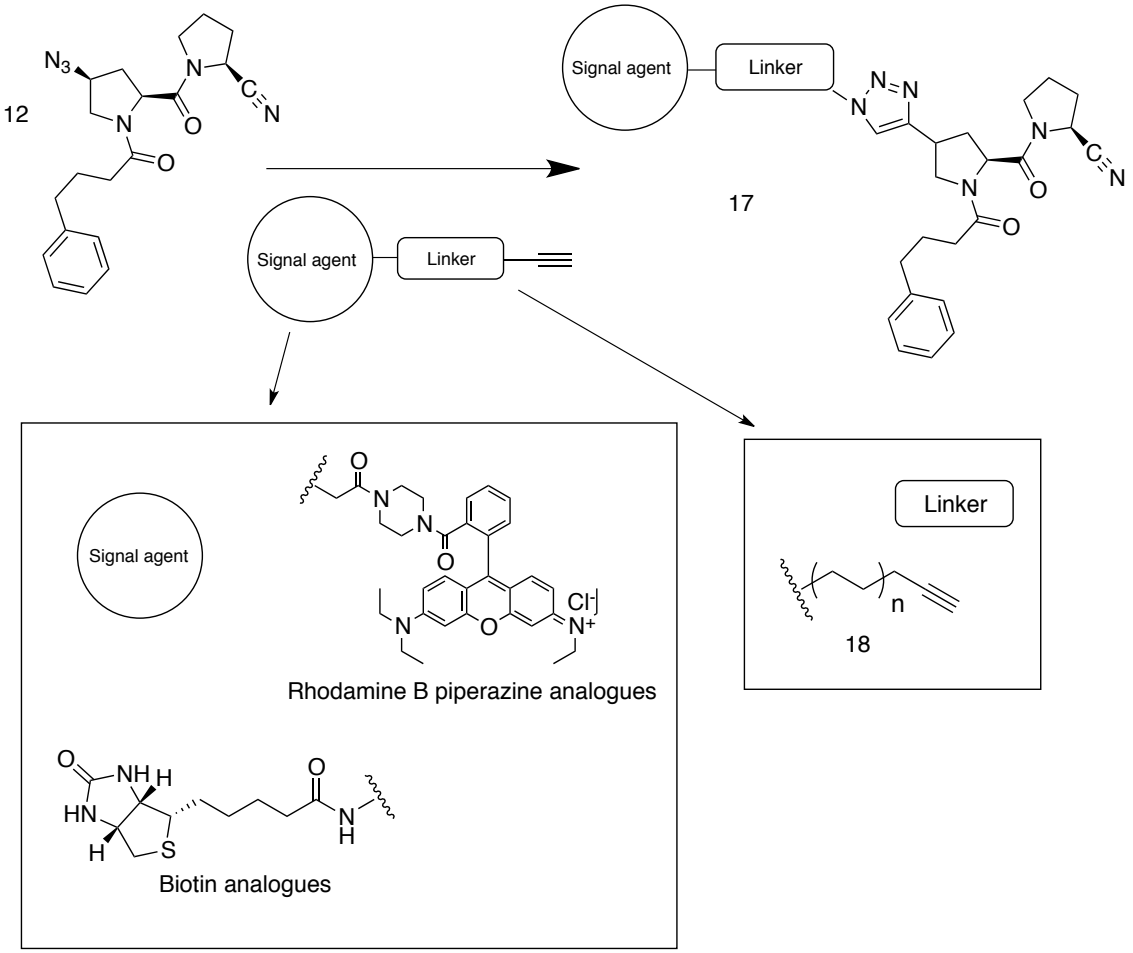



**Fig. 5 General structure for the imaging probe for PREP**

As mentioned before in the objectives, the inhibitor moiety consists of three buiding blocks: (*S*)-pyrrolidine-2-carboxamide hydrochloride (**2**), (*2S,4S*)-4-azido-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (**7**) and 4-phenylbutanoyl chloride (**8**). This project mainly focuses on signal agents for optical imaging. Therefor, either Rhodamine B analogues or biotin analogues were chosen as suitable signal agents.

Rhodamine B piperazine is a water-soluble near infrared (NIR) dye. Rhodamine analogues belong to the family of the xanthenes and are used as laser dyes, fluorescence standards, pigments, fluorescence probes, molecular switches, chemosensors among others because of its high photostability, high fluorescence quantum yield, high absorption coefficient and wide fluorescence in the visible region (Beija, Afonso, & Martinho, 2009).

On the other hand, biotin was chosen because of its high stability *in vivo* provided for the high affinity with avidin (Hnatowich, Virzi, & Rusckowski, 1987). Usually, biotin is used with antibodies but in this case, will be linked to small molecules.

The linker moiety consists of an alkylic chain or a PEG- chain with a terminal alkyne function. The terminal alkyne function is essential for the coupling of the reporter-linker moiety onto the inhibitor building block through a Copper Catalysed Azide-Alkyne Cycloaddition (CuAAC) reaction. This reaction is a variant of the 1,3-dipolar Huisgen addition but it has some advantages over this second one. The CuAAC can be done under mild water-tolerant conditions. Furthermore, it's a selective high-yielding reaction with just a few or no byproducts (Liang & Astruc, 2011).

## 1) Synthesis of the inhibitor

As described in 2012 by Van der Veken et al., the 4-position of the P2-prolyl residue is considered a preferential position to introduce substituents. In this case the substituent introduced in the P2-prolyl is a 4-phenylbutanoil residue meanwhile the P1 is carrying a carbonitrile warhead. This substituent, the 4-phenylbutanoil is not optimal but has been chosen because of its easy introduction and commercial availability. (Van Der Veken et al., 2012a)

The general structure for the inhibitor has been divided in three building blocks as in the Figure 6. The synthesis pathway of this PREP inhibitor will be based on the synthesis pathway described in previous studies made in 2012 by Van der Veken et al. As mentioned before in the introduction, this inhibitor was chosen because of its good results in affinity and $IC_{50}$ values (0.009 µM) for PREP reported in 2012 by Van der Veken et al.

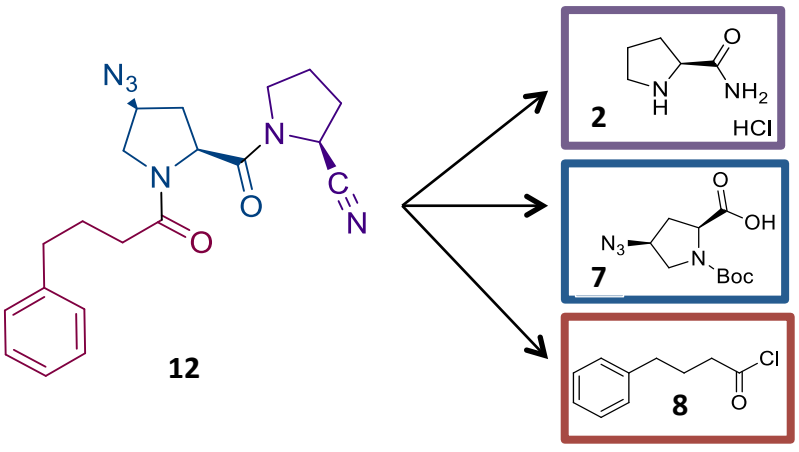
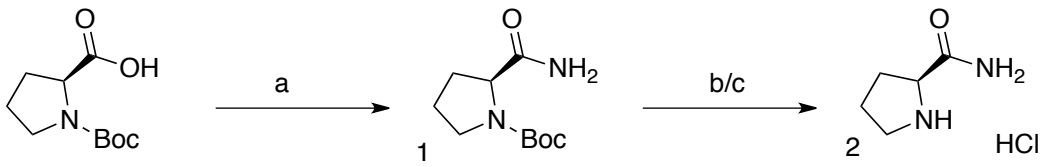



**Fig. 6 General structure of PREP inhibitor divided in 3 building blocks**

## 1st building block:




**Scheme 1. Synthesis pathway for the 1st building block. Reaction conditions: a) DCC (1,1 eq.), HOSu (1,1eq.), $NH_3$ (2eq.), DCM, 0°C, 30 min; b) TFA (20eq.), DCM, rt, 1h; c) HCl in dioxane (8eq.), DCM, rt, on.**

The aim of this step is to form an amide bond in order avoid that the carboxylic acid group reacts in the next coupling step. Furthermore, the amide is needed to form the carbonitrile warhead in the last step.

This first building block was synthesised two times using different reaction conditions for the removal of the Boc in order to compare both ways and use the one with better results for the next steps of the project. L-Boc-proline was used as a starting material in order to make sure that the amide bond was formed in the right position. First of all, the amide bond was formed using HOSu and DCC. After that, a removal of the Boc group was done first by using TFA and then by using a HCl solution in dioxane yielding the compound **2**.

**Table 2. Comparative table of the characteristics for the Boc deprotection using TFA or HCl solution**

| | Method A: TFA | Method B: HCl solution |
|---|---|---|
| **Reaction time** | 1h | Overnight |
| **Work up** | Complex | Simple |
| **Appearance of compound** | oil | White solid |
| **Yield** | 100% | 65,6% |

When comparing both methods for the removal of the Boc group, the reaction is completed faster by using TFA than using an HCl solution. Although the work up for both reactions consists of just one step, it is more difficult to remove the excess of TFA by multiple co-evaporations with diethyl ether. Furthermore, the product was obtained as an oil instead of a solid. While using a HCl solution, resulted in a simpler work up. In this case the product was precipitated by adding a small amount of ether and filtered off. The yield is higher when TFA was used, but in the end, this can be explained by loss of product during the filtration process for method B. When comparing the purity of the compound, the method B was proven to be the better method. Furthermore, the stability of the title compound is better for the formed HCl-salt than for the TFA-salt, because solids are proven to be more stable than oils.

Both compounds (**2**) were characterised by NMR with the following results:

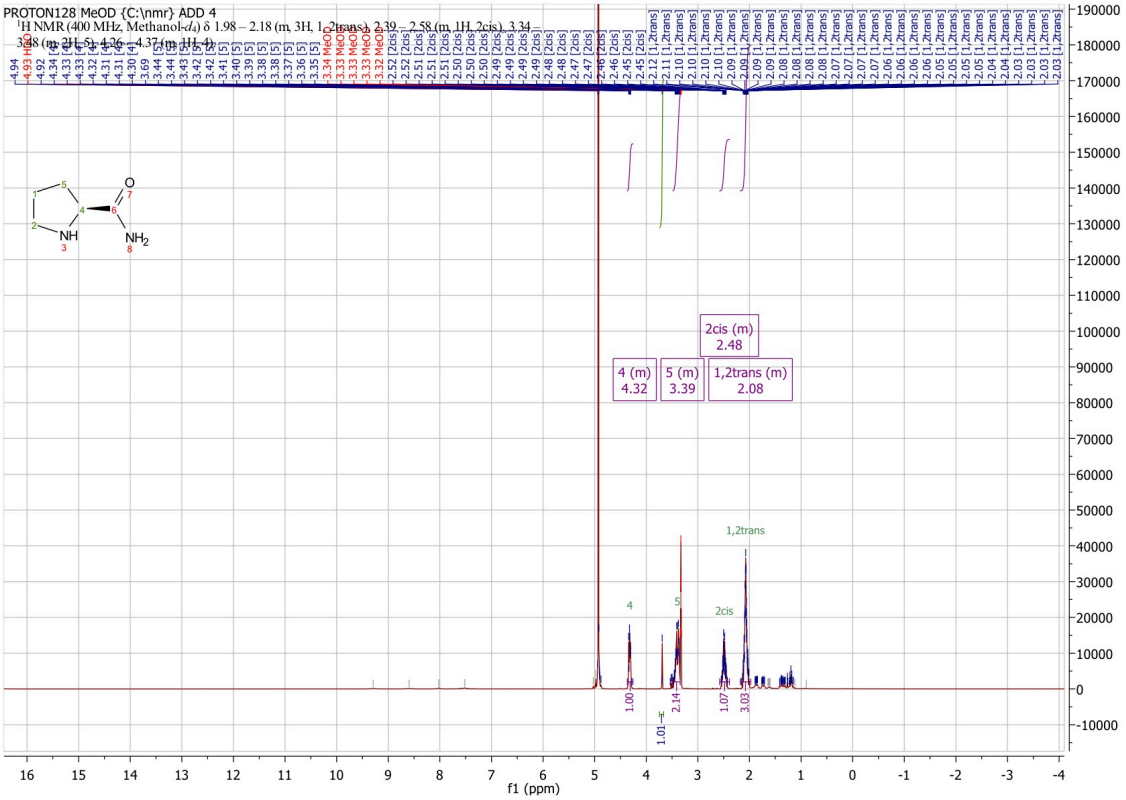



**Fig. 7 H-NMR results for compound 2 using methanol as a solvent - method A**

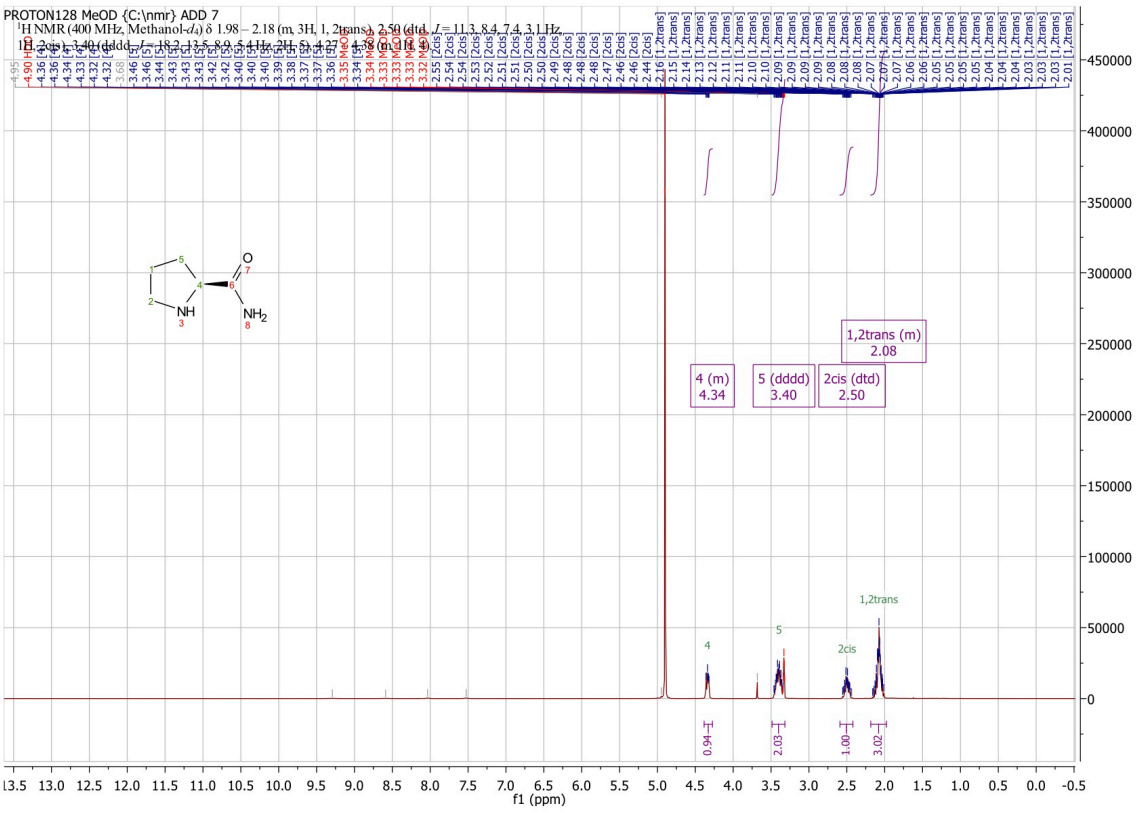
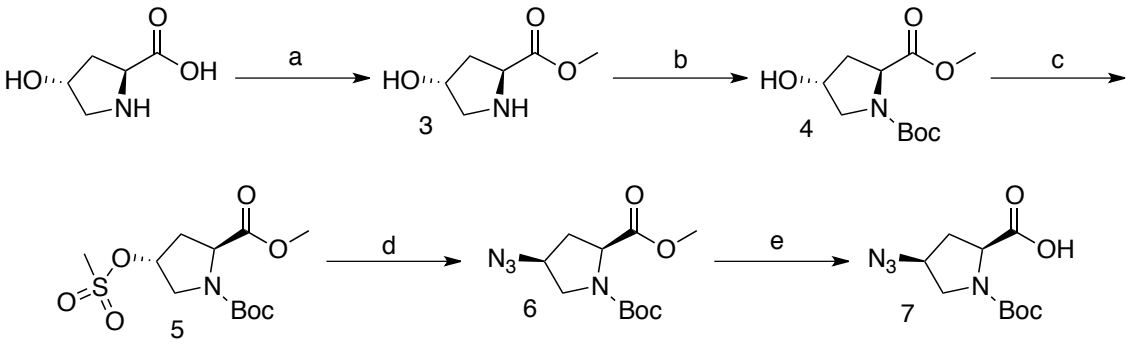



**Fig. 8 [1]H-NMR results for compound 2 using methanol as a solvent - method B**

As we can see in the spectra, the product obtained with the method A has some impurities meanwhile the product obtained with the method B has just a small impurity.

## 2nd building block:




**Scheme 2. Synthesis pathway for the 2nd building block Reaction conditions: a) thionyl chloride (1eq.), MeOH (30eq.), reflux, 2h; b) di-t-butyldicarbonate (1eq.), TEA (2eq.), DCM, rt, 3h c) MesCl (1eq.), TEA (1eq.), DCM, rt, 30 min; d) $NaN_3$ (2eq.), DMF, rt, on; e) KOH (1,05eq.), DCM, rt, on.**

The goal for this step is to form the azide that we need to do the CuAAC at the end to link the signal agent to the inhibitor. The synthesis for this building block started with the formation of a methyl ester using thionyl chloride and MeOH, with a yield of 100%; followed by a protection of the secondary amine with a Boc group in order to make sure that the amine group wouldn't react in the next step. Since the mesylate group is a better leaving group than the hydroxyl function in $S_N2$- type nucleophilic substitution reactions, the hydoxyl group of the prolyl-moiety was mesylated. In the next step, the azide was formed in an $S_N2$- type nucleophilic substitution reaction with $NaN_3$, which lead to a quantative yield. The azide has two nucleophilic ends, which can attack the mesylated carbon forming an alkyl azide. Last step in the synthesis of this building block consisted of an ester hydrolysis with KOH in order to obtain the free carboxyl acid, which is needed for the amide bond formation between this building block and the free amine of the pyrrolidine ring of the first building block. The second building block was obtained with a 93% yield.

All the intermediate compounds were characterised by $^1$H-NMR. The results for the compound (**7**) are shown in Figure 9.

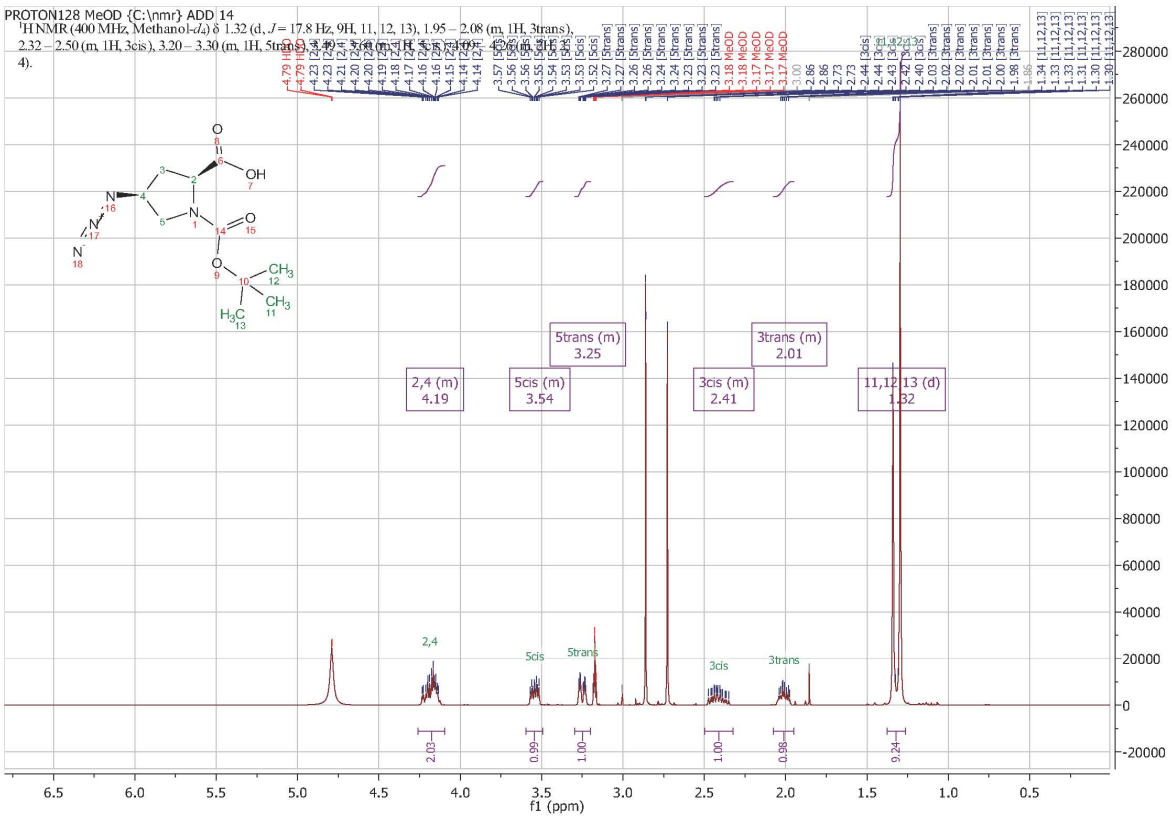
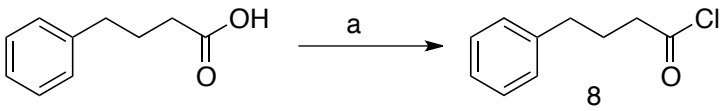



**Fig. 9 $^{1}$H-NMR spectra for compound 7 using methanol as a solvent.**

About these results we can say that there are no impurities but there is still some DMF left (singlets between 2.5 and 3 ppm) after the work up. If we look at the peak for the Boc group (1.32 ppm), we can see that there is splitting that means that there can be different rotamers of the molecule.

## 3$^{rd}$ building block:

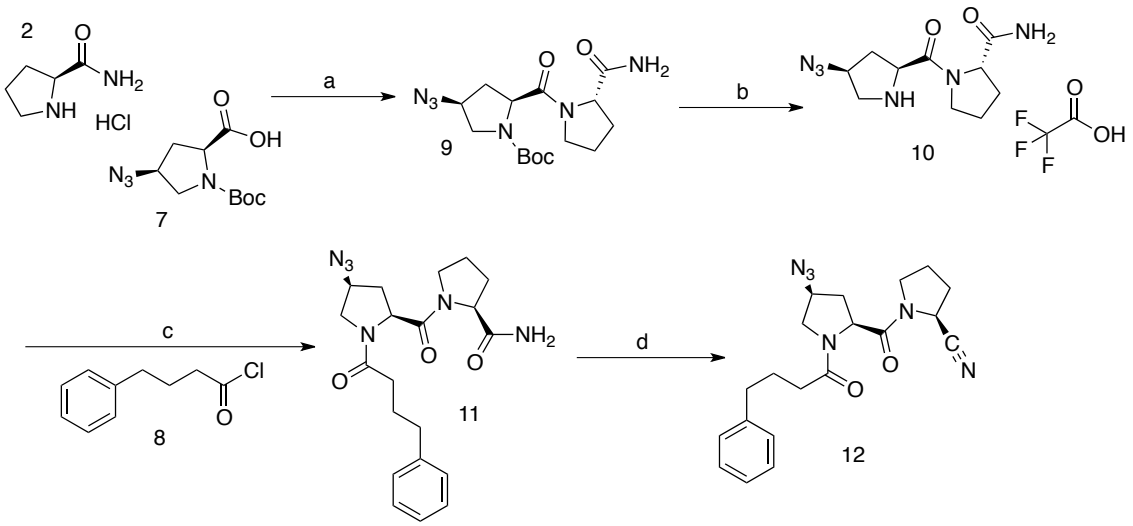



**Scheme 3. Synthesis pathway for the 3rd building block. Reaction conditions: a) sulphurous dichloride (1eq.), 70°C, 70 min.**

In the 3rd building block, 4-phenylbutanoyl chloride was synthesised adding thionyl chloride to the 4-phenylbutanoic acid in order to activate the carboxylic acid to allow rhe formation of the amide bound in the next step. An alternative to the formation of the acyl chloride is to introduce the 4-phenylbutanoic acid onto the inhibitor moiety by using coupling reagents. In this case, the work up will consist of multiple extractions with different aqueous phases and most certainly further purification by flash chromatography in order to get rid of the coupling reagents and the formed side products. While using the acid chloride only requires dry conditions and a base (like

triethylamine), which can be easily removed by washing the organic layer with an acidic aqueous phase. This reaction has a 100% yield.

## Coupling of the three building blocks:




**Scheme 1. Synthesis pathway: coupling of the three building blocks. Reaction conditions: a) HATU (1,05eq.), TEA (2eq.), DMF, rt, on; b) TFA (230eq.), DCM, rt, 45min; c) TEA (2eq.), 0°C, 2h; d) TFA anhydride, pyridine, DCM, 0°C, 1h.**

To complete the inhibitor moiety the three building blocks were couple. First of all, the first and the second building blocks were coupled with HATU. Then, after a Boc removal, the 4-phenylbutanoyl substituent was introduced. Finally, a dehydration of the primary amide group was done in order to obtain the final inhibitor moiety with the carbonitrile warhead.

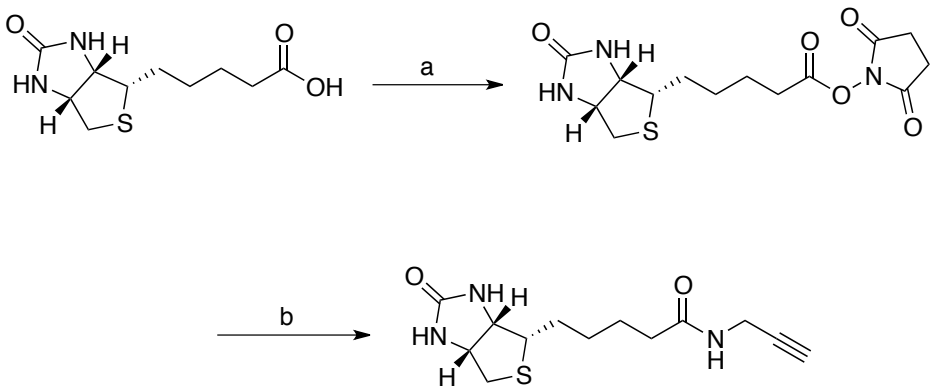
The inhibitor moiety was synthesised twice because the first time there was a racemisation problem. There are two hypotheses to explain the racemisation in the compound **11**. It could be either a problem with the starting material or it could occur in the coupling of the three building blocks. In order to know in which step appeared the problem; the procedure was repeated by using a new starting material and checking the racemisation in every step of the reaction.

First time that the inhibitor was synthesised, two isomers can be found in the UPLC results for the compound **11** because of the racemisation. There are two peaks with the same mass really close one to the other. In order to solve this problem, the compound was purified with a flash chromatography in order to separate both isomers. After that, the column fractions with each isomer were checked by NMR. There were two fractions with different isomers and one fraction with two mixed isomers. The second time, a new starting material was used and there was no

racemisation. In this case, the chromatography was done in the next step for the compound **12** in order to remove some impurities.

## 2) Synthesis of signaling agents

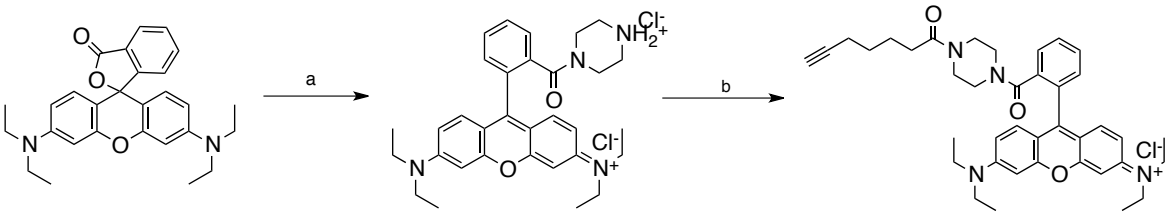
### 2.1) Biotin analogues




**Scheme 2. Reaction conditions: a) D-biotin (1eq.), HOSu (1.2eq.), DCC (1.2eq.), DMF, 70°C, on; b) compound X (1eq.), 2-Propyn-1-amine (1eq.), DIPEA (1eq.), DMF, rt, on.**

In order to connect the biotin with the PREP inhibitor, a linker with triple bound was introduced to the signal agent but first; an NHS ester was formed in order to activate the carboxylic acid for the next reaction. An alternative to this step is to introduce directly the carboxylic acid by using coupling reagents like DCC or HATU but not acid chloride since biotin is quite sensitive to certain reaction conditions.

### 2.2) Rhodamine B piperazine analogues




**Scheme 3. Reaction conditions: a) TMA (1.8eq.), DCM, rt, 2h; piperazine (4eq.), DCM, 65°C, 96h; b) 6-Heptynoicacid (1eq.), DIPEA (3eq.), HATU (1.1eq.), DMF, rt, on.**

First reaction for this step was to introduce the piperazine group to the rhodamine B in order to make it easier to introduce the linker in the next reaction. This reactions were described in 2003 by Nguyen and Francis. The fist reaction has a yield of 74,4%, similar to the 70% yield obtained in the literature (Nguyen & Francis, 2003).

- **Conclusions**

The main goal of this project was to synthesise several molecular imaging probes in order to find the optimal signal agent to follow the evolution of PREP expression levels during epileptogenesis in brain. In order to reach this objective several imaging probes have been synthesised using either rhodamine B piperazine or biotin analogues as signal agents and a PREP inhibitor as a target moiety, which is known to have good affinity for PREP and $IC_{50}$ values.

The inhibitor moiety was synthesised successfully. The yields for the synthesis of the inhibitor are not low and the obtained compounds are quite pure using the synthesis pathway reported in 2012 by Van der Veken et al., but anyway, it can be improved to be optimal by changing the conditions for some reactions. For instance, the Boc deprotection step in the $2^{nd}$ building block was further optimised by using an HCl solution instead of TFA since the obtained compound is has more stability and it is more pure. Also for the coupling reaction of the two proline moieties ($1^{st}$ and $2^{nd}$ building blocks) HATU was used instead of TBTU as in the literature.

About the racemisation problem, after the second synthesis of the inhibitor, we can conclude that the problem was due to the starting material and not due to the synthesis pathway chosen, since the second time that this compound was synthesised using a new starting material there was no racemisation after the coupling step.

We also succeed in the synthesis of both signal agents: rhodamine B piperazine and biotin analogues. But, because a lack of time, we did not get yet the $3^{rd}$ objective of the project but it will be reached during next weeks. Basically, is to introduce the signal agent with the linker on the on the azide through Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC).

This project will continue by following PREP expression during the epileptogenesis in brains of rat model of acquired Temporal Lobe Epilepsy (TLE) and comparing to healthy rat brains. But first, the selectivity and $IC_{50}$ values of the imaging probes for PREP will br determined in the Biochemistry lab.

Neurodegenerative and neuro-inflammation underlies epilepsy symptoms so, the results for these studies will be also useful to understand and examine other neurodegenerative pathologies as well as neurodegeneration processes. Besides, this project can help to find new ways of epilepsy treatment by using PREP-inhibitors.

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Van Der Veken, P., Fülöp, V., Rea, D., Gerard, M., Van Elzen, R., Joossens, J., Augustyns, K. (2012b). P2-substituted N-acylprolylpyrrolidine inhibitors of prolyl oligopeptidase: Biochemical evaluation, binding mode determination, and assessment in a cellular model of synucleinopathy. *Journal of Medicinal Chemistry*. http://doi.org/10.1021/jm301060g