Synthesis of homogeneous $N$-glycosylated- and GPI-anchored peptides for semi-synthesis of the prion protein

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ABBREVIATIONS

i.e. it est

e.g. exempli gratia

°C degree celsius

µm mikrometer

1H-NMR proton nuclear magnetic resonance

AA amino acid

AcBr acetyl bromide

Acm acetamidomethyl

ACN acetonitrile

All allyl

Asn asparagine

Asp aspartic acid

Boc tert-butoxycarbonyl

br broad

brsm by reisolated starting material

BSE bovine spongiform encephalopathy

Bzl Benzyl

CD cluster of differentiation

CJD Creutzfeld-Jakob disease

CNS central nervous system

conc. concentrated

Cys cysteine

d doublet

DNA desoxyribonucleic acid

Dbz 3,4-diamino-benzamide

DCM dichloromethane

dd doublet of doublets

DIC diisopropylcarbodiimide

DIPEA N,N-diisopropyl-ethylamine

DMC 2-chloro-1,3-dimethylimidazolinium chloride

DMF N,N-dimethylformamide

Dmntb 4,5 dimethoxynitrobenzyl
DMS dimethyl sulfide
DMSO dimethylsulfoxid
dt doublet of triplets
DTT dithiothreitol
E1cb Elimination of first order via conjugate base
EDC 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimid
EDTA ethylenediaminetetraacetic acid
EPL expressed protein ligation
ESI-MS electrospray-ionization mass spectrometry
et al. et alli
Et3N triethylamine
EtOAc ethyl acetate
FA formic acid
Fmoc 9-Fluorenylmethoxycarbonyl
Fmoc-OSu 9-Fluorenylmethoxycarbonyl-O-succinimide
FPLC fast protein liquid chromatography
g gram
GdmCl guanidinium chloride
GFP green fluorescent protein
GlcN glucoseamine
GlcNAc N-acetylglucosamine
Glu glutamine
Gly glycine
GPI glycosylphosphatidylinositol
h hour
h hextet
H2 hydrogen
HBTU 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCl hydrochloric acid
HF hydrogen flouride
HFIP 2,2′-hexafluoroisopropanol
Hg mercury
Hmb 2-hydroxy-4-methoxybenzyl
HOBT 1-hydroxy benzo triazol
HPLC  high performance liquid chromatography  
Hz   Hertz  
I₂   iodine  
Ile  Isoleucin  
Ino  myo-Inositol  
J   indirect dipole dipole coupling  
LC-MS liquid chromatography mass spectrometry  
L-Fuc  L-fucose  
LiHMDS  lithium hexa methyl disilizane  
Lys  lysine  
M   Molar solution  
m   multiplet  
m/z  mass per charge  
MALDI-TOF Matrix assisted laser desorption ionization time of flight  
Man  mannose  
Map  4-(dimethylamino)-phenacyl  
mAU  milli absorption units  
MeOH  methanol  
Met  methionine  
min  minutes  
ml  milli liter  
mm  millimeter  
MMBA  4-mercaptomethylbenzoic acid  
mmol  milli mol  
MPAA  4-mercaptophenylacetic acid  
NaN₃  sodium azide  
NaNO₂  sodium nitrite  
NaOMe  sodium methoxide  
Nbz  N-acyl-benzimidazolinone  
NCL  native chemical ligation  
NH₄I  ammonium iodide  
nm  nanometer  
NMP  N-methyl-2-pyrrolidone  
Ox  oxidation
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Oxyma</td>
<td>pthyl cyanohydroxyiminoacetate</td>
</tr>
<tr>
<td>Pd</td>
<td>palladium</td>
</tr>
<tr>
<td>PEG</td>
<td>poly-ethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>protecting groups</td>
</tr>
<tr>
<td>pH</td>
<td>-log [H]+, more precisely a</td>
</tr>
<tr>
<td>PhAcM</td>
<td>Phenylacetamidomethyl</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>PNGaseF</td>
<td>Peptide:N-Glycosidase F</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;C&lt;/sup&gt;</td>
<td>prion protein cellular form</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>prion protein scrapie form</td>
</tr>
<tr>
<td>PyBOP</td>
<td>Benzotriazol-1-yl-oxytritylrorilidinophosphonium-hexafluorophosphate</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>Qtof</td>
<td>quadrupole TOF</td>
</tr>
<tr>
<td>quant</td>
<td>quantitative</td>
</tr>
<tr>
<td>R</td>
<td>rectus</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse phase HPLC</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>rPrP</td>
<td>recombinant prion protein</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>S</td>
<td>sinister</td>
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<tr>
<td>s</td>
<td>singulett</td>
</tr>
<tr>
<td>sat.</td>
<td>saturated</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SGP</td>
<td>sialglycopeptide</td>
</tr>
<tr>
<td>SPFC</td>
<td>solid phase fragment condensation</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid phase peptide synthesis</td>
</tr>
<tr>
<td>t</td>
<td>triplett</td>
</tr>
<tr>
<td>tBu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphin</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFAA</td>
<td>trifluoroacetic acid anhydride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>Thz</td>
<td>Thiazolidine</td>
</tr>
<tr>
<td>TIPS</td>
<td>triisopropylsilane</td>
</tr>
<tr>
<td>Tmob</td>
<td>trimethoxybenzy</td>
</tr>
<tr>
<td>TSE</td>
<td>transmissible spongiform encephalitis</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>v</td>
<td>volume</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
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**SUMMARY**

The prion protein (PrP), an N-glycosylated and GPI-anchored protein, is responsible for transmissible spongiform encephalopathies. The prion pathogenesis is associated with the conformational conversion of its cellular form (PrP$^C$) into the so-called scrapie form (PrP$^S$), an insoluble, partially protease-resistant isoform. PrP$^C$ is a cell surface glycoprotein of 208-209 amino acids of unknown function. The role of the highly heterogeneous N-linked glycosylation is indistinct, resulting in glycosylated species with more than 50 different glycoforms.

To study the role of the PrP glypiation and N-glycosylation, it is of high importance to have access to homogeneous glycosylated PrP. However, with current methods it is difficult to obtain homogeneous glycoproteins from natural sources and the synthesis of glycopeptides and glycoproteins is a very complex task.

The aim of this work was to develop methods to provide access to homogeneous N-glycosylated- and GPI-anchored prion protein fragments for the semi-synthesis of PrP, which allow the evaluation of these modifications in the function, aggregation, replication and infectious properties of the prion protein. Therefore, a combination chemical glycopeptide synthesis and ligation methods was used.

The most convenient method to obtain glycopeptides is the attachment of amino glycans to fully synthetic peptides by having a suitable protecting group pattern easily removable at late stage of the synthesis using the so-called Lanbury aspartylation. The access to appropriate glycan azides, which can be reduced and coupled to the peptide backbone, is thereby essential. In this work, starting from commercially available glucosamine hydrochloride, the synthesis of GlcNAc-azide 12 and its conversion into GlcNAc-amine 19, a building block useful for the synthesis of various glycopeptides, was established using two different routes.

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**Figure 1**: Glycan azides and amine for glycopeptide synthesis using Lansbury aspartylation

Additionally, nonasaccharide-azide 13 was generated chemoenzymatically starting from a sialylglycopeptide extracted from egg yolk powder. Afterwards, the sialic acid residues were cleaved using acidic hydrolysis and the glycan was released with PNGaseF and converted into
the corresponding nonasaccharide-azide 13 using DMC and sodium azide. After reduction, the corresponding nonasaccharide-amine was evaluated for the synthesis of glycopeptides.

A second key requirement for glycopeptide synthesis using Lansbury aspartylation is the orthogonal protection at the aspartic acid side chain, making it available for coupling to an amino glycan while the remaining protecting groups are still intact. Since PrP contains two glycosylation sites close to each other at Asn181 and Asn197, two different orthogonal protected aspartic acid building blocks are required. Thus, to complement the well-established allyl ester, the synthesis of a photolabile protected aspartic acid was established. Starting from commercially available 4-(dimethylamino)-acetophenone and L-aspartic acid the desired Fmoc-Asp(OMap)-OH 11 was synthesized in 7 steps. Thereafter, amino acid 11 was incorporated in the sequence of modelpeptide 36 using Fmoc-SPPS. Building block 11 was introduced without side reactions and the photolabile Map-group was stable during elongation of the peptide. Afterwards, the Map-group was selectively removed by photolysis using a high-pressure Hg-lamp (hν>300 nm) and the free acid function was coupled to amino glycan 19 to give the desired glycopeptide 38. The successful synthesis of 38 proved the applicability of Fmoc-Asp(OMap)-OH 11 for the synthesis of homogeneous glycopeptides.

![Figure 2: Orthogonal protected aspartate building block and model peptides 36 and 38](image)

Four key fragments are required for the semi-synthesis of the prion protein using native chemical ligation: a cysteine modified GPI anchor, the peptide fragment 1 (PrP 214-231), the peptide fragment 2 (PrP 178-213) having a β-mercapto aspartic acid at Asp178 to enable a ligation-desulfurization with the fragment 3 (PrP 23-177), which can be expressed as thioester. The required building blocks to install, a β-mercapto aspartic, are not commercially available. Here, three β-mercapto aspartic acid building blocks were synthesized using a fully protected aspartic acid and a sulfenylating reagent.

In that way, first, Boc-Asp(OtBu,STmob)-OH 8 was synthesized by reaction of a fully protected aspartic acid and STmob-tosyrlate at low temperature and under strong basic conditions to give a β-mercapto functionalized aspartic acid as a mixture of diastereomers. The allyl was removed and pure isomers 8a and 8b were isolated after separation using RP-HPLC. Both isomers were used for the synthesis of fragment 2.
Additionally, a β-mercapto aspartic acid building block having an orthogonal Acm protecting group on the thiol function was synthesized using the same strategy established for 8. For the reaction SAcm-tosylate as sulfenylating reagent was used. The resulting mixture of isomers was, after cleavage of the allyl ester, separated using RP-HPLC. Both isomers, 9a and 9b, were used to synthesize PrP-fragments with an orthogonal protected N-terminal β-mercapto aspartic acid.

![Figure 3: β-mercapto aspartic acid building blocks for ligation-desulfurization strategy](image)

A second orthogonal protected β-mercapto aspartic acid building block was generated, masking the thiol function with a photolabile Map-group using SMap-tosylate. The diastereomeric products, 10a and 10b, were separated after allyl removal using RP-HPLC. The installation of 10 as N-terminal β-mercapto aspartic acid resulted in aspartimide formation. Thereby, the building block was not suitable for SPPS.

The peptide fragment 1 (PrP 214-231) was synthesized using SPPS. To enable thioesterformation for NCL, the peptide was generated as peptide hydrazide and Nbz-peptide. Nbz-peptide 53 was not suitable a thioester precursor and peptide hydrazides were used to obtain the desired thioesters for NCL. To enable sequential NCL in C to N direction hydrazide 56 was synthesized having an unprotected N-terminal cysteine. Additionally, fragment 1 was obtained having an orthogonal protected cysteine for sequential NCL in N to C direction.

![Figure 4: PrP peptide fragment 1 [PrP I (214-231)] thioester precursors (53, 56 and 57) and thioester 61](image)

The peptide-hydrazide 57 was converted into the corresponding thioester 61 and used to investigate the ligation to a cysteine modified GPI-DiMan structure as GPI-mimic. The
The desired ligation product was obtained with an optimized ligation buffer using 6 M GdmCl, 0.1 M Na₂HPO₄, 5 eq MMBA and 30 eq TCEP. After successful ligation, the orthogonal Acm protecting on cysteine of 63 was removed with PdCl₂ and the desired product PrP I [Cys-DiMan] 64 was isolated.

![Figure 5: DiMan anchored PrP I peptides](image)

The synthesis PrP peptide fragment 2 (PrP 178-213) was evaluated using four different strategies. The desired peptides were synthesized using SPPS and were obtained as peptide hydrazide to allow the formation of a thioester for ligation. During the synthesis oxidation of the methionine residues Met205, Met206 and Met213 was observed, which resulted in troublesome purification of the desired peptides. Optimization of the conditions during automated SPPS and installation of pseudoprolines helped improving the purity and total yield of the desired products.

In the first strategy, a sequential ligation-desulfurization strategy in N to C direction was evaluated. Thus, acid labile protected β-mercapto aspartic acid 8 was installed as N-terminal amino acid. Four peptides having a β-mercapto aspartic acid at position Asp178, including three glycopeptides, were synthesized. For the synthesis of glycopeptides Asp181 and/or Asp197 were installed bearing an orthogonal allyl protecting group. After cleavage of the allyl ester, glycosyl amine 19 was attached using Lansbury coupling.

![Figure 6: PrP II (178-213) peptides and glycopeptides using β-mercapto aspartic acid 8](image)
The free thiol functions of Cys179 and β-mercapto Asp178 resulted in intra- and intermolecular disulfide bonds and reduction using TCEP resulted in desulfurization of glycopeptides 70, 71 and 72. Additionally, a small peptide fragment (160-177) of protein thioester (fragment 3) was synthesized as model peptide 73. Peptide hydrazide 73 was successfully transformed into the corresponding thioester 74. Thereafter, β-mercapto aspartic acid containing peptide 68 and thioester 74 were combined using NCL and LC-MS analysis showed the formation of the desired product 75.

![Figure 7: Hydrazide 73, thioester 74 and ligation product 75](image)

Additionally, in a second strategy, fragment 2 (178-213) was synthesized using β-mercapto aspartic acid building block 9. The orthogonal Acm protecting group on the thiol function enables a sequential ligation in C to N direction.

![Figure 8: Hydrazide 77 and glycopeptide hydrazide 79 using β-mercapto aspartic acid 9 and glycopeptide thioester 80](image)

Peptide hydrazide 77 and glycopeptide hydrazide 79 were successfully synthesized. To generate 79 Asp181 was installed with an allyl-protecting group on the side chain, which was removed after peptide assembly and the free β-carboxylate was coupled to glycosyl amine 19 using Lansbury coupling. Glycopeptide hydrazide 79 was successfully converted into the corresponding thioester 80 and tested for ligation to fragment 1.

Solid phase fragment condensation was investigated as third strategy to obtain fragment 2. Two smaller segments of PrP II (179-213), fragment 2A PrP (196-213) and fragment 2B PrP (178-195), were synthesized. Gly195 was chosen as the C-terminal amino acid of fragment 2B to avoid racemization during the coupling. To enable thioester formation and ligation, fragment 2A was synthesized as peptide hydrazide. Additionally, Asp197 was equipped with
an orthogonal allyl- or Map-protecting group for Lansbury coupling after complete SPPS and SPFC and three peptide hydrazides (83, 84 and 85) were synthesized.

Additionally, two peptides for the generation of a fully protected fragment 2B were assembled, including one containing an orthogonal protected Asp181 for possible Lansbury coupling. The cleavage of fragment 2B under mild acidic conditions resulted in a mixture of the fully protected peptide and several semi-protected peptides 87 and 88, which could not be removed to obtain a clean product for condensation with fragment 2A.

Furthermore, a ligation-desulfurization strategy was studied for the synthesis of fragment 2 (178-213). Thus, to obtain the desired product, two peptide hydrazides were synthesized. Fragment 2C (202-213) was synthesized using 8 as N-terminal amino acid, and Asp178 was installed as protected β-mercapto aspartic acid on fragment 2D for sequential ligation in C to N direction.

The synthesis of PrP II (202-213) was accomplished using the conditions optimized for fragment 2. Peptide hydrazide 91 was completed without oxidized methionine residues, in a high crude purity and was easily purified.

The synthesis of PrP fragment 2D (178-201) was investigated with two different strategies. First, the peptide- and glycopeptide hydrazides were obtained having a free N-terminal cysteine. Asp181 and/or Asp197 were installed bearing an orthogonal allyl protecting group to synthesize glycopeptides. After the allyl ester was cleaved, glycosyl amine 19 was attached using Lansbury aspartylation.
The low solubility of fully protected β-mercapto aspartic acid thioester limited the successful ligation to the peptide- and glycopeptide hydrazides 92, 95, 97 and 99. Additionally, fragment 2D was synthesized using 9 to have Acm protected glycopeptide hydrazides. Asp181 and/or Asp197 had an allyl protecting group that was removed after successful synthesis and glycosyl amine 19 was coupled to generate the desired glycopeptide hydrazides using Lansbury coupling. The hydrazides were successfully converted into thioesters, which were obtained in a mixture with the corresponding thiolactone, formed with the free thiol function of Cys179.

In order to obtain fragment 2, the ligation between the desired glycopeptide thioesters and β-mercapto aspartic acid containing peptide 91 was optimized. A previously used ligation buffer without additional thiol resulted in fast hydrolysis of the thioester and thiolactone and the ligation product was not observed. A ligation buffer containing 5 eq. MMBA resulted in the formation of disulfides involving 91 and MMBA without the formation of a ligation product. Finally, the desired products were observed by adding a more reactive thiol (MPAA) at a lower concentration. Unfortunately, the conversion was very low, and pure products 113, 114 and 115 could not be separated from hydrolyzed starting material and disulfides 91 formed with MPAA.
In summary, to gain access to a homogeneous N-glycosylated GPI-anchored prion protein a strategy using sequential native chemical ligation of protein-, peptide- and glycopeptide fragments was investigated. The synthesis of the required building blocks for glycopeptide synthesis and for a ligation-desulfurization strategy was successful, and the building blocks were used to generate the desired peptide- and glycopeptide thioesters. The ligation of the C-terminal PrP fragment 1 (214-231) was effective using an optimized buffer system and cysteine modified GPI-DiMan structure, but the ligation to a full GPI remains to be optimized. Additionally, PrP fragment 2 (178-213) was obtained in non-, mono- and diglycosylated forms using SPPS and a ligation-desulfurization strategy. The desired thioesters were successfully used in different ligation reactions. However these peptides showed low conversion and further improvements in future applications are required. Thus, a protection of the thiol function of Cys179 can be beneficial. The desired glycopeptides were generated bearing a glucosamine residue, but the installation of a nona-saccharide using Lansbury aspartylation was not effective. A strategy to elongate the obtained glycopeptides enzymatically using a glycan oxazolidine remains to be investigated.
**ZUSAMMENFASSUNG**

Das Prion Protein (PrP) ist ein GPI-verankertes Glykoprotein, das für übertragbare schwammartige Hirnleiden (Transmissible Spongiforme Enzephalopathie [TSE]) verantwortlich ist. Die Pathologie der Prionerkrankung wird mit der konformellen Veränderung der zellulären Form (PrP<sup>C</sup>) der Prion Proteins in die sogenannte Scrapie Form (PrP<sup>Sc</sup>) des Proteins assoziiert. PrP<sup>Sc</sup> ist unlöslich und teilweise resistent gegen über Proteasen. PrP<sup>C</sup> ist ein Zelloberflächenglykoprotein das aus 208-209 Aminosäuren besteht und dessen Funktion nicht bekannt ist. Mehr als 50 verschiedene Zuckerformen können am PrP verankert sein, wobei die Rolle dieser hochgradig heterogenen N-gekoppelten Glykosylierungen unklar ist.


Die Zielsetzung dieser Arbeit war es Methoden zu entwickeln um Zugang zu homogenen GPI-verankerten Prion Glykoproteinen zu haben und dadurch den Einfluss der verankerten Kohlenhydrate auf die Funktion, auf die Aggregation, auf die Verbreitung und auf die infektiösen Eigenschaften des Prion Proteins zu untersuchen. Für diese Aufgabe wurde eine Kombination aus chemischer Glykopeptidsynthese und Ligationsmethoden genutzt.

enzymatische Spaltung mit PNGaseF freigesetzt und in Gegenwart von DMC und Natriumazid in das entsprechende Nonasaccharid Azid 13 umgewandelt

Nach der Reduktion zum Nonasaccharid Amin, wurde das Molekül für die Glycopeptidsynthese getestet.

Für die Semisynthese des Prion Proteins durch native chemische Ligation werden vier Schlüsselfragmente benötigt: ein mit einem Cystein modifizierten GPI-Anker, das Peptidfragment 1 (PrP 214-231), das Peptidfragment 2, welches die Installation einer β-Mercaptyl Asparaginsäure an Position Asp178 benötigt um eine Ligations- und Entschwefelungsstrategie zu nutzen. Außerdem wird Protein fragment 3 (PrP 23-177) als mit Proteinthioester benötigt.


Außerdem wurde ein β-Mercaptyl Asparaginsäurebaustein erzeugt der eine orthogonale Acm-Schutzgruppe an der Thiolfunktion trägt. Dafür wurden dieselben Bedingungen genutzt die bereits für Baustein 8 etabliert wurden unter Verwendung von SAcm-Tosylat als Sulfonierungsreagenz. Die resultierenden Diastereomere wurden nach Allylesterspaltung durch RP-HPLC getrennt und beide Isomere, 9a und 9b, wurden für die Synthese von Prion Peptidfragmenten genutzt die eine orthogonal geschützte N-terminale β-Mercaptyl Asparaginsäure besitzen.

Abbildung 3: β-Mercaptyl Asparaginsäurebausteine für Ligations- und Entschwefelungsstrategien

Zusätzlich wurde ein zweiter orthogonal geschützter β-Mercaptyl Asparaginsäurebaustein mit derselben Strategie hergestellt, wobei die Thiolfunktion mit der lichtsensitiven Map-Gruppe geschützt wurde. Für die Reaktion wurde SMap-Tosylat als Sulfonierungsreagenz verwendet. Nach der Allylesterspaltung wurden die Isomere mit Hilfe RP-HPLC getrennt. Leider führte
die Installation von Baustein 10 zu Aspartimidbildung und diese β-Mercaptyl Asparaginsäure konnte nicht für die Peptidfestphasensynthese genutzt werden.


Nach der erfolgreichen Synthese von Prionproteinhdyrazid 57 wurde das Peptid in den entsprechen Thioester 61 umgewandelt und mit einer Cystein modifizierten GPI-Dimannose ligiert. Der Ligationspuffer wurde optimiert und mit der Nutzung von 6 M GdmCl, 0.1 M Na2HPO4, 5 eq MMBA und 30 eq TCEP wurde das gewünschte Ligationsprodukt 63 hergestellt. Anschließend wurde die Acm-Schutzgruppe mit Hilfe von PdCl2 entfernt und PrP I [Cys-DiMan] 64 wurde isoliert.

und Met213 als Nebenreaktion beobachtet, wodurch die Reinigung der Peptide verkompliziert wurde. Die Bedingungen für die Peptidfestphasensynthese wurden optimiert und durch die Installation von Pseudoprolinen wurden die Reinheit und die Ausbeute der Produkte verbessert.


![Abbildung 6: PrP II (178-213) Peptide und Glycopeptide mit β-Mercaptyl Asparaginsäure 8](image)


![Abbildung 7: Hydrazid 73, Thioester 74 und Ligationsprodukt 75](image)

In der zweiten untersuchten Strategie wurde Fragment 2 (178-213) unter Nutzung des β-Mercaptyl Asparaginsäure Bausteins 9 synthetisiert. Hier ermöglichte die orthogonale

Abbildung 8: N-Terminus β-Mercaptyl Asparaginsäure 9 modifiziertes Hydrazid 77 und Glykopeptid Hydrazid 79 und Glykopeptidthioester 80


Abbildung 9: Synthetisierte Fragmente 2A für die SPFC mit orthogonalen Schutzgruppen zur möglichen Installation von Glykosylierungen
Die Glykosylierungsstelle an Asparaginsäure 181 im Fragment 2B wurde zusätzlich mit einer orthogonalen Allyl-Schutzgruppe ausgestattet.

![Abbildung 10: Synthetisierte resingebundene Fragmente 2B für die SPFC](image)

Obwohl, die beiden Peptide in hoher roher Sauberkeit synthetisiert wurden, resultierte die Abspaltung von 87 und 88 unter milden sauren Bedingungen in einer nicht trennbaren Mischung von vollgeschützten und mehreren teilentschützten Peptiden. Aufgrund des Mangels an aufgereinigtem Material war die Untersuchung der Kondensation mit Fragment 2A nicht möglich.


![Abbildung 11: Fragment 2C (202-213) 91](image)

Fragment 2C 91 wurde mit den optimierten Bedingungen aus früheren Synthesen von Fragment 2 dargestellt und durch nicht beobachtete oxidierte Methionine in hoher Sauberkeit isoliert.


Ein Ligationspuffer mit 5 Äquivalenten MMBA, resultierte in der Formierung von Disulfiden zwischen 91 und MMBA und somit nicht zur Bildung des Produktes. Letztendlich, wurde das Produkt mit Hilfe eines Puffers isoliert der das reaktive Thiol MPAA in geringer Konzentration aufwies. Unglücklicherweise war die Umsetzung sehr gering und die
Ligationsprodukte 113, 114 und 115 konnten nicht von Verunreinigungen und Nebenprodukten gereinigt werden.

Abbildung 14: Ligationsprodukte zur Darstellung von Fragment 2

INTRODUCTION

1. GLYCOPROTEINS

The majority of proteins are post translationally modified including phosphorylation, lipidation, or glycosylation, whereby the attachment of carbohydrates is the most abundant alteration in nature. Post translational modifications change the physical, chemical, and biochemical properties of proteins, influencing their stability, folding, function, and conformation and are essential for protein activation. Thus, much attention has been given to these modifications. Glycosylation introduces an immense diversity to the modified protein that exists as a mixture of so called glycoforms, molecules having the same peptide sequence with different carbohydrate structures. Glycoproteins are basic to many important biological processes including fertilization, immune defense, viral replication, parasitic infection, cell growth, cell-cell adhesion, degradation of blood clots and inflammation. The importance of understanding the functions of glycoproteins is also motivated by their role played in many human diseases like autoimmune infections and cancer. Furthermore, it has been suggested that the naturally occurring mixtures of glycoforms provide a spectrum of activities that can be seen as fine-tuning. N-linked protein glycosylation has a role in “quality control” of protein synthesis; proteins possessing an incorrect glycosylation state tend to fold wrong, suggesting a role for added glycans as indicator of correct protein structure. Although this and other functions have been attributed the glycosylation of proteins, the role of the carbohydrate structure in the protein function is still poorly understood. This is mainly due to the lack of sufficient amounts of pure homogeneous glycoproteins.

1.1 Functions of Oligosaccharides

Biological roles of oligosaccharides seem to have a very broad spectrum, varying from trivial tasks to those that are crucial for function, development, growth, and survival of an organism. Even within a given group of proteins, such as cell surface enzymes, effects of altering glycosylation are highly variable and unpredictable. Additionally, it is observed that the same modification in glycosylation can have different effects on the protein activity in vivo and in vitro. Carbohydrate modifications can change properties of the attached protein by altering the stability, protease resistance, or quaternary structure. Characteristic carbohydrate moieties which are existent in cell adhesion molecules, tumor-associated antigens, viral or bacterial invasion targets and blood group determinations are most
commonly presented on the cell surface and involved in binding processes of molecular recognition systems in the form of glycoproteins.\textsuperscript{18}

\section*{1.2 Diversity of Oligosaccharides}

The four major classes of bio-macromolecules are lipids, nucleic acids, carbohydrates and proteins. Nucleic acids and proteins have only one type of linkage, resulting in an almost solely linear structure. In comparison, carbohydrates differ in two important characteristics: Monosaccharides are linked by an acetal linkage which is found in either equatorial or axial configuration and the hydroxyl group used to form a disaccharide is not unique, ranging from simple epimers to regioisomers of primary and secondary hydroxyl groups. Moreover single monosaccharides can maintain these linkages to several others units, introducing a second degree of complexity, \textit{i.e.} branching. Oligosaccharide structures are surpassing in the conveyed density of information. Precise differences in the nature of linkages between two pyranoses (e.g. 1-2, 1-3, 1-4, 1-6) oppose with the linear nature of proteins and nucleic acids. While DNA, with a basis set of 4, and amino acids, with a basis set of 20, may create a biological language for information transfer of 4096 and 6.4\times10^7 “words”, carbohydrates can create more than 1.05\times10^{12} variations.\textsuperscript{17, 19} The biosynthesis of oligosaccharides involves a series of enzymatic reactions that are not driven by a template or controlled by transcription. Therefore, varying conditions like substrate activity or enzymatic activity can easily affect this process. As a result, glycoproteins can have many different glycoforms.\textsuperscript{20}

The majority of carbohydrates are attached to lipids or proteins and are present having many different structures, such as biantennary, triantennary and tetraantennary patterns. Additionally, the oligosaccharides exhibit a site-specific diversity.\textsuperscript{21} Variations in the combination of the possible linkages and substitutions lead to thousands of related carbohydrate structure. This is known as microheterogeneity and is an unique and important property of natural glycoproteins.\textsuperscript{22-23} The elements of glycoproteins are very similar, but show a high degree of diversity in detailed structures and can thus be seen as “natural libraries”.\textsuperscript{24}

\section*{1.3 Types of Oligosaccharide linkages}

Among the known carbohydrate-protein linkages (\textit{C}-, \textit{N}-, \textit{O}-, and \textit{S}-linkages), most common are \textit{N}- and \textit{O}-linked glycoproteins. Regarding the \textit{O}-linked type, small oligosaccharides are attached to either serine or threonine usually having an \textit{N}-acetyl-\textit{α}-D-galactosamine as first unit. These sequences of small oligosaccharides are classified into 8 groups.\textsuperscript{9} The more
complex N-linked type consists of large and branched oligosaccharides, that are further divided into three classes, *i.e.* complex, hybrid and high mannose type.\(^3\) *N*-glycans share a common Man\(_3\)GlcNAc\(_2\)-Asn structure and vary in the degree of branching, the terminal structures in the side chain and the substitution of the core pentasaccharide.\(^{25}\) The high-mannose *N*-glycan contains only D-mannose (Man) residues attached to the core. Complex *N*-glycans have antennae or branches attached to the core, like the addition of D-Gal, *N*-GlcNAc, L-Fuc, sialic acid or sulfate. The amount of antennae in mammals reach numbers between two and four. Hybrid *N*-glycans have both, Man residues on the Man(α1,3) arms of the core and one or two more antennae containing complex glycans attached to the Man(α1,6) arm.\(^{26}\)

### 2. Glycosylphosphatidylinositol (GPI)

An additional modification present in eukaryotic proteins is the anchoring to a GPI. The GPI anchor is bound to the C-terminus of a protein attaching it to the cell membrane (Scheme 1). Throughout all eukaryotic cells GPs have a conserved structure, which consists of three mannose residues, a glucosamine, and a myo-inositol connected to a phospholipid.\(^{27-28}\) To this core structure, various modifications depending on tissue, cell-type and organism can be found. Most prominent are addition of monosaccharide units such as galactose and mannose to Man II, branching at either C3 or C4 position of Man I, additional phosphorylations and acetylations. More precisely, mammalian GPI present a specific additional phosphorylation at Man I and a β-linked GalNAc structure is commonly attached at C4 of Man I.\(^{29}\) The role of the length of the lipid chain and its saturation is heavily discussed in literature.\(^{27,30-32}\) The exact composition of the fatty acids strongly influences the interaction within regions of elevated cholesterol and sphingolipid content.\(^{33}\)

![Scheme 1](image_url): General structure of GPs: the black core can bear different appendages depending on species, tissue and cell-type origin
Other than its primary role of anchoring proteins to the outer leaflet of the biomembrane, functions of the GPI are difficult to prove and investigate. Considering the conservation of GPIs and that simpler ways of anchoring a protein exist, one can assume involvement in a number of mechanisms in and between cells. Certain GPIs play a role in microdomain formation, protein sorting, diagnostic and cross reacting antigens and exert immunomodulatory effects. However, these roles are not to be generalized and were only proofed for distinct GPIs at a time.

3. CHEMICAL SYNTHESIS OF PEPTIDES, GLYCOPEPTIDES AND PROTEINS

3.1 Synthesis of Glycoproteins

As aforementioned, the study of the biological aspects of N-glycosylation requires the synthesis of homogeneous N-glycoproteins. In general, the methods to obtain well-defined glycoproteins can be summarized in three different groups as depicted in Scheme 2.

Transglycosylation (A, Scheme 2) involves the enzymatic manipulation of an expressed glycoprotein with a glycosidase followed by an enzymatic coupling of the desired oligosaccharides. A homogeneous glycoprotein can as well be obtained by sole chemical synthesis. Small peptides are synthesized by solid phase peptide synthesis, and the oligosaccharide is introduced as a building block, or by coupling it to the appropriate protected peptide. Afterwards, the peptide fragments are coupled by chemical ligation.
strategies (B, Scheme 2). This method however is limited to small proteins. The two mentioned strategies are combined in a third approach (C, Scheme 2), where the non-glycosylated protein part is expressed and glycosylated peptide fragments are generated by chemical synthesis. The different fragments are connected by native chemical ligation.

3.2 Solid Phase Peptide Synthesis (SPPS)

Synthetic peptides are used ubiquitously for studies in biology, biochemistry, chemical biology, medicinal chemistry, and many other areas of research and industry. Solid phase peptide synthesis (SPPS) is the predominant method for preparation of peptides. It was introduced by Merrifield et al. in 1963 revolutionizing the field. Elongating the peptide chain on an insoluble solid support has obvious benefits: the immobilized intermediate peptide can be separated from soluble reagents simply by filtration and washing, thus saving time and labor compared to solution phase synthesis. In SPPS, the Nα-function is protected orthogonally to the side chain functionalities. After every coupling step the temporary α-amino protecting group is removed to couple the next amino acid. This deprotection-coupling process is repeated until the desired sequence is completed. Additionally, most steps in SPPS can be conducted in an automated fashion, making this approach even more convenient. For the orthogonal protection of the Nα-function and side chain functional groups two strategies, namely Boc/Bzl- and Fmoc/tBu-SPPS, are used. In Boc-SPPS the tert-butoxycarbonyl (Boc) group is used for temporary protection of the α-amino group, which is usually removed with neat trifluoroacetic acid (TFA) or TFA in dichloromethane (DCM). For more permanent protection of the side-chains of trifunctional amino acids a range of benzyl-based (Bzl) protecting groups have been developed. However, this protecting strategy requires the use of hydrogen fluoride (HF) or strong acids as trifluoromethanesulfonic acid for the deprotection of the side-chain protecting groups, which can lead not only to several byproducts but are also dangerous to handle. Additionally, the conditions in the Boc/Bzl strategy are not suitable for the synthesis of glycopeptides, since the glycosidic linkages are sensitive to strong acids. In Fmoc-SPPS the α-amino group is masked with the base-labile 9-Fluorenlymethoxycarbonyl (Fmoc) group, which is removed by base, usually using 20% piperidine in DMF. The side chain is protected through acid-labile protecting groups, typically tert-butyl- and trityl-based. Both, the linker to the resin and side chain protecting groups are cleaved in the final step by using 90-95% TFA and scavengers to capture the resulting carbo-cations. Since TFA is only used after the peptide assembly, this
approach is conveniently used for the synthesis of glycopeptides and phosphorylated peptides. The principal of Fmoc-SPPS is shown in Scheme 3.

During Fmoc-deprotection the Fmoc-group undergoes an E1cb mechanism. The key step is the initial deprotonation of the benzylic position to generate an aromatic cyclopentadiene-type intermediate, which rapidly eliminates to form dibenzofulvene. With the excess of base, dibenzofulvene forms a piperidine adduct, which strongly absorbs UV light, offering a possibility to monitor the reaction progress and coupling yields (Scheme 4).34

The approach of SPPS, however, has its limitations. During chain assembly, byproducts arising from incomplete reactions, side reactions, or impure reagents accumulate on the resin and contaminate the final product. Regarding product purification, these impurities and deletion sequences hamper the process significantly. Usually, desired sequence and deletion sequences have very similar properties, complicating the required separation. Nevertheless, many strategies to overcome these issues have been published.34 For example are the choice of the solid support as well as the resin loading important parameters for the success of the synthesis. Additionally, solid-phase fragment condensation (SPFC)36 and native chemical ligation (NCL)37-38 emerged as convenient methods to couple smaller peptide fragments, overcoming mentioned limitations of SPPS.
3.3 Native chemical ligation

Solid phase peptide synthesis is significantly limited by the maximum number of amino acids that can be coupled in a single process. Depending on the sequence, resin loading and coupling conditions, peptides and small proteins with up to 50 amino acids can be synthesized. However, due to incomplete deprotection steps, coupling reactions and side reactions side products accumulate reducing the yield of the desired peptide and narrow the solid-phase methodology. Native chemical ligation (NCL) emerged as a powerful approach to overcome this limitation. It allows the coupling of two unprotected peptides in aqueous buffer.\(^3\)\(^8\) The strategy requires a C-terminal thioester and an N-terminal cysteine, which undergo a chemoselective transthioesterification followed by a rapid intramolecular S→N acyl shift to form the native amide bond. The mechanism of NCL is depicted in Scheme 5.

![Scheme 5: Native Chemical Ligation](image)

3.4 Native chemical ligation without Cysteine: Ligation-desulfurization chemistry

Since the first introduction of native chemical ligation as strategy for the synthesis of proteins more than 20 years ago\(^3\)\(^8\) this strategy continues to be fundamental for chemical synthesis and modification of proteins. Conventionally, NCL relies on the presence of an appropriately placed cysteine residue, which has a relatively low abundance of 1.1% in naturally occurring proteins. To overcome this limitation strategies have been introduced.\(^3\)\(^9\) The main focus thereby lies on the development of new N-terminal cysteine surrogates and of new C-terminal acyl donors.\(^3\)\(^9\)\(^-\)\(^4\)\(^0\) Cysteine surrogates use a similar reaction pathway to native chemical ligation, but they can either be removed or manipulated to generate other proteinogenic amino acids at ligation site.

One of the most extended methods is the ligation-desulfurization strategy. This concept was first demonstrated by Yan and Dawson and involves the reductive desulfurization of cysteine.
after a ligation reaction to give a native alanine residue. The concept of employing unnatural amino acid derivatives bearing a suitably positioned auxiliary represents a crucial extension to the native chemical ligation methodology. After a ligation using thiol amino acid building blocks, the thiol moiety is removed to give the desired amino acid residue at the ligation position (Scheme 6).

A great number of thiol-derived amino acids for the ligation-desulfurization strategy have been reported including: phenylalanine, valine, lysine, threonine, leucine, proline, glutamine, arginine, aspartic acid, glutamic acid and tryptophan. Ligation-desulfurization chemistry using these β- or γ-thiol building blocks has been successfully employed in the synthesis of a number of complex peptide and protein targets. However, the challenging synthesis of suitably protected thiol amino acid building blocks is currently hindering the widespread adoption of this strategy. Additionally, a shortcoming of this strategy is that in most cases the desulfurization chemistry is not chemoselective in the presence of other thiol moieties. Thus, it is required to protect native cysteine residues to avoid conversion to alanine upon treatment with reductive or radical desulfurization conditions. To overcome this drawback, chemoselective ligation-deselenization protocols have been described. The observed chemoselectivity in the deselenization of seleno-cysteine in the presence of cysteine inspired the synthesis of unnatural, selenol-derived amino acid derivatives. However, to date only γ-selenoprolina and β-selenophenylalanine have been successfully employed in chemoselective ligation-deselenization reactions in the presence of unprotected thiols. Furthermore, a chemoselective ligation-desulfurization procedure at β-thiol aspartic acid in the presence unprotected cysteine residues is known. The C-S bond in β-mercapto aspartic acid is significantly weaker than the C-S bond of cysteine, enabling a selective desulfurization of β-mercapto aspartic acid in the presence of cysteine.
3.5 Methods for the synthesis of peptide thioesters

It is necessary to install a C-terminal thioester for native chemical ligation and for ligation-desulfurization strategies. However, the modification of the C-terminal acid function to form a thioester for the NCL is one of the biggest challenges. Therefore, particular effort is and has been made to generate strategies that allow easy access to these products.³⁸, ⁴⁸

Dawson Nbz as thioester precursor

A strategy to form peptide thioesters in situ after the cleavage of the peptide from the resin as an N-acyl-benzimidazolinone (Nbz) was described by Dawson and coworkers (Scheme 7).⁴⁹

Starting from a 3,4-diamino-benzamide (Dbz) resin, the peptide is assembled using standard Fmoc-SPPS. Notably, after acylation of one of the amino groups on the linker, the remaining free amino group in the aromatic position 3 or 4 becomes hindered or deactivated, making further protection unnecessary. After completion of the peptide, the C-terminus is activated through acylation of the remaining amino group with p-nitrophenylchloroformate. Adding a base for activation an intramolecular attack of the anilide to form the resin-bound benzimidazolidine is promoted. The Nbz-peptide is cleaved from the resin with TFA and scavenger. After thioester formation by addition of a thiol in buffer at pH 7, the thioester can either be isolated or directly reacted with a cysteine containing peptide.

Synthesis of peptides thioester from fully protected peptide fragments

A direct formation of peptide thioesters uses fully protected peptides to convert the C-terminal carboxylic acid into a thioester (Scheme 8).⁵⁰ The peptide is synthesized on a very acid labile trityl resin, and released from the solid support by hydrolysis under mild acidic conditions like 20% HFIP in DCM or 1% TFA in DCM. Thereby, all side chain protecting groups remain
intact. After thioester formation using PyBOP, DIPEA and a thiol, the protecting groups are removed with TFA and the unprotected peptide thioester can be used for NCL.

**Scheme 8:** Synthesis of peptides thioester from fully protected peptide fragments

However, problems with this strategy arise when the C-terminal acid is sterically hindered or the peptide shows a low solubility and it is not possible to form a thioester. To avoid racemization of the C-terminal amino acid during activation low temperatures are used, which might lead to incomplete conversion during thioester formation. Additionally, amino acids with very acid labile protected side chain functions like histidine can lose the protecting group, resulting in side reactions during the thioester formation. Unfortunately, separation of the desired fully protected peptides and partially deprotected peptides is not possible due to their almost identical properties and size.

**Peptide hydrazides as thioester precursor**

A different strategy to generate a peptide thioester precursor directly from the solid support is the synthesis of peptide hydrazides. To prepare the required hydrazide carboxylate the hydroxyl group of a Wang resin is activated with p-nitrophenyl chloroformate and N-methyl morpholine and subsequently reacted with hydrazinium hydroxide (Scheme 9).

**Scheme 9:** Synthesis of Wang hydrazide resin

After complete elongation, the peptide is cleaved from the solid support with TFA. The unprotected peptide is further reacted to the desired thioester using an oxidation with NaNO₂ and followed by a reaction with a thiol. Mechanistic studies showed that during the oxidation step the peptide hydrazide is converted into an azide using NaNO₂ at pH 3, which is directly
reacted with a thiol (MPAA or MMBA) to form a thioester at pH 7. The peptide thioester can be isolated and used for native chemical ligation (Scheme 10).

An advantage of using peptide hydrazides as thioester precursors is the applicability in sequential native chemical ligation. Previously, sequential NCL of multiple peptide fragments was performed in C to N direction, while the N-terminal cysteine group was protected. A new approach, conducting sequential NCL in N to C direction, using peptide hydrazides was introduced by Liu et al. in 2011. The key to achieve a sequential NCL with peptide hydrazides is to oxidize one peptide at pH 3, add a thiol at pH 7 to convert it into a thioester and then add the second peptide hydrazide. With this strategy orthogonal protection of cysteine and additional deprotection/purification steps are unnecessary. Scheme 11 below shows sequential NCL in N-to-C direction using peptide hydrazides.

**Peptide sulfonamides as thioester precursor**

The sulfonamide method is another used approach to generate peptide thioesters by Fmoc-SPPS. The method involves displacement of the peptide fragment with a thiol from an activated peptidyl-sulfamylbutyryl resin. Unsuitably, the method has a number of drawbacks, involving incomplete acylation of the resin bound sulfonamide, incomplete activation of the sulfonamide, incomplete thiolysis, due to inherent low reactivity of the activated sulfonamide towards thiols, and poor solvation of the resin bound protected peptide.
This method was improved by Burlina et al.,\textsuperscript{52} who described a double linker strategy, involving anchoring of the sulfonamide linker to a standard acid labile resin (Scheme 12). Using the new approach the \(N\)-peptidylsulfonamide can be released from the resin by TFA. Thus, monitoring of the synthesis progress by the extent of methylation is possible. The unprotected \(N\)-peptidyl-\(N\)-methyl-sulfonamide that is released from the resin by TFA treatment can be used directly for NCL reactions without prior conversion to a thioester, eliminating poor yields associated with on-resin thiolysis.

### 3.6 Solid-Phase Fragment Condensation (SPFC)

In addition to native chemical ligation a different strategy, the solid-phase fragment condensation is an efficient alternative to overcome shortcomings of stepwise SPPS (Scheme 13). In SPFC the advantages of both, solution synthesis, such as the possibility of purification of intermediate peptides, and solid-phase synthesis, \textit{e.g.} rapid reaction, high yield and easy removal of excess reagent, are combined.\textsuperscript{36,53}

In SPFC the target peptide is divided in fragments, where the \(C\)-terminal fragment is attached to the resin and the \(N\)-terminal fragment is a fully protected peptide in solution. Both fragments are combined using standard SPPS activation protocols and cleaved from the solid support to give the desired peptide. The \(C\)-terminal fragment is synthesized using Fmoc-SPPS on a resin bearing an acid labile linker.\textsuperscript{36}
The N-terminal fragment can be approached in two routes by synthesis on solid support. Either a very acid labile linker to cleave the fully protected peptide is used or acid stable side-chain protection like Bzl where global deprotection is achieved with HF-cleavage can be employed.

In SPFC the proper choice of fragments is one of main consideration. Facile synthesis of the fragments and a rapidly proceeding condensation reaction, with high yield, under mild conditions and most importantly without racemization are crucial requirements. On that account it is necessary to choose a fragment with non-racemizing C-terminal amino acid like proline or glycine. Steric hindrance and poor solubility hampering the coupling reaction demand proper length of individual fragments. Usually fragments of the length from 5 to 10 amino acids are chosen and side reactions caused by unstable side chain protecting groups are best avoided by employing few amino acids with functionalized side-chains. In summary SPFC is a powerful tool to gain access to peptides that are difficult to generate with standard SPPS, given the desired peptide sequence can be divided in suitable fragments.

3.7 Orthogonal protecting groups in SPPS

To achieve clean and selective formation of new bonds during a reaction appropriate protecting groups of functional groups are mandatory. Decent protecting groups have different requirements that need to be taken into account when selected for a reaction. Installation selectivity and feasibility, stability to a broad range of reaction conditions and smooth removal are properties for a good protecting group. The concept of orthogonality is fundamentally important in the field of chemical synthesis. It describes two or more protecting groups that belong to independent classes and can be removed by distinct mechanisms and conditions. Thus, protecting groups can be removed in any order and in the presence of each other since selective deprotection is administered by alternative cleavage mechanisms, usually under milder conditions.

The development of new protecting groups has been deeply tied to peptide synthesis, since protection is mandatory for the assembly of these polyfunctional molecules. Masking side chains of different amino acids with orthogonal protecting groups is necessary for the manipulation of the functionality before cleavage and final deprotection. It is also required, if a certain side chain functionality is involved in the manipulation after the final cleavage. For Fmoc-SPPS orthogonal protecting groups need to be stable to the basic conditions (20% Piperidine in DMF) repeatedly used for Fmoc removal and acidic conditions (90-95% TFA and scavenger) used for final cleavage and deprotection. Additionally, the removal of
orthogonal protecting groups before the final cleavage has to proceed with conditions that leave standard amino acid protecting groups intact.

**Orthogonal protection of cysteine**

To use peptidethioester fragments in sequential native chemical ligation it is necessary to protect the N-terminal cysteine of the thioester to avoid self-aggregation or cyclization caused by an intramolecular reaction with the thioester moiety. Installing the N-terminal cysteine as Thiazolidine (Thz) (Figure 15) has been widely used for this purpose. The required Boc-L-Thioprine building block is commercially available and can be easily installed using standard conditions for SPPS. After complete ligation, the Thz-peptide is converted into a Cys-peptide using 0.2 M methoxyamine hydrochloride at lower pH. Noteworthy, this strategy has been used for one-pot synthesis of peptides, thereby avoiding difficult and time consuming purification steps of intermediates.

![Figure 15: L-Boc-Thioprine](image)

Other commonly used orthogonal protecting groups for cysteine are Acetamidomethyl (Acm) and Phenylacetamidomethyl (PhAcm) (Figure 16). The required amino acid building blocks are commercially available and they can be coupled to the peptide chain using standard SPPS conditions.

![Figure 16: Acetamidomethyl (Acm) and Phenylacetamidomethyl (PhAcm) protected cysteine](image)

Both protecting groups (Acm and PhAcm) can be removed with mercury salts (Hg[II]) to give the free thiol and with iodine (I2) to give the disulfide peptide. Additionally, PhAcm can be removed enzymatically with penicillin aminohydrolase, giving the possibility to remove it in the presence of Acm. Recently, it was shown by Brik et al. that Acm can efficiently be removed in a Palladium-assisted one-pot deprotection. The strategy uses water soluble palladium salts in a buffer system or in water and releases the free thiol after treatment with dithiothreitol (DTT).

**Orthogonal protection of aspartic acid**

Synthesizing glycopeptides using the conditions proposed by Lansbury et al. it is necessary to orthogonally protect the aspartic acid residue to be modified with the N-glycan. Frequently,
the side chain acid function is protected with allyl (All) (Figure 17). The protecting group can be removed under palladium catalyzed reactions and is stable towards acidic and basic conditions. With all other side chain protecting groups intact, the released acid can now be coupled to an amino glycan to give the desired N-glycopeptide.

Figure 17: Allyl protected aspartic acid

3.8 Side reactions in SPPS

An often neglected topic when introducing solid phase peptide synthesis are side-reactions. Those certainly occur and common side-reactions are well documented. Generally, they can be avoided by appropriate selection of protecting groups and resin linker. However, some unwanted reactions are more difficult to avoid and require special mentioning.

Aspartimide formation

A side-reaction most likely to be encountered in routine synthesis is aspartimide formation. The probability of this reaction is highly sequence dependent, but occurs most frequently with peptides containing the Asp(OrBu)-Xaa motif, where Xaa=Asn(Trt), Gly, Ser, Thr. The reaction can be catalyzed under either acidic or basic conditions. Thus, the process is observed during final deprotection and chain elongation in Fmoc-SPPS. The reaction involves an attack of the nitrogen attached to the α-carboxyl group of aspartic acid or asparagine on the side-chain ester or amide group respectively. Aspartimide formation is also a widely encountered side-reaction during Lansbury coupling for the synthesis of glycopeptides. The formation of the resulting five-membered imide is accompanied by epimerization. Moreover, the newly formed aspartimide can suffer further reactions, such as hydrolysis to give the corresponding α- and β-aspartidyl peptides, or the ring-opening that is observed by applying piperidine during Fmoc-removal, forming α- and β-piperidides (Scheme 14).

Scheme 14: Aspartimide formation during SPPS or Lansbury Coupling

Addition of 0.1M HOBt to the deprotection mixture can help to prevent this unwanted side reaction to some extent. However, complete protection against aspartimide formation is
only possible by temporary protection of the nitrogen of the amino acid preceding the Asp or Asn residue. This can be achieved by incorporating 2-hydroxy-4-methoxybenzyl (N-Hmb) protected amino acid before the problematic aspartic acid or asparagine residue.\textsuperscript{72-73} Yet, for residues other than glycine this approach is tedious and causes racemization when coupling backbone protected dipeptides.\textsuperscript{69, 74} Recently, a convenient approach to overcome aspartimide formation during synthesis of \(N\)-linked glycopeptides via solid-phase aspartylation was introduced by Unverzagt and coworkers.\textsuperscript{69} The method takes advantage of the glycosylation consensus-sequence (Asn-\(\chi\)aa-Ser/Thr; \(\chi\)aa≠Pro) by installing a pseudoproline (Ψpro) dipeptide corresponding to the Xaa-Ser/Thr units. This residue efficiently suppresses the formation of aspartimides by constraining this peptide part (Figure 18).

![Figure 18: Cis-conformation of pseudoproline containing peptides](image)

The \textit{trans} conformation of Xaa-Ser/Thr is converted into a stable \textit{cis} conformation when pseudoproline dipeptides are incorporated into the sequence during SPPS. Thereby aspartimide formation is drastically suppressed.\textsuperscript{69, 75}

**Oxidation of Methionine**

A major problem associated with peptide synthesis is the oxidative degradation of oxidation sensitive amino acids. Together with cysteine, methionine is one of the easily oxidized amino acids.\textsuperscript{76} The thioether side chain function of methionine has a low oxidation potential and is particularly sensitive to oxidation. It can form sulfoxides [Met(O)] and sulfones [Met(O\(_2\))] making it more hydrophilic than the native form, which is considered interchangeable with other hydrophobic amino acids like leucine or valine. The process of oxidation involves a two electron transfer mechanism (Scheme 15).

![Scheme 15: Oxidation of methionine](image)

The natural \(L\)-methionine can produce two possible diastereoisomers in its oxidized form, the (\(R,S\))-Met(O) and the (\(S,S\))-Met(O). Thus, after each oxidation two different products can be observed. The oxidation is strongly dependent on the protein substrate, shown by the fact that in methionine rich proteins only a certain number of the residues can be oxidized.\textsuperscript{77}
Unfortunately, only the number of oxidized methionine can be determined using mass spectrometry but the exact position usually remains unclear.\textsuperscript{78} The amino acid sequence surrounding the methionine and/or conformational changes possibly play a role in the oxidation process.\textsuperscript{76} However, a consensus sequence for methionine oxidation has yet to be identified. Reduction of Met(O) \textit{in vivo} is performed by two enzymes, \textit{i.e.} Met(O) reductases, MsrA and MsrB, which are respectively responsible for the reduction of (S,S)-Met(O) and (R,S)-Met(O). Unfortunately, for \textit{in vitro} use these enzymes are not beneficial, since they are extremely expensive and require specific experimental conditions. Thus, halides are commonly used as reducing agents.\textsuperscript{79-81}

This protocol uses much more cost efficient reagents, but it lacks enzyme stereoselectivity. However, both strategies are time consuming and require troublesome peptide purification with consequent decrease in peptide yield. The reduction of Met(O) can be performed during or after the cleavage. In the latter case, the sequence and other functionalities have to be taken into account when choosing the conditions. Often observed side reactions during Met(O) reduction are the formation of disulfide bridges of unprotected cysteine residues and the dimerization of tryptophan. Moreover, the hydrolysis of peptide thioesters is a common side reaction, using iodine salts for the reduction due to I\textsubscript{2} generated during sulfoxide reduction. To circumvent this reaction, addition of dimethylsulfide (DMS) is necessary (Scheme 16).\textsuperscript{79, 81}

\textbf{Aggregation during SPPS}

Since the introduction of solid phase peptide synthesis an enormous number of synthetic peptides have been produced. Many efforts were made to improve the methodology by enhancing the number of used protecting groups, support systems, activation methods, and the automation of protocols. However, limitations still exist and peptide synthesis is encumbered by different problems. Despite extensive work to optimize SPPS conditions, some peptides are resistant to efficient assembly in good yield. Poor solvation of the growing peptide chain
and limited solubility of fully protected peptide fragments often lead to incomplete coupling reactions.\textsuperscript{37, 82-83} Intermolecular hydrophobic aggregation of the protected peptide chains and/or formation of secondary structures such as $\beta$-sheets are usually the reason for these undesired physicochemical problems.\textsuperscript{84-85} It is suggested that aggregation may be caused by either one of three possible scenarios (Figure 19). While the optimal case of fully solvated system is depicted in scenario A, scenario B shows a self-association through intramolecular hydrogen bonds. Thus, interaction between the medium and the amino termini is minimized, and the accessibility of reagent is limited. Another possibility is the poor solvation of the polymer backbone, as displayed in scenario C, hindering reagent access to the peptide chain. The third possibility, scenario D, comprises interchain association, which produces a cross-linked matrix, reducing mobility and accessibility in comparison to scenario A.

![Figure 19](image)

\textbf{Figure 19}: Scenarios depicting the possible solvation or aggregation of a polymer bound peptide sequence.

Regardless of whether aggregation is due to inter- or intra-molecular associations, steric hindrance of the $N$-terminus is increased, which consequently leads to complications with Fmoc-removal and subsequent acylation. Different possibilities have been introduced to overcome these problems, among them the use of chaotropic salts, different solvent compositions, or increased temperature are commonly used.\textsuperscript{86-87} The introduction of an amide protecting group within the peptide chain has shown to prevent associations involving hydrogen-bonding.\textsuperscript{88} The earlier introduced pseudoprolines ($\Psi$-prolines) have a pronounced effect upon the peptide backbone. Pseudoprolines are serine and threonine derived oxazolidine and cysteine derived thiazolidine derivates, which show a great structural similarity with proline itself, as shown in Scheme 17.
The Ψ-Pro dipeptide structure introduces a conformational change to the peptide backbone and prevents peptide aggregation. The Ψ-proline containing building blocks are commercially available and can be coupled without racemization according to standard procedures. Furthermore, oxazolidine- or thiazolidine-containing dipeptides have a much greater polar character than conventionally protected serine, threonine, or cysteine derivate. Thus, pseudoprolines act as a polar protecting technique contributing to higher solvation of the growing peptide. Additionally, Ψ-prolines have the potential to simplify the purification of peptide fragments and to increase the segment coupling kinetics. Pseudoprolines have enabled the synthesis of otherwise inaccessible polypeptides by solubilizing hydrophobic protected segments and preventing self-association of peptide fragments in convergent strategies or in chemoselective approaches. Moreover, to avoid aggregation, the choice of solid support during SPPS is crucial. Traditionally, solid phase synthesis has relied on polystyrene-based resins for the synthesis of all kinds of peptides. Due to their hydrophobicity these resins have certain limitations. For the synthesis of complex peptides poly(ethylene glycol) (PEG)-based resins are often found to give superior results. The PEG-based ChemMatrix® resins have better swelling properties than polystyrene resins, allowing a more efficient stepwise solid-phase synthesis.

### 3.9 Synthesis of Glycopeptides

For the synthesis of glycoproteins using NCL and EPL, one crucial step is the attachment of the glycan to the glycopeptide. In general, three different methods appear to be the most useful and have been frequently used. The first method (A), depicted in Scheme 18, introduces the oligosaccharide during SPPS using a glycosylamino acid cassette. However, after incorporation of larger glycans, further elongation of the peptide is complicated, since side reactions, involving free hydroxyl groups, can occur. Moreover, solubility and reactivity of the peptide are affected in a negative manner.
Sequential elongation of glycopeptides is more demanding compared to peptides without modifications, especially when longer or difficult sequences are targeted. Therefore, installation of glycans in the last step of the synthesis is often employed as a better alternative. By this method, peptides obtained by automatic peptide synthesis are directly modified on the solid support. The required glycosyl amine is coupled to an aspartate of an otherwise fully protected peptide using the so-called Lansbury aspartylation (B, Scheme 18). Thus, an orthogonal protecting group for aspartic acid is needed. The main drawback of this approach, is the high probability of aspartimide formation throughout the coupling. However, this side reaction can be efficiently reduced by use of pseudoprolines at the Ser/Thr residues of the consensus sequence Asp-Xaa-Ser/Thr (Xaa≠Pro).

The third strategy (C, Scheme 18) to synthesize glycopeptides bearing large glycans relies on an enzymatic method. Endo-β-N-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) is used to transfer the oligosaccharide of high-mannose type and complex-type N-glycan oxazoline substrates en bloc onto various acceptors having an N-acetylgalcosamine residue.

Thus, glycopeptides with only a small GlcNAc residue have to be synthesized, thereby avoiding the complications and side reactions of strategy A.

### 3.10 Complex N-Glycan-Asn and N-Glycans from Egg Yolk

For the chemical synthesis of glycopeptides, the availability of N-glycan-Asn derivatives and N-glycans has to be addressed. Recently, egg yolk was described as a source for a sialoglycopeptide (SGP). This disialylated undecasaccharide can be degraded to valuable Fmoc-Asn building blocks, to a glycan amine and to a glycan oxazoline.
The generation of \(N\)-glycan-Asn building blocks for a stepwise synthesis of glycopeptides using egg yolk extracted material starts with pronase digestion of the isolated SGP. Afterwards, the free amine of the released asparagine is protected with Fmoc and the sialic acid residues are hydrolyzed under acidic conditions using diluted hydrochloric acid (A, Scheme 19).\(^{91, 94-95}\) For the synthesis of complex glycans-amines useful for Lansbury coupling the sugar moiety is cleaved from SGP with PNGase F. Afterwards, sialic acid is cleaved with neuramidase and the anomeric hydroxyl group is converted into an azide using 2-chloro-1,3-dimethylimidazolinium chloride (DMC) and sodium azide under basic conditions in water. The azide is reduced to enable coupling to the acid function of aspartic acid (B, Scheme 19).\(^{69, 91}\) Glycan oxazolidines for the enzymatic production of glycopeptides are synthesized by cleavage of the glycan from SGP with Endo-M and treatment with DMC in water under basic conditions (C, Scheme 19).\(^{91-92, 96}\)

### 3.11 Synthesis of GPI anchored Peptides, Glycopeptides and Proteins

It is essential to have access to structurally well-defined GPI-anchored proteins and glycoproteins to study the role of the GPI. Due to microheterogeneity and laborious purification protocols, isolation of structurally defined samples is difficult to achieve from natural probes. Therefore, different synthetic and semi-synthetic strategies have been developed to generate GPI-anchored proteins. Reported methods include regioselective chemical condensation, native chemical ligation (NCL) and enzymatic ligation strategies.\(^{97-98}\)
Chemical total synthesis of GPI-anchored peptides and glycopeptides

One strategy to generate homogeneous GPI-anchored peptides and glycopeptide is based on a conventional, regioselective condensation between a properly protected GPI-anchor and a fully protected peptide or glycopeptide depicted in Scheme 20.

The strategy was shown first in the synthesis of GPI-anchored peptide 2, a partial structure of the human sperm CD52 antigen by Guo and coworkers.99 A GPI, protected with carboxybenzyl and benzyl groups and a free amino group and a dipeptide having a free C-terminal acid were coupled using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDC) and N-hydroxybenzotriazole (HOBt). Under these conditions the desired product was obtained without racemization. The final deprotection was completed in two steps; using dimethylamine to remove Fmoc and the carboxybenzyl and benzyl groups were removed in a Pd-catalyzed hydrogenolysis.100

This strategy was later used by the same group to successfully synthesize a skeleton structure of sperm antigen CD52,100 showing the first chemical synthesis of a natively linked GPI-anchored glycopeptide. The required fully protected glycopeptide was assembled on an acid-sensitive 2-chlorotrityl resin using Fmoc solid phase peptide synthesis, which was released from the solid support with 10% acetic acid in DCM. The coupling to the protected GPI was performed with EDC and HOBt as condensation reagents. With the desired product in hand the protecting groups were removed in two steps. A Pd-catalyzed hydrogenolysis was used to remove the carboxybenzyl and benzyl groups on the GPI and the amino acid side chains were deprotected with 15% trifluoroacetic acid (TFA) (Scheme 21).
**Synthesis GPI-anchored peptides and proteins via NCL**

A powerful tool for the synthesis of large GPI-anchored proteins is NCL, a method that is widely used for the chemical synthesis of proteins. In this method, a peptide- or protein thioester can be ligated with any molecule carrying an N-terminal cysteine residue under mild conditions.

This strategy to obtain GPI-anchored proteins was first explored by Nakahara and coworkers by ligating a peptide thioester to a GPI-analog. In a similar strategy, the Bertozzi group used expressed protein ligation (EPL) to couple a full-size green fluorescent protein (GFP) to cysteine-containing GPI analog. NCL was also used for the synthesis of a recombinant prion protein (rPrP) attached to a GPI anchor by the group of Seeberger (Scheme 22). In this semisynthetic approach, the GPI anchor was equipped with a cysteine and the rPrP thioester, prepared by an intein system. Both molecules were connected by native chemical ligation.
reaction. Following this approach, the first protein anchored to a complete GPI was synthesized.

Scheme 22: Synthesis of a GPI-anchored prion protein

Synthesis of GPI-anchored peptides, glycopeptides and proteins via enzymatic ligation

A strategy that uses sortase A (SrtA), a bacterial transpeptidase derived from *Staphylococcus aureus*, was developed by Guo and coworkers to ligate peptides, glycopeptides and proteins with GPIs. SrtA recognizes a protein C-terminal signal, a pentapeptide LysProXaaThrGly (Xaa is variable) known as sorting signal. SrtA reacts with this sorting signal peptide to generate a thioester at carboxyl group of Thr, and links the protein C-terminus to the N-terminus of a cell wall peptidoglycan. Testing the applicability of this strategy it was observed that when a short peptide and a simple GPI analog were reacted the reaction only hydrolyzed the peptide. However, when the GPI analog was modified with one or two glycine residues attached to the phosphoethanol unit, the reaction proceeded rapidly and gave the desired conjugates. After establishing the reaction conditions and proving the concept, SrtA was used to synthesize several analogs of the GPI-anchored human antigens CD52 and CD24. Additionally, this enzyme-catalyzed ligation was used to generate a GPI-anchored glycopeptide with a partial sequence of MUC1 and a GPI-anchored GFP.

Scheme 23: Synthesis of GPI-anchored peptides, glycopeptide and proteins using SrtA
4. PRION PROTEIN

Prions have drawn immense research interest for many years accounted to their unique properties, composition and for being the causing agent of transmissible spongiform encephalitis (TSEs) like bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD). Prions have no significant nucleic acid (genes) and TSEs are explained by the widely accepted “prion-only” hypothesis. According to the hypothesis, an abnormal form of one of the body’s own proteins cellular prion protein (PrPC) is transformed to an alternative form, PrPSc, the so called scrapie form of the protein. It is suggested that PrPSc operates as a template which forwards the conversion of PrPC to the scrapie form. The two isoforms differ in their conformation and aggregation, whereby the new formed PrPSc is the infectious aggregate causing neurodegenerative diseases. The cellular prion protein is a GPI anchored membrane protein and contains two N-linked glycosylation sites, which are variable in their carbohydrate structure.

4.1 Prion Diseases

Prion diseases are infectious, fatal neurodegenerative diseases that include BSE and scrapie in animals and CJD in humans. The transmission of BSE to humans to cause CJD represents the urgent need to understand the molecular basis of prion propagation, pathogenesis, and the barriers limiting inter-mammalian transmission. Although prion diseases are rare neurodegenerative disorders, due to the unique biology of the transmissible agent prion, remarkable attention is given to this illness. The classes of prion diseases can be grouped in genetic, infectious, or sporadic disorders, all of which involve modification of the prion protein. The prion concept explains how a single disease can have genetic or infectious etiology. Prions are defined transmissible particles that are devoid of nucleic acid and composed exclusively of a modified prion protein (PrPSc). The term “prion” is derived from proteinaceous and infectious. Prions are composed entirely of a protein that adopts an abnormal conformation and is therefore defined as infectious protein. In a posttranslational process, PrPC is converted into the β-sheet rich PrPSc, thereby introducing profound changes in the physicochemical construct of PrP. Prions encipher their strain-specific in the tertiary structure of PrP.
A great deal of effort has been put into understanding the chemical nature of the infectious agent, testing the validity of the protein-only mechanism. Much less attention has been given to the question of how PrPSc causes the central nervous system (CNS) pathology. Frequently discussed are three hypotheses explaining how changes in PrPC function can cause neurodegeneration.119-120 The most widely discussed hypothesis, the gain-of-function hypothesis, proposes that the prion pathology is attributed to a toxic “gain-of-function” mechanism (1, Scheme 24). It assumes that PrPSc possesses a novel toxic property that is not related to the normal, physiological function of PrPC. An alternative hypothesis, the “loss-of-function” hypothesis (2, Scheme 24), suggests that PrPC holds biological activity that is lost upon conversion to PrPSc or when in contact with PrPSc. For instance, the loss of anti-apoptotic activity of PrPC would lead directly to neuronal death. The “subversion-of-function” hypothesis (3, Scheme 24) proposes that the interaction with PrPSc converts PrPC from a transducer of neuroprotective signals into a transducer of neurotoxic signals.

Genetic and biochemical evidence shows that the conversion of PrPC to PrPSc develops through the formation of a PrPC/PrPSc complex. Two models explain the conformational conversion of PrPC to PrPSc, taking the “protein only” hypothesis into account (Scheme 25).1 The first model (1, Scheme 25) is the “template-directed refolding” hypothesis, predicting an interaction between exogenously introduced PrPSc and endogenous PrPC, which is induced to transform itself into further PrPSc. Spontaneous conversion from PrPC into PrPSc may be prevented by a high-energy barrier.1 The second model (2, Scheme 25), the so called “seeded nucleation” hypothesis, proposes that PrPC and PrPSc are in reversible thermodynamic equilibrium, where only a minimum amount of PrPSc exists. The monomeric are mounted into a highly ordered seed and further monomeric PrPSc can be recruited and eventually aggregate to amyloid. Due to fragmentation the number of nuclei would increase to recruit further PrPSc, resulting in apparent replication of the agent.121
Data suggest that PrP\textsuperscript{SC} might not be directly responsible for neurodegeneration. The development of an effective therapy for these fully penetrant disorders is needed. Interfering with the conversion of PrP\textsuperscript{C} into PrP\textsuperscript{SC} seems to be the most attractive therapeutic target\textsuperscript{122}. Understanding how PrP\textsuperscript{C} unfolds and refolds into PrP\textsuperscript{SC} is also of great importance to study other degenerative illnesses. Knowledge of PrP\textsuperscript{SC} formation may also help developing effective therapies for the more common neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease\textsuperscript{118}.

### 4.2 Structure of the Prion Protein

The normal cellular prion protein (PrP\textsuperscript{C}) is a glycoprotein containing 209 AA (23-231) with \( \alpha \)-helices as the predominant feature of the secondary structure\textsuperscript{117}. One disulfide bond is formed between Cys179 and Cys214. The two \( N \)-linked glycosylation sites are at Asn181 and Asn197, whereby the Asn181 glycan is attached to an \( \alpha \)-helix and the Asn197 glycan located on a loop\textsuperscript{123}. The large size of the oligosaccharides, their dynamic properties and the flexible linkage to asparagine result in shielding of a large region of the surface of PrP. Both glycans cover orthogonal faces of the protein and sterically hinder either intermolecular protein-protein interaction or intramolecular interactions involving residues 1-90. Major questions relate to the relevance of the glycoform distribution, as defined by glycan site occupancy, to strain type and disease transmission. Glycan analysis has shown that prion protein contains at least 52 different carbohydrate structures, which consist of a subset of brain sugars, and that glycan processing is site specific. PrP\textsuperscript{Sc} from the brains of syrian hamsters contains the same set of glycans as PrP\textsuperscript{C}, but higher proportion of tri-and tetra-antennary sugars\textsuperscript{124}.
Prion protein is usually, but not perpetually, attached to the neuronal cell membrane via a GPI anchor at Ser231. The GPI anchor, which is modified with sialic acid, may allow the prion protein to be mobile in the lipid bilayer. It is indicated by models that the polar C-terminus makes very few noncovalent interactions between the protein and the GPI. This provides the protein with considerable dynamic freedom relative to the membrane. On the composition of the acyl chain only limited data exists. It contains stearic acid, a saturated C18:0 fatty acid, which would be expected to promote lipid order in the membrane. All proteins with a GPI signal sequence expressed in the same cell initially receive the same anchor. However, GPI anchors are frequently modified during transition to the cell surface. A relatively unusual modification is the addition of sialic acid but no role for this feature has been proposed yet.

**Figure 20**: Proposed structure of PrP<sup>C</sup>; a) The GPI anchor is shown in two orientations b) two orthogonal views showing the areas of the protein surface covered by the glycans at Asn181 (orange) and Asn197 (yellow)
AIM OF THE THESIS

The aim of this work was to develop methods to generate homogeneous N-glycosylated- and GPI-anchored prion protein fragments for the semi-synthesis of the prion protein, which allow the evaluation of these modifications in the function, aggregation, replication and infectious properties of the prion protein. This goal can be achieved by a combination of chemical synthesis, protein expression and ligation methods.

The synthesis of the prion protein, containing 209 amino acids, two glycosylations at Asn181 and Asn197 and a GPI-anchor at Ser231, can be performed by sequential native chemical ligation of protein and peptide fragments. An analysis of the PrP composition suggests that the prion protein can be divided in three fragments. Fragment 3 (23-177) [PrPIII], without glycosylation, can be obtained as a thioester through protein expression using an intein system. It is important to note that with this intein system thioester formation at C-terminal aspartic acids leads to cyclization and has to be avoided. Thus, Asp178 can be installed as unnatural β-mercapto amino acid as N-terminal amino acid on the C-terminal fragment (178-231), making a ligation desulfurization strategy possible. The fragment 178-231 of PrP, containing the carbohydrates can be obtained by chemical synthesis using SPPS. However, it is known that the longer the peptide-chain the more side reactions, incomplete coupling steps or aggregation scenarios can take place.

Thus, this fragment can be divided in fragment 2 (178-213) [PrPII], which consists of 36 amino acids and involves the carbohydrate moieties, and fragment 1 [PrPI], containing 18 amino acids. Cys214 is the ligation position for these two fragments. A fourth fragment is the
GPI-anchor containing a cysteine residue on phosphoethanolamine making it useful for NCL. The obtained fragments can be combined via native chemical ligation and ligation desulfurization strategies as depicted in Scheme 26.

The synthesis of glycopeptides and glycoproteins is a very complex task. The challenge of synthesizing complex oligosaccharides is further complicated by the fact that peptide chemistry is inconsistent with carbohydrate chemistry. For instance, the final deprotection of the oligosaccharide can influence the peptide structure and the strong acids used to remove the glycopeptide from the solid support can affect some glycosidic bonds. To gain access to a homogeneous glycosylated and GPI-anchored protein, different obstacles have to be addressed.

For the assembly of peptides on solid support using Fmoc based chemistry, the modification of the C-terminal to form a thioester for the NCL is one of the biggest challenges. Therefore it is crucial to find the optimal conditions for thioester formation and choose the appropriate strategy; either the Dawson linker, peptide hydrazides or peptide sulfonamides as thioester precursor or the use of fully protected peptides for thioester formation. As a part of this work, a variation of the mentioned techniques will be evaluated and optimized to gain access to the desired peptides.

Additionally, to combine the expressed protein thioester and the chemically synthesized peptide and glycopeptide fragments a ligation-desulfurization strategy has to be followed. The required β-mercapto aspartic acid (8) will be synthesized according to the published protocol. However, to also allow a ligation in C to N direction the thiol requires orthogonal protection. Thus, a new β-mercapto aspartic acid having either Acm (9) or a photolabile 4-(dimethylamino)phenacyl (Map) ester as sulfur protecting group (10) is designed and accordingly generated.

![Figure 21: β-mercapto aspartic acid building blocks for ligation-desulfurization strategy](image)

The attachment of the glycan is a fundamental step. In this work, the glycopeptides will be obtained using the Lansbury aspartylation, which connects the sugar to an aspartate after the complete peptide is assembled. A key requirement for glycopeptide synthesis using Lansbury aspartylation is the orthogonal protection of the aspartic acid side chain to make it available for coupling while the remaining protecting groups are still intact. Since PrP contains two glycosylation sites closely to each other at Asn181 and Asn197, a second
orthogonal protecting group, which is removable in presence of the commonly used allyl protecting group on aspartic acid, is needed. This second orthogonal handle, will allow the installation of one glycosylation at a time and install two different glycans at each glycosylation site. Unfortunately, no such group is established. Thus, the synthesis of an aspartic acid building block having a photolabile Map protection on the side chain (11) will be explored.

Figure 22: Orthogonal protected aspartate building block

A second key requirement for glycopeptide synthesis is the synthesis of an appropriate glycan building block that can be attached to the peptide. Amino glycans are not stable and therefore have to be generated in situ. In contrast, the according glycan azides are stable upon storage and for that reason the required mono-saccharide (12) and nona-saccharide (13) will be synthesized using chemical and chemoenzymatic methods.

Figure 23: Glycan azides for glycopeptide synthesis using Lansbury aspartylation
RESULTS AND DISCUSSION

1. SYNTHESIS OF GLYCAN BUILDING BLOCKS FOR GLYCOPEPTIDE SYNTHESIS

For the synthesis of N-glycopeptides using the convergent strategy, it is necessary to have access to unprotected glycosyl amines that can be coupled to a free acid side chain of aspartic acid of the otherwise fully protected peptide. However, glycosyl amines are unstable upon prolonged storage and therefore are generated in situ from the corresponding glycosyl azides.

1.1 Synthesis of N-acetylglucosamine-azide 12

To synthesize N-glycopeptides, N-acetylglucosamine-azide (β-D-GlcNAc-azide), the simplest glycan-azide, is typically used to establish reactivity, selectivity and reaction conditions. Moreover, it gives N-glycopeptides to investigate the role of smaller glycans and can also be elongated using enzymatic methods to obtain larger N-glycans. Until recently, β-D-GlcNAc-azide was not commercially available for a reasonable price, and therefore, had to be synthesized.

Starting from the hydrochloride of D-glucosamine, N-acetylglucosamine 16 was synthesized in a two stepped strategy. Generating 16 directly from D-glucosamine with sodium methoxide and acetic anhydride only gave the product in low yields. Thus, peracetylated N-acetylglucosamine 15 was synthesized in quantitative yield by treating D-glucosamine hydrochloride 14 with acetic anhydride in pyridine. 15 was O-deacetylated using sodium methoxide in methanol to obtain 16 in 84% yield. Thereafter, 16 was reacted with acetyl chloride in the presence of a catalytic amount of HCl to give the desired peracetylated N-acetylglucosamine-chloride 17 in 45% yield. A substitution of the chloride with an azide was completed by treating 17 with sodium azide in DMF and gave 18 in 93% yield.

Additionally, a second strategy with only two reaction steps was evaluated to overcome shortcomings, e.g. difficult purification and incomplete conversion, of the first strategy.
Scheme 28: Synthesis of 18: a) pyridine, Ac₂O, quant., b) TMS-N₃, Sn(IV)Cl₄, EtOAc, 0°C, 68%

The second approach (Scheme 28) started from peracetylated N-acetylglucosamine 15, which was reacted with TMS-azide in the presence of Tin(IV)chloride in anhydrous ethyl acetate to give the desired product 18 in 62% yield. Noteworthy, the use of anhydrous ethyl acetate instead of anhydrous DCM gave significantly higher yields due to increased solubility of the reaction mixture.

Scheme 29: Synthesis of 20: a) NaOMe, MeOH, 4 h, 58%, b) Pd/C, H₂↑, MeOH 2 h

In the last step (Scheme 29) azide 18 was O-deacetylated with sodium methoxide in methanol to yield 12 in 68%. 12 can be stored and was reduced when required to generate the corresponding amine by treatment with hydrogen in the presence of palladium catalyst. Amino glycan 19 was directly used for the synthesis of several N-glycopeptides.

1.2 Isolation of a sialylglycopeptide from egg yolk powder

The chemical synthesis of carbohydrates has made significant process in recent years and can be performed using diverse methods including an automated fashion. However, the synthesis of complex glycans needed for glycopeptides is still a time-consuming and tedious process. Fortunately, to overcome these limitations, glycopeptides containing defined complex glycans can be extracted from egg yolks. Using this method, complex bianntenary N-glycans and asparagine building blocks containing complex bianntenary N-glycans can be obtained.

Initial reports described the isolation of a sialylglycopeptide from fresh eggs by separating insoluble proteins and lipids by precipitation with phenol. Here, however, a method well-known from glycomic analysis in large scale using egg yolk powder was used. The isolation started with suspension of 300 g dry egg yolk powder (corresponding to ~38 egg yolks) in 900 ml water and 450 ml methanol. The mixture was divided in 40 ml fractions and centrifuged for 10 min at 3500 rpm (thermo scientific Heraeus Multifuge X3R centrifuge with a Thermo TX-100 rotor). To each fraction 10 ml chloroform were added and the mixture was centrifuged again 10 min at 3500 rpm (thermo scientific Heraeus Multifuge X3R centrifuge
with a Thermo TX-100 rotor). The phase containing the water soluble compounds with the large oligosaccharides was concentrated.

![Figure 24: Chromatographic profile of the elution of sialylglycopeptide 20 on a Sephadex G50 column.](image)

The resulting slurry was suspended in 100 mM ammonium acetate and chromatographed on a Sephadex G50 column (Figure 24). All fractions were analyzed using MALDI-TOF. The fractions eluting between 240 ml and 320 ml contained the desired sialylglycopeptide (SGP) 20 and were combined and lyophilized. After size exclusion chromatography, 400 mg crude 20 was obtained. This quantity corresponds to a yield of approximately 8.4 mg crude glycopeptide/egg yolk.

### 1.3 Synthesis of the biantennary nonasaccharide azide 13

To attach complex glycans to a peptide using Lansbury coupling, it is necessary to either protect the free acid group of sialic acid or to remove the sialic acid residues to avoid side reactions. In this work, the sialic acid residues were removed. Having 20 in hand, the synthesis of nonasaccharide azide 13 was started by acidic hydrolysis of 20 using an aqueous 10 mM TFA solution,\(^\text{91, 95}\) to which TFA was added until a final pH of 3 was reached. The mixture was incubated at 80°C until MALDI-TOF analysis indicated completion of cleavage of the sialic acid residues (Scheme 30). After 18 hours both sialic residues were cleaved and the nonasaccharide containing hexapeptide 21 was purified on a superdex 30 size exclusion column (Figure 25).
Unfortunately, even though MALDI-TOF analysis indicated full hydrolysis, a small amount of complex glycopeptides with one sialic acid were observed. However, both glycopeptides could be separated to give 21 in a yield of 38%.

Having the desired and pure 21, the compound was treated with PNGaseF in ammonium acetate buffer for 16 hours to release nona-saccharide 22 (Scheme 31). The glycan was separated from the peptide using a SiliaSep™ C18 cartridge by eluting the polar product which does not bind to the column with water. The remaining peptides bind to the C18 material. The pure product 22 was observed in 44% yield. In the next step, 22 was converted into nona-azide 13 by reacting it with 2-chloro-1,3-dimethylimidazolinium chloride (DMC) and sodium azide in the presence of lutidine at 2°C for one week. Nona-azide 13 was
purified on a Sephadex G25 eluting with 5% ethanol in water to give the desired product in 82% yield. $^1$H-NMR analysis of the product showed nearly identical chemical shifts compared to reported data$^{69}$ and verified the success of the purification and reactions.

![Chemical Structures](image)

**Scheme 31: Synthesis of 13:** a) PNGase, 100 mM ammonium acetate, 37°C, 16 h, 44%, b) DMC, NaN$_3$, lutidine, 2°C, 7 days, 82%

### 1.4 Synthesis of Fmoc-Asn-Nonasaccharide

For the linear synthesis of glycopeptides using SPPS an Fmoc protected asparagine containing the N-glycan is required. This building block can be generated from SGP. After extraction from egg yolk, the glycopeptide was digested with pronase to release the desired glycosyl amino acid 23 (provided by Dr. Sebastian Götze). To remove the sialic acid residues from glycosylated asparagine, 23 was treated with a 10 mM TFA solution (pH 3) at 80°C. The removal is necessary to avoid side reactions with the acid function of sialic acid during activation in SPPS. The reaction was carefully monitored with MALDI-TOF and when both sialic acids were removed, the mixture was lyophilized. Purification on a Sephadex G25 eluting with water containing 20% acetonitrile gave the desired product 24 in 94% yield (Scheme 32).

To protect the N$^\alpha$-function of asparagine, 24 was reacted with 9-Fluorenymethyl $N$-succinimidyl carbonate (Fmoc-OSu) and triethylamine in a 1:1 mixture of dioxane and water. After MALDI-TOF showed complete conversion of the starting material, the reaction mixture was neutralized with acetic acid, lyophilized and purified on a SiliaSep™ C18 cartridge using 5, 10, 15 and 20% acetonitrile in water. Due to the presence of the hydrophobic Fmoc protecting group, the fractions eluting with 10 and 15 % contained the desired product. The fractions were combined, lyophilized and analyzed by MS and NMR. All NMR signals were in accordance with the reported data.$^{95}$ The desired product 25 was generated in 51% yield.
1.5 Conclusion for synthesis of glycan building blocks for glycopeptide synthesis

The synthesis of appropriate amino glycans or glycosylated amino acids for glycopeptide synthesis is an essential and challenging task in the field of glycoprotein synthesis. Depending on the selected strategy, an Fmoc-Asn-glycan for sequential coupling, a glycosyl amine for Lansbury aspartylation or a glycan-oxazolidine for enzymatic methods has to be synthesized. For the synthesis of GlcNAc-azide 12, two strategies were evaluated to generate peracetylated compound 18. Both synthesis pathways were successful and gave the desired product. However, using Sn(IV)Cl₄ and TMS-azide to generate the product directly from peracetylated glucosamine resulted in a significant higher yield with fewer synthesis steps. After reduction with Pd under hydrogen atmosphere GlcNAc-amine 19 was used for the synthesis of various glycopeptides.

The generation of nonasaccharide-azide 13 by chemoenzymatic methods from SGP 20 extracted from egg yolk was also successful. The method for extraction of the desired glycopeptide 20 was modified to a simplified and less toxic strategy. Instead of using fresh eggs, egg yolk powder was used to obtain glycopeptide 20. Additionally, to remove the undesired proteins and lipids a precipitation with methanol was used to replace the reported treatment with phenol. After purification by size exclusion chromatography 8.4 mg crude
SGP/egg yolk were observed, which is equal to published yields using a more complex process.\textsuperscript{135} The sialic acid residues were cleaved using acidic hydrolysis to give 21 without further cleavage of additional sugar moieties. Digestion of the obtained glycopeptide with PNGaseF released the unprotected sugar, which was converted into the corresponding nona-azide 13 using DMC and sodium azide in one-step.\textsuperscript{69, 134}

Additionally, Fmoc-Asn-nona 25 was synthesized from glycosylated asparagine 23. After acidic hydrolysis of the sialic acid residues the N\textsuperscript{\alpha}-function of asparagine was Fmoc protected and the building block can be used in SPPS according to established protocols.

2. SYNTHESIS OF A PHOTOLABILE PROTECTED ASPARTIC ACID FOR LANSBURY ASPARTYLATION

Orthogonal protecting groups are an enormous asset in the synthesis of complex molecules such as natural products. In Fmoc based solid phase peptide synthesis researchers take advantage of using acid labile permanent protecting groups on the side chains and an acid labile linker and base labile Fmoc as temporary protection on the N\textsuperscript{\alpha} function. To modify specific side chains on a synthetic peptide, it is crucial to use protecting groups that are stable during peptide elongation and easily removable without affecting any other protecting groups. To couple glycans selectively to a free aspartic acid residue on a synthesized peptide all other amino or acid functions require protection to avoid side reactions. An established protecting group for this purpose is allyl, which is stable to TFA and basic conditions and can be removed with palladium.\textsuperscript{68-69, 136} For the installation of the two glycosylation sites contained by fragment 2 it is necessary to have an additional orthogonal protecting group.

Considering the available protecting groups, a photolabile protection on aspartic acid would serve impeccably the necessities, though no such protecting group stable during SPPS is known.\textsuperscript{57} 4,5-dimethoxynitrobenzyl (Dmnb) has been described as photocleavable protecting group on aspartic acid. However, it has been shown that building blocks bearing this protecting group are not only difficult to introduce, they are also prone to aspartimide formation under basic conditions used for Fmoc removal in SPPS.\textsuperscript{137} In contrast, photoremovable protecting groups to mask the \(\alpha\)-carbonyl function of amino acids have been described\textsuperscript{138-139} and might also be used for side chain of aspartic acid. The 4-(dimethyl)-aminophenacyl (Map) was selected as an ideal protecting group for the protection of aspartic acid because it has a good stability against TFA and can be easily removed by UV-light.\textsuperscript{138, 140} Aspartic acid having this protection are not commercially available, therefore synthesis of Fmoc-Asp(OMap)-OH was designed for this purpose.
2.1 Synthesis for Fmoc-Asp(OMap)-OH 11

To obtain the desired Fmoc-Asp(OMap)-OH building block 11 for SPPS starting from unprotected L-aspartic acid, a selective protection of the side chain acid function was necessary before Fmoc protection of the amine. This process was performed using a method to prepare β-ester of aspartic acid introduced by Feijen et al. in 1982. This method uses amino acid copper(II) complexes and the corresponding halides of the protecting group for alkylation.

The required Map-bromide 28 can be synthesized in a two stepped high yielding fashion by bromination and debromination of 4-(dimethylamino)-acetophenone.

To start the synthesis 4-(dimethylamino)-acetophenone 26 was brominated in concentrated sulfuric acid with bromine to give 2,2-dibromo-1-(4-(dimethylamino)phenyl)-ethanone 27 as a green solid in 90% yield. To debrominate product 27 diethylphosphite in THF was used and gave the desired Map-bromide 28 as a greenish yellow solid in 43% yield (Scheme 33).

Scheme 33: Synthesis of 28: a) H$_2$SO$_4$, Br$_2$, rt, 14 h, 90%, b) diethylphosphite, TEA, THF, 0°C to rt, 14 h, 43%

Having the suitable halide 28 in hand, L-aspartic acid was converted into a copper(II) complex 30 using copper(II)acetate in water, with a yield of 90% as a blue solid. After the formation of a L-aspartic acid copper (II) complex 30, N,N,N’,N’-Tetramethylguanidinium was added to the complex to form the corresponding salt and to increase the solubility in DMF/water (10:1). Map-bromide 28 was added directly to this solution and reacted for 14 hours. Decomplexation of 31 with ethylenediaminetetraacetic acid (EDTA) in water gave H-Asp(OMap)-OH 32 in 60% yield over three steps. Finally, protection of the free amine using FmocOSu in THF/water (1:1) delivered the desired building block 11 in 70% yield. Notably, the whole synthesis was conducted without purification by column chromatography and gave the desired product in 40% yield over 5 steps (Scheme 34).
2.2 Glycopeptide synthesis using Fmoc-Asp(OMap)-OH

Having the desired aspartic acid building block 11 in hand, its applicability for Fmoc-SPPS and the following release from the side chain was investigated. For this purpose decapeptide 36 taken from the prion protein was used as a model peptide. To avoid aspartimide formation it is necessary to install a pseudoproline dipeptide before the Fmoc-Asp(OMap)-OH. Thus the sequence to be evaluated was as follows: Fmoc-Thr(tBu)-Thr(tBu)-Gly-Glu(tBu)-Asp(Map)-Phe-Thr(psiMe,Mepro)-Glu(tBu)-Thr(tBu)-OH. Starting from a commercially Trityl-ChemMatrix® 33 (loading: 0.37 mmol/g) the resin was brominated using 10% AcBr in anhydrous DCM under argon atmosphere for four hours. Afterwards, the first amino acid (Fmoc-Thr(tBu)-OH) was coupled to the activated resin using DIPEA as base. The resin loading of 35 was determined using Fmoc-quantification, which gave a resin loading of 0.35 mmol/g (Scheme 35).

The peptide was manually elongated in a fritted syringe. After each coupling step Fmoc was removed using 20% piperidine in DMF twice for 5 and 20 minutes. The couplings were carried out using 5 molar equivalents amino acid, HOBT, HBTU and DIPEA in a 1:1:1:2 ratio, except for the costly pseudoproline building block, where only 2 molar equivalents were used. All amino acids were coupled twice for 20 minutes each. The pseudoproline building block and the Fmoc-Asp(OMap)-OH amino acid were coupled twice for 60 minutes.
After coupling the final amino acid a test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) as cleaving solution. LC-MS analysis showed a single product, which was assigned to the desired deca peptide 36. Noteworthy, the photosensitive Map protecting group was stable towards the coupling conditions and Fmoc removal and could be installed without complications. Additionally, the photolabile protection leads to a negative signal of Map-protected molecules on the UV-trace at 280 nm (Figure 26), allowing a convenient monitoring of its removal.

The photolysis in pure ethanol as proposed by Ueda et al.\textsuperscript{138} was not possible due to the low swelling of the resin in this solvent. Therefore, in a first attempt, the removal of the Map-group from 36 was conducted in pure DCM using a continuous flow system equipped with a high-pressure Hg-lamp ($h\nu>300$ nm, flow rate 1 ml/min, 18 ml loop, radiation time 18 min). Under these conditions, photolysis showed to be incomplete. Thus the solvent was exchanged to a mixture of 1:1 DCM/ethanol keeping the same conditions mentioned to give the desired product 37. To validate the success and completeness of the photocleavage a test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v). LC-MS analysis showed complete removal of the Map side chain protection of aspartic acid and the desired peptide 37 was observed as only product (Figure 27). During the photo cleavage no side reaction, such as aspartimide formation was observed. Thus, the building block Fmoc-Asp(OMap)-OH was proven as a beneficial handle for orthogonal protection of the aspartic acid side chain in Fmoc SPPS and deprotection.
Having the desired peptide with a free aspartic acid side chain in hand, the next step was the coupling of an amino sugar. Deca peptide 37 was coupled using Lansbury coupling with amino glycan 19. This was accomplished using PyBOP and DIPEA as a base in DMF as coupling reagents. The reaction gave the desired glycopeptide 38 as a single product without aspartimide formation (Figure 28).

With the successful synthesis of 38, the photolabile Map-protection of aspartic acid (Fmoc-Asp(OMap)-OH) was proven advantageous. Additionally, the installation of pseudoproline dipeptides avoided aspartimide formation during Lansbury coupling for the synthesis of this glycopeptide as proposed by Unverzagt and coworkers.69

2.3 Conclusion for synthesis of a photolabile protected aspartic acid for Lansbury aspartylation

Starting from commercially available 4-(dimethylamino)-acetophenone and L-aspartic acid the desired Fmoc-Asp(OMap)-OH 11 was synthesized in 7 steps. The protecting group was obtained following the method of Diwu and coworkers.142 Using the copper(II)-complex of aspartic acid 30 and the corresponding halide β-ester 28, the side chain was selectively
protected using a method introduced by Feijen and coworkers. After decomplexation of using EDTA, H-Asp(OMap)-OH was observed in 60% yield over three steps. The N\textsuperscript{α} function of Asp was protected with Fmoc to furnish the desired Fmoc-Asp(OMap)-OH. After the successful synthesis of, the building block was used for the generation of glycopeptide. 11 was incorporated in the sequence of peptide. During the coupling and peptide elongation using Fmoc-SPPS no aspartimide formation and no deletion sequences were observed, proving building block to be optimal for SPPS and to be far superior to the known Fmoc-Asp(ODmb)-OH. Additionally, the removal of the Map-group was selective using a high-pressure Hg-lamp (hv>300 nm) in flow and a retention time of 18 minutes. The free acid function was coupled with amino glycan to give a model glycopeptide showing that Fmoc-Asp(OMap)-OH can be used for the synthesis of glycopeptides with Lansbury aspartylation.

3. SYNTHESIS OF ASPARTIC ACID BUILDING BLOCKS FOR LIGATION-DESULFURIZATION STRATEGIES

Native chemical ligation is a powerful tool for the total synthesis of proteins. However, requirement of a cysteine residue and its relatively low abundance of up to 1.1% of amino acid residues in natural occurring proteins demand access to cysteine surrogates. To overcome this shortcoming the use of unnatural β- or γ-mercapto amino acids for ligation-desulfurization is a reliable method. Unfortunately, β- or γ-mercapto amino acids are not commercially available, making chemical synthesis of these unnatural amino acids necessary. As described in the retrosynthesis, synthesis of the prion protein requires installation of a β-mercapto aspartic acid at Asp178 to enable ligation to the expressed protein thioester.

To enable ligation in N to C direction the thiol of β-mercapto aspartic acid can be synthesized using an acid labile protection. For a possible ligation in C to N direction the thiol requires an orthogonal protecting group. Therefore, β-mercapto aspartic acid was as well synthesized with Acm and Map as a thiol protection group.

3.1 Synthesis of Boc-Asp(OtBu)-OAllyl

The synthesis of the desired aspartic acid building blocks was started with allyl protection of the free acid function of commercially available Boc-L-Asp(OtBu)-OH by reaction with allyl bromide in DMF under basic conditions. The desired product was observed in quantitative yield.
Having the desired fully protected aspartic acid building block 40 in hand, three β-mercapto amino acids were synthesized.

3.2 Synthesis of Boc-Asp(OtBu;STmob)-OH 8

The protocol for the synthesis of required trimethoxybenzyl (Tmob) protected β-mercapto aspartic acid (Boc-Asp(OtBu;STmob)-OH 8) was published by Payne and coworkers (Scheme 38).47 The synthesis was started by generating required sulfenylating reagent 43 for the envisaged β-aspartyl enolate sulfenylation. 2,4,6-trimethoxybenzyl alcohol 41 was reacted with TFA in MeOH at 0°C to generate an activated ester which was directly treated with potassium toluene thiosulfonate 42. Unfortunately, using this strategy the desired product was not observed.

A stereocontrolled protocol for protected β-mercapto aspartic acids using a similar sulfenylating reagent was also published by Shibata et al. (Scheme 39).128 and the strategy was used to synthesize 43.

The synthesis was started by treating 2,4,6-trimethoxybenzyl alcohol 41 with TEA at room temperature in acetone and DCM. The mixture was cooled to 0°C and reacted with TFAA to form an activated ester, which was directly converted into the required sulfenylating reagent 43 by using potassium toluene thiosulfonate 42. The desired product 43 was observed in 72% over two steps.

Having both, a fully protected aspartic acid 40 and sulfenylating reagent 43 in hand, the next reaction was approached.
Aspartic acid building block 40 was dissolved in anhydrous THF and activated with LiHMDS at -78°C to form the corresponding dianion. 43 was added and after work up and purification by silica column chromatography product 44 was observed in 76% yield as a mixture of inseparable stereo isomers. Thereafter, the allyl ester was cleaved using Pd(PPh₃)₄ in DCM to give 8 in 80% yield (Scheme 40).

Here, separation of the isomers was successful using RP-HPLC (on Macherey-Nagel Nucleodur C18 Pyramid), giving 8a and 8b in a 1:2 ratio (determined by analytical HPLC of crude 8 [Figure 29]). The stereochemistry of 8a and 8b was confirmed by analysis of ¹H-NMR coupling constants. Large differences in coupling constants between Hα and Hβ of 8a (J=7.7 Hz) and 8b (J=9.6 Hz) suggest that 8a is the erythro diastereomer, which is inconsistent with the stereochemical selectivity reported. As reported, during ligation both isomers show the same reactivity and therefore can be used in ligation reactions.

The generated β-mercapto aspartic acid can be used for ligation desulfurization protocols in N to C direction. To also enable sequential native chemical ligation in C to N direction, protection of the thiol is required. Since Tmob is acid labile, cleavage during the final cleavage and deprotection step in Fmoc-SPPS using TFA will occur. Yet, it is also possible to ligate a protected Boc-Asp(OtBu,STmob) to PrP fragment 2 (179-213), since amino acid 179 is a cysteine. Thus a Boc-Asp(OtBu,STmob) thioester is required.
Using PyBOP and DIPEA, 8a formed an activated ester that was directly reacted with benzyl mercaptan in DMF to give the required thioester 45 in quantitative yield (Scheme 41).

### 3.3 Synthesis of Boc-Asp(OtBu;SAcm)-OH

In addition to the previously introduced ligation strategy, ligation in C to N direction is also possible when the thiol function on the N-terminal amino acids bears an orthogonal protection group. A well described orthogonal protection group for thiols in Fmoc-SPPS is acetamidomethyl (Acm).

The corresponding synthetic route was started with the generation of sulfenylating reagent 47 (Scheme 42).

\[
\text{Scheme 42: Synthesis of 47: a) TEA, acetone, rt, 15 min, b) TFAA, 0°C to rt, 16 h, 52% over 2 steps}
\]

\(N\)-(hydroxymethyl)-acetamide 46 was activated with DIPEA and TFAA in acetone and reacted with potassium 4-methylbenzenesulfonothioate 42 according to the protocol published by Shibata and coworkers. The sulfenylating reagent 47 was isolated in 52% yield.

\[
\text{Scheme 43: Synthesis of 9a and 9b: a) LiHMDS, anhydrous THF, -78°C, 2 h, 14%, b) Pd(PPh_3)_4, neat DCM, rt, 1 h, 57%}
\]

To synthesize the required orthogonal protected β-mercapto aspartic acid, protected aspartic acid 40 was activated with lithium hexamethyldisilazide (LiHMDS) in THF at -78°C and reacted with sulfenylating reagent 47 to give 48 as a mixture of stereo isomers in 14% yield. Afterwards allyl was removed with Pd(PPh_3)_4 in DCM and product 9 was obtained in 57% yield (Scheme 43).

The stereo isomers were separated using RP-HPLC (on Macherey-Nagel Nucleodur C18 Pyramid), giving 9a and 9b in a 2:3 ratio (determined by analytical HPLC of crude 9 [Figure 30]). Unfortunately, assigning the stereochemistry using \(^1\)H-NMR was not possible since multiplets were observed for the coupling constants between Hα and Hβ.
3.4 Synthesis of Boc-Asp(OtBu;SMap)-OH 10

Additionally, an orthogonal protected β-mercapto having a photolabile group masking the thiol function was synthesized.

To synthesize sulfenylating reagent 49, bromide 28 was reacted with potassium salt 42 in DMF to give the desired product in 38% yield (Scheme 44).\(^{143}\)

After activation of aspartic acid 40 with LiHMDS in THF at -78°C, reaction with 49 to yield 50 as a mixture of stereo isomers was successful in 62%. In the next step, the ally protecting group was removed with Pd(PPh\(_3\))\(_4\) in DCM to give product 10 in 72% yield (Scheme 45).

Using RP-HPLC the stereo isomers were separated (on Macherey-Nagel Nucleodur C18 Pyramid), giving 10a and 10b in a 3:7 ratio (determined by analytical HPLC of crude 10 [Figure 31]). Assigning stereochemistry using \(^1\)H-NMR failed due to inseparable impurities in the first fraction.
3.5 Conclusion Synthesis of Aspartic Acid Building Blocks for Ligation-Desulfurization Strategies

The synthesis of the prion protein requires installation of a β-mercapto aspartic acid at Asp178 to enable a ligation-desulfurization strategy with the expressed protein thioester (PrP 23-177). The required building blocks are not commercially available and three β-mercapto aspartic acid building blocks were synthesized, including two bearing an orthogonal protecting group on the thiol function.

In a first attempt, known Boc-Asp(OtBu,STmob)-OH 8 was synthesized according to the strategy published by Payne and coworkers.47 After the synthesis route for the required sulfenylating reagent 43 was unsuccessful, a route published by Shibata et al.128 was adapted to generate required 43. Finally, fully protected aspartic acid 40 was treated with LiHMDS at low temperature and reacted with 43 to give an inseparable mixture of diastereomers 44 in 72% yield. After palladium(0)-catalyzed removal of the allyl ester, the isomers 8a and 8b were separated using RP-HPLC. However, the published stereocontrol of the reaction was not reproducible. The isomers were observed in a 1:2 ratio in favor of the anti-isomer. Subsequently, both isomers were used for SPPS.47

A second β-mercapto aspartic acid with Acm as orthogonal protecting group on the thiol function was synthesized. Required sulfenylating reagent 47 was synthesized according to the route established for 43 in 52% yield. Unfortunately, a very low reactivity was observed in the next step and the diastereomeric mixture of 48 was observed in 14% yield. After allyl removal with palladium(0), the isomer 9a and 9b were separated using RP-HPLC and showed a 2:3. The syn- as well as the anti-isomer were used for SPPS.
A third strategy for a β-mercapto aspartic acid with the photolabile Map as orthogonal protecting group, masking the thiol, was evaluated. Sulfenylating reagent 49 was generated by reacting bromide 28 and potassium 4-methylbenzenesulfonothioate 42 in DMF in 38%. By reacting 40 with 49 after treatment with LiHMDS, desired 50 was observed as a mixture of stereo isomers in 62%. The allyl ester was cleaved with palladium(0) and the isomer 10a and 10b were separated using RP-HPLC to give the products in a 3:7 ratio.

4. SYNTHESIS OF PrP FRAGMENT 1 (PrPI [214-231])

A fully protected fragment 1 was evaluated for the synthesis of a thioester in a previous work (master thesis). Therefore, fragment 1 was synthesized on an acid labile 2-Cl-trityl linker bound to a ChemMatrix®. The desired peptide was synthesized successfully. However, it was not possible to convert the product into a thioester using the conditions proposed by Kajihara and coworkers. Optimization efforts by changing the temperature, reaction time and coupling reagents showed no formation of the desired product. Thus, for this work different strategies were examined.

4.1 Dawson Nbz as thioester precursor

Starting from a commercially available Dawson Dbz NovaSyn® TGR resin 51 the desired peptide was synthesized using the conditions described by Dawson et al. in a fritted syringe. Yet, LC-MS analysis showed that using HBTU and HOBT as coupling reagents, the formation of the desired sequence was not observed. Optimization efforts by changing the solvent from DMF to NMP showed no influence on that result. This observation led to the assumption, that the proposed coupling reagents are not suitable for SPPS using a Dawson linker. In a new effort, the synthesis of the desired peptide was evaluated using commercially available Dawson Dbz NovaSyn® TGR resin 51 on the microwave assisted peptide synthesizer Liberty Blue™. ethyl-cyano(hydroxyimino)acetate (Oxyma pure) and N,N'-diisopropylcarbodiimide (DIC) were used as coupling reagents and cysteine was installed as L-thioproline, making the product useful for sequential NCL (Scheme 46).

![Scheme 46: Synthesis of 53](image)

**Scheme 46: Synthesis of 53**: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C 2. 2x 20% piperidine, 90°C, b) 50 mM p-nitrophenylchloroformate, DCM, 1 hour, c) 0.5M DIPEA, DMF, 15 min, d) TFA/water/TIPS (190:5/5, v/v/v)
To control the success and completion of coupling steps a test cleavage and LC-MS analysis were performed. Finally, using DIC and Oxyma as coupling reagents the desired Dbz-peptide 52 was observed in a high crude purity (Figure 32).

![Figure 32: LC-MS analysis of 52](image)

Having the desired peptide in hand, the linker was activated through acylation of the amine group with \( p \)-nitrophenylchloroformate. After addition of DIPEA, an intramolecular attack of the anilide by the resin-bound benzimidazolidine was promoted (Scheme 46). The Nbz-peptide was cleaved from the resin with TFA/water/TIPS (190/5/5, v/v/v). Successfully performing cyclisation, the desired product was synthesized in a high crude yield and starting material 52 was completely converted into Nbz-peptide 53 (Figure 33).

![Figure 33: LC-MS analysis of 53](image)

Regrettably, upon purification the Nbz-peptide 53 was hydrolyzed completely. Therefore, the thioester formation was performed without further purification. Unfortunately, all efforts to convert the Nbz-peptide into a peptide thioester failed. The thioester was not formed using the conditions proposed by Dawson et al. [6 M GdmCl (Gdm=guanidine), 200 mM Na$_2$HPO$_4$, 200 mM 4-Mercaptophenylacetic acid (MPAA) and 20 mM TCEP·HCl]
(TCEP=tris(carboxyethyl)phosphine) at pH 7]. A pH adjustment to 7.5 lead to hydrolysis of the starting material and no thioester was observed. Thus, an alternative route, using peptide hydrazides as thioester precursor, was approached.

4.2 Peptide hydrazides as thioester precursor

The synthesis of fragment 1 as peptide hydrazide 56 was started with the preparation of the required hydrazine carboxylate Wang resin. Therefore, hydroxymethyl polystyrene was activated with \( p \)-nitrophenyl chloroformate and \( N \)-methyl morpholine (MMP) and the obtained carbonate was reacted with hydrazinium hydroxide to give the desired solid support for Fmoc based SPPS (Scheme 47).

\[
\text{Wang resin} \xrightarrow{\text{a, b}} \text{Wang resin} \xrightarrow{\text{NHNH}_2} \text{solid support}
\]

Scheme 47: Synthesis of hydrazide carboxylate Wang resin 55: a) \( p \)-Nitrophenyl chloroformate, NMP, DCM, 0°C to rt, 16 h, b) hydrazinium hydroxide, DCM/DMF (1:1), 0°C to rt, 16 h

4.4 Fragment 1 for ligation in C to N direction

The first amino acid, (i.e. serine) was coupled using PyBOP and DIPEA as coupling reagents. Fmoc quantification gave a loading of 0.63 mmol/g. The desired peptide was synthesized with MW-SPPS using the automated peptide synthesizer Liberty Blue\textsuperscript{TM} with Oxyma and DIC as coupling reagents. All amino acids were coupled twice for 6 minutes at 90°C and Fmoc was removed twice using 20% piperidine in DMF for 1 minute at 90°C (Scheme 48). For ligation to fragment 2 in N to C direction the N-terminal cysteine was introduced without orthogonal protection.

\[
\text{PrP 215-231} = \text{VTQYQKESQAYYDGRRSS}
\]

Scheme 48: Synthesis of 56: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C 2. 2x 20% piperidine, 90°C, b) TFA/water/TIPS (190/5/5, v/v/v)

The PrP I hydrazide 56 was cleaved from the resin with TFA/water/TIPS (190/5/5, v/v/v). The elongation performed smoothly and gave the desired product containing minor deletion sequences. The reaction was reproducible and the peptide was purified using a RP-HPLC semi-preparative C18 column (Synergi Hydro RP C18 or YMC hydrosphere RP C18). The pure product 56 was obtained in 31% yield and was used for NCL to fragment 2 (Figure 34).
4.5 Fragment 1 for ligation in N to C direction

To perform sequential NCL in C to N direction and to ligate fragment 1 with a GPI, it was necessary to orthogonally protect the N-terminal cysteine on PrP I to avoid side reactions. However, installation as L-thiopropoline was not performed, since side reactions during oxidation of the hydrazide occur. Therefore, S-acetamidomethyl (Acm) was used to mask the thiol function. The desired peptide 57 was synthesized with MW-SPPS using the automated peptide synthesizer Liberty Blue™ with Oxyma and DIC as coupling reagents. All amino acids were coupled using the conditions previously described for the synthesis of 56. After complete elongation, peptide hydrazide 57 was cleaved with TFA/water/TIPS (190/5/5, v/v/v) from the resin (Scheme 49).

Scheme 49: Synthesis of 57: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C 2. 2x 20% piperidine, 90°C, b) TFA/water/TIPS (190/5/5, v/v/v)

The desired product 57 was synthesized in a high crude purity. The crude peptide hydrazide was transformed into a thioester using NaNO₂ at pH 3 as oxidative condition and 4-(mercaptomethyl)benzoic acid (MMBA) as thiol. The benzylic thiol MMBA was preferred over MPAA, since the thioester formed shows a similar reactivity but is more stable. Despite being commercially available, because of its high price, MMBA was synthesized in a two stepped synthesis from methyl 4-(chloromethyl)benzoate (Scheme 50).

Scheme 50: Synthesis of 60: a) thiourea, water, reflux, 1 h; b) 10 N NaOH, water, reflux, 1 h, 98%
Methyl 4-(chloromethyl)benzoate 58 and thiourea were suspended in water and refluxed for one hour to give Methyl 4-(isothiuroniummethyl)benzoate 59. Hydrolysis was performed with 10 N NaOH in the refluxing reaction mixture for one hour. Afterwards the mixture was acidified to pH 1 with concentrated HCl to precipitate the desired product 60. After filtration MMBA 60 was lyophilized and the pure product was observed in 98% yield.

To obtain the thioester 61, crude peptide 57 was dissolved in NaNO₂ containing diazotation buffer and reacted at pH 3 at -10°C. After 20 minutes MMBA in 1 M NaOH was added. The pH was raised to 7 and the reaction was incubated for 30 minutes (Scheme 51). The product was immediately purified using gel filtration (Superdex™ peptide 10/300) with 20% ACN in water. A quantitative conversion was observed.

![Scheme 51: Synthesis of 61](image)

**Scheme 51: Synthesis of 61**: a) 6 M GdmCl, 0.2 M Na₂HPO₄, 0.02 M NaNO₂, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min, 25%

After purification using a RP-HPLC semi preparative C18 column (Synergi Hydro RP C18 or YMC hydrosphere RP C18) the pure product 61 was obtained in 25 % yield (Figure 35). The purified thioester 61 was used for NCL to a cysteine modified GPI anchor.

### 4.6 Ligation of 61 to cysteine modified Di-Mannose

To evaluate the feasibility of a ligation between thioester 61 and a cysteine modified glycan a ligation with Cys-DiMan 62 was used. First, a ligation buffer with a composition of 6 M GdmCl, 0.1 M Na₂HPO₄, 0.05 M MPAA, 5 mM TCEP at pH 7 was used (Scheme 52). This buffer system is widely used for NCL of peptides and glycopeptides. However, under these conditions product 63 was not observed and only traces of disulfide 63a were detected.
Efforts to isolate product 63a using a Superdex™ peptide 10/300 gel filtration column with 20% ACN resulted in an inseparable mixture of 63a and MPAA. Occurrence of disulfide 63a and the slow reaction indicates the possibility that also Cys-DiMan 62 formed a disulfide with MPAA. The concentration of TCEP in the ligation buffer was enhanced to circumvent this possible oxidation. Unfortunately, the addition of more TCEP to reduce the disulfide resulted in decomposition of the starting materials. To overcome this issue, less reactive MBAA was used as thiol in the ligation buffer. Product formation was not observed as well.

Since the low efficiency of the reaction was attributed to the formation of disulfides in this buffer system, the concentration of thiol and TCEP were adjusted according to the amount of thioester 61 and Cys-DiMan 62 used. The ligation was conducted using a ligation buffer with 6 M GdmCl, 0.1 M Na2HPO4, 5 eq MMBA and 30 eq TCEP (Scheme 53). The progress of the reaction was monitored using HPLC.
Already after one hour the formation of desired product 63 was observed (Figure 36) and to bring the ligation to completion the reaction was incubated for additional 23 hours. HPLC analysis showed complete consumption of thioester 61 after a total reaction time of 24 hours. Additionally to desired product 63, disulfide 63b was observed (Figure 37).

The ligation product was purified using gel filtration (Superdex™ peptide 10/300) with 20% ACN in water to give 63 and 63b in 84% combined yield. The mixture of 63 and 63b was used for the next reaction without separation. With the desired product 63 and the corresponding disulfide 63b in hand, removal of Acm was approached.

A drawback, despite its great utility and wide applicability of the Acm protecting group is its difficult removal. Often, extended reaction times (6–15 h) and harsh conditions, such as the use of Hg(OAc)₂ in aqueous medium containing 50–90% acetic acid or the use of I₂ in 80–95% acetic acid are required. Recently, Brik and coworkers published an efficient palladium-assisted deprotection to remove the Acm moiety on cysteine in water. Different
Pd(2) complexes were established. However, PdCl₂ in 6 M Gn-HCl, pH 7 at 37 °C showed the fastest reaction rate and removed Acm quantitatively within five minutes after dithiothreitol (DTT) treatment to quench the reaction. Additionally, the reaction can be performed in pure water. Thus 63 and 63b were dissolved in water and 10 eq. PdCl₂ were added (Scheme 54). The reaction was incubated at 37°C and the removal was monitored with HPLC. A small amount was taken from the reaction mixture, quenched with DTT and analyzed using HPLC.

After 4 hours starting material 63 was still observed and the reaction was incubated for additional 12 hours. Finally, the reaction was completed and after quench with DTT, PrP I (Cys-DiMan) 64 was purified using gel filtration (Superdex™ peptide 10/300) with 20% ACN in water. Unfortunately, isolation of pure compound by gel filtration was not possible. Rather, 64 was obtained in a mixture with unknown contaminations. The impurities were removed using semi preparative RP-HPLC (YMC hydrosphere RP). Finally, the desired product PrP I (Cys-DiMan) 64 was observed (Figure 38). However, significant loss of product during final purification gave 64 in only 5% yield.

After the buffer system for the ligation was established and the successful removal of Acm using PdCl₂ was shown, a one-pot ligation and Acm removal reaction was evaluated (Scheme 55).
The ligation of thioester 61 with Cys-DiMan 62 was conducted using the previously described optimized conditions. Fortunately, the reaction was reproducible and ligation was complete within 24 hours as indicated by HPLC analysis. Again, the desired product 63 was observed together with the corresponding MMBA disulfide 63b. To the products 63 and 63b in ligation buffer 10 eq. PdCl₂ were added and the reaction was incubated at 37°C. A small amount was taken from the reaction mixture, quenched with DTT and analyzed using HPLC, which showed complete removal of Acm within 30 minutes. The reaction was quenched using DTT and purified using Superdex™ peptide 10/300 gel filtration column with 20% ACN. The faster conversion of the reaction is attributed to the better solubility of PdCl₂ in 6 M GdmCl buffer. Unfortunately, 64 was again obtained in a mixture with unknown contaminations. To remove semi-preparartive RP-HPLC (YMC hydrosphere RP) was used and the desired product PrP I [Cys-DiMan] 64 was observed in 6% yield (Figure 38).

**Figure 38: LC and MALDI-TOF analysis of 64** [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H₂O (0.1% TFA) in 30 min]

### 4.7 Ligation of 61 to cysteine modified monolipidated GPI

After the conditions for ligation of thioester 61 and Cys-DiMan 62 were optimized and resulted in a fast reaction, forming the desired product, the same conditions were applied for the ligation of 61 and a cysteine modified monolipidated GPI (Cys-GPI 4) (Scheme 56). The process of the reaction was monitored using HPLC. Unexpectedly, product formation was not observed within the first 24 hours. Even with an incubation time of one week, no product was detected.
To facilitate the formation of GPI-anchored PrP 63, the reaction temperature was elevated to 37°C. Unfortunately, only the formation hydrolyzed thioester 61 was observed.

4.8 Conclusion for synthesis of PrP Fragment 1 (PrP\([214-231]\))

Fragment 1 for the synthesis of a GPI-anchored prion protein was synthesized using SPPS. To enable native chemical ligation a C-terminal thioester of this fragment is required and two strategies for peptide thioester synthesis were evaluated. The peptides were synthesized using DIC and Oxyma on a microwave assisted peptide synthesizer Liberty Blue™. First, a commercially available Dawson Dbz NovaSyn® TGR resin was used to generate an Nbz-peptide as thioester precursor. The desired Dbz-peptide 52 was generated in high crude purity and on resin converted in Nbz-peptide 53. This thioester precursor 53 was not stable during purification and conversion into the corresponding thioester resulted only in hydrolysis of Nbz-peptide 53.

Thereafter, peptide hydrazides were evaluated as thioester precursors. To enable ligation with fragment 2 peptide hydrazide 56 was synthesized using SPPS in 31% yield. Additionally, fragment 1 was synthesized as peptide hydrazide masking cysteine with an orthogonal Acm protecting group on the thiol function. The crude hydrazide 57 was obtained in 60% yield and converted into desired thioester 61 in 25% yield.

Having 61 in hand, ligation reactions to a cysteine modified GPI-mimic 62 and a cysteine modified mono-lipidated GPI 4 were evaluated. With an optimized buffer for the ligation, the reaction of 61 and 62 went rapidly and was complete within 24 hours. DiMan anchored 63 was observed in combination with the corresponding MMBA disulfide product 63b in 84% yield. The removal of Acm was conducted in water at 37°C with PdCl\(_2\) to give the desired
product in 5% yield. Additionally, a one-pot ligation and Acm removal reaction was evaluated to give the desired product 64 in 6% yield.

With the optimized buffer system the ligation of mono-lipidated GPI 4 and thioester 61 was evaluated. Even after a prolonged incubation time of one week and elevated temperature at 37°C the reaction was not proceeding and only hydrolyzed thioester started to form.

5. SYNTHESIS OF PRP FRAGMENT 2 (PRP II [178-213])

For the synthesis of fragment 2 (178-213) complementary considerations have to be taken into account. Fragment 2 contains 35 amino acids, including the possibly glycosylated Asn181 and Asn197 and β-mercapto Asp178. The installation of a β-mercapto aspartic acid unit at Asp178 is required for further ligation with an expressed protein thioester. To generate the required thioester of fragment 2, the peptide was synthesized as a hydrazide using hydrazide carboxylate Wang resin 55. To generate a homogeneous GPI-anchored and glycosylated prion protein, sequential native chemical ligation in two directions can be considered. To enable a ligation in N to C direction, β-mercapto Asp178 without orthogonal protection and Asp178 was installed using Boc-Asp(O_{tBu},STmob)-OH 8. After ligation to an expressed protein thioester, the hydrazide can be converted into the corresponding thioester and ligated to fragment 1. Contrariwise, a sequential ligation strategy in C to N direction demands an orthogonal protection of the thiol function on β-mercapto Asp178 to avoid selfligation of the fragment. Thus, for the ligation strategy Asp178 can be installed using Boc-Asp(O_{tBu},SAcm)-OH 9 or Boc-Asp(O_{tBu},SMap)-OH 10. After SPPS fragment 2 can be converted into a thioester and ligated to fragment 1.

Independent of the ligation direction, installation of glycans at Asn181 and Asn197 can be performed by Lansbury coupling. Therefore, an orthogonal protection of Asp181 and/or Asp197 is required on the β-carboxylate using either the commercially available Fmoc-Asp(OAll)-OH or Fmoc-Asp(OMap)-OH 11. The prion protein exists in non-, mono- and di-glycosylated forms. To evaluate the influence of the attached glycan, fragment 2 was synthesized without glycan and with a glycan at Asn197 and/or Asn181.

5.1 Optimization of the Synthesis of Fragment 2

Initial attempts to synthesize fragment 2 have shown the demanding nature of the sequence, which is prone to aggregation. Thus, to avoid aspartimide formation during Lansbury coupling and aggregation during SPPS three pseudoprolines were installed (Figure 39).
Starting from Wang hydrazide resin 55, fragment 2 was synthesized using SPPS on the automated microwave assisted peptide synthesizer Liberty Bule™. The first amino acid, methionine, was coupled using PyBOP and DIPEA and a loading of 0.58 mmol/g was observed according Fmoc-quantification. The investigation of the synthesis of fragment 2 required various optimizations. In a first examination, the same coupling conditions optimized for fragment 1 (56 and 57) were used. All amino acids were double coupled a five-fold excess for 6 minutes at 90°C and Fmoc was removed twice using 20% piperidine in DMF for 1 minute at 90°C. Yet, under these conditions the desired product was only produced containing a significant amount of deletion sequences. To overcome this issue, the reaction time was prolonged while lowering the temperature during coupling. As a result, all amino acids were coupled twice at 50°C for 10 minutes. Unfortunately, no significant improvement compared to the previous outcome was observed.

Further investigation revealed that the high swelling of the resin leads to insufficient coverage of the resin with coupling solution and deprotection solution. Thus, after completion of common sequence PrP (Fmoc198-213) 66 (Scheme 57; Figure 40), the resin was divided in half to increase the volume of the reaction mixture. Thereafter, all amino acids were coupled twice in a ten-fold excess for 6 minutes at 90°C. Fmoc was removed twice using 20% piperidine in DMF for 1 minute at 90°C. Moreover, Arg208 had to be coupled three times to afford the peptide in a well purity.

Additionally, the pseudoproline dipeptides Fmoc-L-Phe-L-Thr[Ψ(Me,Me)Pro]-OH, Fmoc-L-Val-L-Thr[Ψ(Me,Me)Pro]-OH and Fmoc-L-Ile-L-Thr[Ψ(Me,Me)Pro]-OH were coupled manually in a fritted syringe using PyBOP and DIPEA as coupling reagents. The dipeptides were coupled twice for 20 minutes in a two-fold excess. A Kaiser test was performed to ensure the completeness of the coupling.
An additional side reaction was observed when Asp(OMap) or Asp(OAll) were installed at position Asp197. During prolonged synthesis aspartimide formation was observed. To circumvent this side reaction, 0.1M HOBt was added to the deprotection solution. Unfortunately, no change of the outcome was observed. Thereafter, the temperature during deprotection was lowered to room temperature and Fmoc was removed using three times 20% piperidine in DMF for five minutes without microwave irradiation. With these conditions the formation of the aspartimide side product was significantly lowered, but still observed. Finally, also the temperature during coupling was reduced and all amino acids were coupled twice in a ten-fold excess for 10 minutes at 50°C aspartimide formation was completely avoided.

In summary, for all peptides the following conditions were applied: amino acids were coupled twice in a five-fold excess for 6 minutes at 90°C and Fmoc was removed twice using 20% piperidine in DMF for 1 minute at 90°C, but when Asp(OMap) or Asp(OAll) was installed at position Asp197 temperature during coupling was reduced to 50°C and the temperature during deprotection was lowered to room temperature.

### 5.2 Strategy 1: Fragment 2 for N to C Ligation

To perform native chemical ligation between the thioester of fragment 3 and fragment 2, β-mercapto Asp178 residue was installed using Boc-Asp(OtBu,STmob)-OH 9. Additionally, Asp181 and/or Asp197 were equipped with an orthogonal protecting group to enable Lansbury coupling after complete SPPS.
Starting from 66, fragment 2 was synthesized using SPPS on the automated peptide synthesizer Liberty Bule™ using the optimized conditions. Four different peptides were generated to give non-, mono- and diglycosylated structures.

### 5.3 Synthesis of fragment 2 for N to C Ligation without glycosylation

The synthesis of 67 was successfully completed using the conditions optimized for fragment 2 (Scheme 59).

Having 67 in hand, Boc-Asp(OtBu;STmob)-OH 9 was coupled manually in a 3.6 fold excess in a fritted syringe using PyBOP and DIPEA as coupling reagents. After 16 hours, a Kaiser test confirmed complete coupling and the product was cleaved from the solid support with TFA and scavengers (Scheme 60).

The crude product was analyzed using HPLC and MALDI-TOF and confirmed the success of the synthesis. However, after coupling of the final amino acid the purity of the desired product decreased, as a result of inter- and intramolecular interactions of the N-terminal β-mercapto aspartic acid.

Peptide 68 contains two free thiol functions, one at β-mercato Asp178 and one at Cys179. Both thiols can form intra- and intermolecular disulfides. To investigate whether the dramatic change of the HPLC profile is indeed a result of the formation of disulfides, TCEP was added to the peptide containing solution before HPLC analysis. HPLC-analysis showed a slight improvement of the analytic profile of 68 (Figure 41).
Figure 41: LC-MS analysis of 68 with and without additional TCEP [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70%ACN in H2O (0.1% TFA) in 30 min]

Still, several peaks were observed. In fact, analysis of the MS data showed that one or more of the three methionine residues in fragment 2 oxidized during the synthesis. The thioether side chain function of methionine is particularly sensitive to oxidation, since it has a low oxidation potential and can form sulfoxides [Met(O)]. The natural L-methionine can produce two possible diastereoisomers in its oxidized form, the (R,S)-Met(O) and the (S,S)-Met(O). Thus, after each oxidation two different products can be observed. Taking into account that 68ox contains three methionine residues, a variety of oxidized species can be present in the crude peptide.

To reduce the sulfoxide moieties, 68ox was treated with NH₄I and dimethyl sulfide (DMS) in TFA (Scheme 61). After quenching the reaction with a saturated ascorbic acid solution, the peptide was purified using gel filtration (Superdex™ peptide 10/300) with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18). After reduction, peptide hydrazide 68 was treated with 10 eq. TCEP to reduce the disulfides and thereby simplifying the purification. Purified 68 was obtained in 5% yield (Figure 42).
5.4 Synthesis of fragment 2 for N to C Ligation with glycosylation at Asn197 and/or Asn181

Starting from 66, peptide 69 was synthesized with an orthogonal protected aspartic acid at position Asp197 and Asp181 using commercially available Fmoc-Asp(OAll)-OH. The peptide was elongated using the conditions optimized for fragment 2 on the automated peptide synthesiser (Scheme 62).

A test cleavage was performed and analyzed using HPLC and MALDI-TOF, confirming the success of the synthesis. As detected in the case of 68, also 69 was observed containing intramolecular and intermolecular disulfides and oxidized methionine residues. Thereafter, the allyl ester was cleaved to give a free aspartic acid side chain. Using Pd(PPh₃)₄ and phenylsilane as scavenger in DCM, the reaction went smoothly and the protecting group was completely removed. Amino glycan 19 was coupled to the released carboxylate using PYBOP and DIPEA in DMF/DMSO.

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**Scheme 62: Synthesis of 69:**
a) Fmoc-SPPS: 1. 2x 10 eq. Fmoc-AA-OH, 10 eq. DIC/Oxyma (0.1M DIPEA), 50°C 2. 3x 20% piperidine, rt, b) 8, PyBOP, DIPEA, DMF, rt, 16h

**Scheme 63: Synthesis of 70:**
a) Pd(PPh₃)₄, phenylsilane, DCM, 2 h, quant. b) 19, PyBOP, DIPEA, DMF/DMSO, 16 h, quant., c) TFA/water/TIPS (190/5/5, v/v/v)
The reaction was complete after 16 hours and crude glycopeptide 70 was cleaved from the resin with TFA/water/TIPS (190/5/5, v/v/v). Two additional PrP (178-213) glycopeptides were synthesized using the same conditions. Glycopeptide 71 having a GlcNAc unit at Asn181 and glycopeptide 72 having a GlcNAc unit at Asn197.

![Figure 43: Glycopeptide 71 and 72](image)

To reduce the sulfoxide moieties of the oxidized glycopeptides 70, 71 and 72 were treated with the same conditions shown for peptide 68. The glycopeptides were purified using the same conditions previously optimized for peptide 68. Unfortunately, LC-MS analysis of the glycopeptides revealed that the glycopeptides were not stable towards the treatment with TCEP before purification.

![Figure 44: Desulfurized glycopeptides 70a, 71a and 72a](image)

All three glycopeptides were observed as desulfurized products (70a, 71a and 72a [Figure 44]) and were suitable for the investigation of ligation conditions and the semi-synthesis of PrP.

![Figure 45: LC-MS analysis of purified 70a](image)
5.5 Synthesis of PrP Fragment 3 (PrP III [160-177])

To evaluate the reactivity of fragment 2 and to explore the optimal conditions for the ligation to protein thioester PrP (23-177), a small peptide thioester was used as a model peptide.

\[ \text{PrP 160-177} = \text{VYYRPVDQYSNQNNFVH} \]

**Scheme 64: Synthesis of 73**

a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C  
2. 2x 20% piperidine, 90°C  
b) TFA/water/TIPS (190/5/5, v/v/v)

The first amino acid, *i.e.* histidine, was coupled using PyBOP and DIPEA as coupling reagents. Fmoc quantification gave a loading of 0.56 mmol/g. The desired peptide was synthesized using SPPS with the previously described and optimized conditions for the synthesis of fragment 1. PrP III hydrazide 73 was cleaved from the resin with TFA/water/TIPS (190/5/5, v/v/v) (Scheme 64). The elongation was performed smoothly and gave the desired product containing minor deletion sequences. Desired product 73 was purified using RP-HPLC (YMC hydrosphere RP C18 column). After purification, peptide hydrazide 73 was isolated in 25% yield (Figure 46).

**Figure 46: LC-MS analysis of 73** [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H2O (0.1% TFA) in 30 min]

Peptide hydrazide 73 was transformed into a thioester using NaNO₂ at pH 3 as oxidative condition and MMBA at pH 7 (Scheme 65). Surprisingly, the reaction showed only a 25% conversion.

**Scheme 65: Synthesis of 74**

a) 6 M GdmCl, 0.2 M Na₂HPO₄, 0.02 M NaNO₂, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min, 10% (40% brsm)
Since hydrazide 73 was recovered, it can be assumed that the oxidation was incomplete before MMBA was added and the pH was adjusted to 7. After thioester formation 74 was purified using gel filtration (Superdex™ peptide 10/300) with 20% ACN in water and RP-HPLC on an YMC hydrosphere RP C18 column. Purified thioester 74 was isolated in 10% yield (40% brsm) (Figure 47).

5.6 Ligation of peptides 74 and 68

The ligation between thioester 74 and β-mercapto aspartic acid containing peptide 68 was evaluated. Since synthesizing 68 was rather troublesome, thioester 74 was used in a twofold excess to ensure full conversion.

Scheme 66: Synthesis of 75: a) 6 M GdmCl, 0.2 M Na₂HPO₄, 5 eq. TCEP, 37°C, 2 days

The ligation was conducted using a buffer with 6 M GdmCl, 0.2 M Na₂HPO₄ and 5 eq. TCEP at pH 7 as proposed by Payne and coworkers. However, learning from the quick desulfurization during purification of β-mercapto aspartic acid containing peptides 70, 71 and 72, the concentration of TCEP was lowered. The reaction was incubated at 37°C and monitored with LC-MS.
Figure 48: LC-MS analysis of the ligation between 68 and 74 [LC on Agilent 1100: HydroSphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H2O (0.1% TFA) in 30 min]

Within 24 hours peptide 68 was completely consumed and LC-MS analysis showed traces of the desired product (Figure 48). The reaction was incubated for additional 24 hours and purified using gel filtration (Superdex™ peptide 10/300) eluting with 20% ACN in water. Unfortunately, only an inseparable mixture of hydrolyzed thioester 74, thioester 74 and ligation product 75 were found.

5.7 Conclusion and Outlook for synthesis of fragment 2 using strategy 1

For the synthesis of fragment 2 using strategy 1 the segment 178-213 was synthesized as one peptide fragment. The generation of the required peptides and glycopeptides containing a β-mercapto aspartic acid at Asp178 was successful but suffered from significant side reactions and problems during purification. Optimization of the conditions during automated SPPS and installation of pseudoprolines improved the purity and total yield of the desired products. However, oxidation of the methionine residues Met213, Met206 and Met205 could not be avoided during peptide assembly and all peptides were reduced after synthesis.

For the synthesis of fragment 2 using strategy 1, four peptides having a β-mercapto aspartic acid at position Asp178, including 3 glycopeptides, were synthesized. For the synthesis of glycopeptides Asp181 and/or Asp197 were installed bearing an orthogonal allyl protecting group. After cleavage of the allyl ester with palladium, amino glycan 19 was attached using Lansbury coupling. The in general successful synthesis was hampered greatly by the formation of oxidized side products. Additionally, Cys179 was involved in unexpected side reactions. Using strategy 1, the free thiol functions of Cys179 and β-mercapto Asp178 formed intra- and intermolecular disulfide bonds. Thus before purification, the peptide were treated with TCEP and peptide 68 was successfully isolated after RP-HPLC purification.
Unfortunately, using the same conditions for the reduction of the disulfides of glycopeptides 70, 71 and 72 desulfurization was observed and the glycopeptides 70, 71 and 72 were isolated as desulfurized products. Thus, only 68 was obtained and further evaluated for ligation to fragment 3. In future applications, it should be beneficial to mask the thiol function of Cys179 with Acm to avoid the formation of intramolecular disulfides and thereby simplifying purification (Scheme 67).

Additionally, a small fragment (160-177) of protein thioester (fragment 3) was synthesized as peptide hydrazide 73. After purification, peptide 73 was transformed into the corresponding thioester 74 in 10% yield.

The ligation of modelpeptide thioester 74 and β-mercapto aspartic acid containing peptide 68 was conducted in a buffer system with a low TCEP concentration. Although LC-MS analysis showed the formation of the desired product, the yield of the ligation was low and the desired product could not be isolated from the hydrolyzed starting material.

5.8 Strategy 2: Fragment 2 for C to N Ligation

In order to explore a sequential ligation strategy in C to N direction β-mercapto Asp178 was installed having an orthogonal protecting group using Boc-Asp(OtBu;SAcm)-OH 9. After successful ligation the Acm group can be removed and further ligation to an expressed protein thioester can be investigated. Additionally, the β-carboxylate of Asp181 was equipped with an orthogonal protecting group to enable Lansbury coupling after complete SPPS.

5.9 Synthesis of fragment 2 for C to N Ligation without glycosylation

The synthesis of peptide 76 with an orthogonal protected β-mercapto aspartic acid at position Asp178 started from peptide-resin 67. Boc-Asp(OtBu;SAcm)-OH 9 was coupled manually in
a two-fold excess in a fritted syringe using PyBOP and DIPEA as coupling reagents. A Kaiser test confirmed that no free amine groups remained and the coupling was complete. Thereafter, product 76 was cleaved from the solid support with TFA and scavengers (Scheme 69).

Scheme 69: Synthesis of 76: a) 9, PyBOP, DIPEA, DMF, rt, 16h, b) TFA/water/TIPS (190/5/5, v/v/v)

The product was analyzed using LC-MS and MALDI-TOF and confirmed the success of the synthesis.

Figure 49: LC-MS analysis of 76 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H2O (0.1% TFA) in 30 min]

As described for peptide hydrazide 68, the synthesis suffered from significant oxidation of methionine residues hampering the purification using RP-HPLC. To avoid multiple purification steps using RP-HPLC was crude peptide hydrazide 76 (Figure 49) directly converted into the corresponding thioester 77, and reduced afterwards.

Scheme 70: Synthesis of 77: a) 6 M GdmCl, 0.2 M Na2HPO4, 0.02 M NaN3O3, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min, 10% (40% brsm) c) NH4I, DMS, TFA, 0°C, 20 min

Thioesterformation was conducted using NaN3O2 at pH 3 as oxidative condition and MMBA at pH 7.144 Due to the oxidized side products, purification with RP-HPLC was not beneficial at this point and the peptide thioester 77 was reduced with NH4I and DMS in TFA.79 Unfortunately, after reduction an inseparable mixture was observed and pure thioester 77 could not be isolated. Careful investigation revealed that during thioesterformation a thiolactone formed involving the free thiol function of Cys179. Peptide thioester 77 and its
corresponding thiolactone were not separable from undesired impurities (i.e. deletion sequences). Thus, purification of the peptide hydrazide before thioester synthesis is necessary to obtain a clean thioester for ligation.

5.10 Synthesis of Fragment 2 for C to N Ligation with Glycosylation at Asn181

Starting from peptide-resin 66 the desired peptide was synthesized using the conditions optimized for fragment 2. Thereafter, Boc Asp(OBu;SAcm)-OH 9 was coupled manually in a twofold excess in a fritted syringe using PyBOP and DIPEA as coupling reagents (Scheme 71). A Kaiser test confirmed complete coupling.

Desired product 78 was observed together with deletion sequences and the complete sequence with one or more oxidized methionine residues (Figure 50).

Notably, when the thiol function of β-mercapto Asp178 was masked with Acm, the formation of disulfides was avoided, indicating the properties of β-mercapto Asp178 greatly influence the formation of disulfides. Thereafter, the allyl ester of Asp181 was cleaved from the peptide-resin 78 and the released carboxylate was coupled to amino glycan 19 using the conditions described for the synthesis of 70 (Scheme 72).
The desired product was found together with oxidized peptide species as was observed previously. To reduce the sulfoxide moieties, 79 was treated with NH4I and DMS in TFA. After quenching the reaction with a saturated ascorbic acid solution, the peptide was purified using gel filtration (Superdex™ peptide 10/300) eluting with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18) to give pure peptide hydrazide 79 in 5% yield (Figure 51).

Peptide hydrazide 79 was transformed into a thioester 80 using NaNO2 at pH 3 as oxidative condition and MMBA at pH 7 (Scheme 73)."
Thioester 80 was purified using gel filtration (Superdex™ peptide 10/300) with 20% ACN in water. The conversion resulted in a mixture of desired thioester 80 and thiolactam 81 that resulted from a transthioesterification between the C-terminal thioester and the mercapto group of the side chain from Cys179 (Figure 52). Thioester 80 and thiolactam 81 exist in equilibrium and can both be used for NCL.144

5.11 Ligation of peptides 80/81 and 56

The ligation between 80/81 and peptide 56 was evaluated.

The ligation was conducted using a buffer the optimized buffer with 6 M GdmCl, 0.2 M Na₂HPO₄, 5 eq. MMBA and 30 eq. TCEP at pH 7 (Scheme 74). The ligation was monitored using HPLC. After 24 and 72 hours HPLC analysis showed that the starting materials were not consumed and the reaction was not proceeding. Thus, MPAA was added to form a more reactive thioester via transthioesterification. After additional 24 hours, LC-MS analysis showed complete consumption of thioester 80 and the reaction mixture was purified using gel filtration (Superdex™ peptide 10/300) eluting with 20% ACN in water. Unfortunately, desired product was not observed and only hydrolyzed thioester was found.
5.12 Conclusion and outlook for synthesis of fragment 2 using strategy 2

The synthesis of PrP fragment 2 having an orthogonal protected β-mercapto aspartic acid Asp178 for strategy 2 was conducted using the conditions optimized for fragment 2. Installation of pseudoprolines improved the purity and total yield of the desired products. Yet, the methionine residues Met213, Met206 and Met205 oxidized during peptide assembly and reduction of the peptides was necessary. Thereby, peptide hydrazide 77 and glycopeptide hydrazide 79 were successfully synthesized. As previously described, the general successful synthesis was greatly hampered by the formation of oxidized side products. Additionally, the free thiol function of Cys179 resulted in formation of a thiolactam during the synthesis of the thioesters. This side product complicated the purification of the crude thioesters. Thus, glycopeptide hydrazide 79 was purified before thioester formation. Finally, with this optimized strategy desired product 80 was isolated as a mixture with the corresponding thiolactam 81. Unfortunately, additional purification steps reduced the final yields. Therefore, for future experiments protection of Cys179 should be considered to avoid this side reaction and facilitate isolation of pure products (Scheme 75).

Scheme 75: New Retrosynthetic analysis of strategy 1: fragment 2 for C to N ligation

The reactivity of thioester 80 was investigated in a native chemical ligation reaction to peptide fragment 56. However, independent of the reactivity of the thioester used the desired product could not be observed and only hydrolyzed product was found.

5.13 Strategy 3: Synthesis of fragment 2 via SPFC

The synthesis of fragment 2 (178-213) suffered from different side reactions, e.g. deletion sequences and oxidation of the product, when employing strategy 1 and 2. Interestingly, for the synthesis of PrP (Fmoc198-213) 66 these side reactions were not observed. Taking this observation into account, it was clear that the synthesis of fragment 2 in smaller segments should be beneficial. Hence, a strategy using solid phase fragment condensation was designed for the synthesis of fragment 2. For SPFC it is important to choose suitable fragments. Especially, it is important to avoid racemization during fragment coupling, whereby proline and glycine are ideal C-terminal amino acids.
Thus, PrP II (178-213) was separated in two peptide segments: fragment 2B PrP (178-195) and fragment 2A PrP (196-213), having Gly195 as the C-terminal amino acid of fragment 2B (Scheme 76).

To enable thioester formation and ligation to fragment 1, fragment 2B was synthesized as peptide hydrazide. Fragment 2A was synthesized on an acid labile trityl-ChemMatrix® resin, thereby allowing cleavage from the solid support under mild acidic conditions, leaving all side chain protecting groups intact. Additionally, Asp181 and/or Asp197 were equipped with an orthogonal protecting group for Lansbury coupling after complete SPPS and SPFC.

### 5.14 Synthesis of fragment 2A

The synthesis of peptide 83 was started from peptide 66. Amino acid Glu196 and Asn197 were coupled manually using PyBOP as coupling reagent and DIPEA as base. Fmoc was removed with 20% piperidine in DMF (Scheme 77). After complete assembly, a test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v). LC-MS analysis showed the success of the synthesis of peptide hydrazide 83 and only minor deletion sequences were observed (Figure 53).

---

**Scheme 76: Retrosynthetic analysis of strategy 3: fragment 2 via SPFC**

**Scheme 77: Synthesis of 83:** a) Fmoc-SPPS: 1. 2x 10 eq. Fmoc-AA-OH PyBOP/DIPEA 2. 2x 20% piperidine
In addition, peptide fragment 2A was generated bearing an orthogonal protected aspartic acid. After coupling of fragment 2A and 2B, the orthogonal protecting group can be removed and Lansbury coupling will give access to the desired glycopeptide. First, Asp197 was installed having an allyl side chain protection to enable Lansbury coupling (Scheme 78). The peptide hydrazide 84 was synthesized with the same conditions as used for 83.

Scheme 78: Synthesis of 84: a) 1. 2x 10 eq. Fmoc-AA-OH, 10 eq. PyBOP 20 eq. DIPEA 2. 2x 20% piperidine, b) 2x 10 eq. Fmoc-AA-OH, 10 eq. PyBOP 20 eq. DIPEA

A test cleavage using TFA/water/TIPS (190/5/5, v/v/v) was performed. LC-MS analysis showed desired peptide 84 was synthesized successfully in high crude purity (Figure 54).
Additionally, fragment 2A was synthesized having a photolabile protecting group at Asp197 using Fmoc-Asp(OMap)-OH 11 was coupled manually in a twofold excess using PyBOP as coupling reagent and DIPEA as base (Scheme 79). A testcleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and the peptide was analyzed using LC-MS, which showed desired peptide 85 was generated in high crude purity (Figure 55).

**Scheme 79: Synthesis of 85**

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction Conditions</th>
</tr>
</thead>
</table>
| a)   | 1. 2x 10 eq. Fmoc-AA-OH, 10 eq. PyBOP 20 eq. DIPEA  
2. 2x 20% piperidine, |
| b)   | 2x 2 eq. 11,  2eq. PyBOP 4 eq. DIPEA |

**Figure 55: LC-MS analysis of 85**

LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H2O (0.1% TFA) in 30 min

### 5.15 Synthesis of fragment 2B

To start, commercially available trityl-ChemMatrix® (loading: 0.6-1.2 mmol/g) was brominated using 10% AcBr in anhydrous DCM under argon atmosphere for four hours. Thereafter, the first amino acid (Fmoc-Gly-OH) was coupled to the activated resin using DIPEA as base. After coupling, the resin loading was determined using Fmoc-quantification of 86, which gave a loading of 0.65 mmol/g (Scheme 80).

**Scheme 80: Bromination of trityl linker 33 and coupling of first amino acid**

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>10% AcBr in DCM, 4h</td>
</tr>
<tr>
<td>b)</td>
<td>Fmoc-Gly-OH, DIPEA, 14h</td>
</tr>
</tbody>
</table>

Two peptides were synthesized with MW-SPPS using the coupling conditions, which were previously optimized for fragment 2.
First, fragment 2B [Fmoc179-195] was generated installing asparagine at Asn181. The N-terminal Fmoc protecting group was left intact to enable the synthesis of a fully protected peptide (Scheme 81). After the desired sequence was completely assembled, a test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v). LC-MS analysis proved the successful synthesis of 87 (Figure 56).

Scheme 81: Synthesis of 87: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C
2. 2x 20% piperidine, 90°C

The desired peptide 87 was observed in a high crude purity and only minor deletion sequences were found.

Additionally, a second fragment 2B was synthesized, installing Asp181 with an orthogonal allyl protecting group (Scheme 82).

Scheme 82: Synthesis of 88: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C
2. 2x 20% piperidine, 90°C

The synthesis was performed using the same coupling conditions as for 87. After complete assembly of peptide 88 a test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and analyzed using LC-MS, which proved the effective synthesis of 88 in a high crude purity with minor deletion sequences (Figure 57).
5.16 Cleavage of fully protected fragment 2B [179-195]

Having fragment 2B, 87 and 88, in hand, the cleavage conditions to obtain fully protected peptides were evaluated.

The resin was treated three times with 10 ml 1% TFA in DCM for 3 min and immediately quenched with DIPEA in DCM. The resin was washed with DCM and methanol and concentrated under reduced pressure. The resulting solid was dissolved in DCM and extracted with sat. NaCl-solution and water. The organic layer was dried over NaSO₄, concentrated under reduced pressure and crude peptide 89 was obtained in 39% yield.

![Diagram of peptides 87, 88, and 89]

Scheme 83: Synthesis of 89: a) 1% TFA in DCM, 3x3min

Fully protected peptide 89 was analyzed using MALDI-TOF and ESI-MS (Q-Tof). Unfortunately, a variety of side products with one or more missing protecting groups was observed. To remove the partially deprotected peptides, peptide 89 was purified on a Sephadex LH-20 size exclusion column eluting with 20% DCM in methanol. Nevertheless, separation of fully protected and partially protected was not possible. With the mixture of fully and partially protected peptide 89 a condensation reaction with fragment 2A was not beneficial.

To release fully protected 90 having an orthogonal allyl protecting group at Asp181, the same cleavage conditions as introduced for 89 were used.
The fully protected peptide 90 was analyzed using MALDI-TOF and ESI-MS (Q-Tof). The desired product was observed as a mixture with partially deprotected products. Removal of partially deprotected peptides using a Sephadex LH-20 size exclusion column eluting with 20% DCM in methanol was not successful and coupling to fragment 2A was not possible.

### 5.17 Conclusion and outlook for synthesis of fragment 2 using strategy 3

The synthesis of fragment 2 was evaluated using solid phase fragment condensation. PrP fragment 2A (196-213) was successfully synthesized. Three different peptide hydrazides were obtained, including two peptides containing an orthogonal protecting group on Asp197. Only few deletion sequences were observed and no oxidized methionine residues were found. Peptide hydrazides 83, 84 and 85 can be coupled to fully protected fragment 2B using SPFC, thereby giving the desired PrP II (178-213) without oxidized side products. Additionally, after successful coupling to fragment 2B, allyl- or Map-protecting groups can be removed to synthesize the desired glycopeptide using Lansbury coupling.

Additionally, two PrP fragments 2B (179-195) were generated, including one peptide having an allyl protecting group on Asp181. Both peptides, 87 and 88, were observed in a high crude purity with only minor deletion sequences. Unfortunately, when the peptide was cleaved from the resin using mild acidic conditions, the cleavage of several side chain protecting groups was observed. The mixture of partially deprotected and fully protected peptides was inseparable and SPFC could not be performed.

In comparison to strategy 1 and 2, the desired peptides for the synthesis of fragment 2 using SPFC were synthesized in a high crude purity. However, cleavage of the two fragments 2B with all side chain protecting groups intact was not possible.

Thus, in future applications, it would be beneficial to synthesize smaller fully protected peptides. For example, this can be achieved by using additional fragments having pseudoproline Ile182Thr183 and Val189Thr190 as C-terminal amino acids.\(^{132}\)
5.18 Strategy 4: Synthesis of Fragment 2 using a Ligation-Desulfurization Strategy

PrP 2 (178-213) was synthesized using SPPS as described in strategy 1 and 2. However, the synthesis suffered from various side reactions, including oxidation of different methionine residues and deletion sequences. As described in strategy 3, these shortcomings can be overcome by segmenting fragment 2 into smaller peptides that are easier to synthesize and purify. Therefore, a ligation-desulfurization strategy was evaluated. Thus, a ligation position using a β- or γ-mercapto amino acid is necessary. These unnatural amino acids are not commercially available and the synthesis involves a multi-step strategy. Fortunately, Boc-Asp(OtBu,STmob)-OH 8 was already synthesized for installation at Asp178 and could also be used at Asp202 to facilitate a second ligation-desulfurization step.

Thus, to synthesize fragment 2 using a ligation-desulfurization, the sequence was separated in two fragments: fragment 2C PrP (202-213) with Boc-Asp(OtBu,STmob)-OH 8 installed as N-terminal amino acid and fragments 2D PrP (178-204). Fragment 2D contains the two glycosylation sites and Asp178 is installed as orthogonal protected β-mercaptop aspartic acid (Scheme 85). To enable ligation, both fragments were obtained as hydrazides to facilitate thioester formation.39, 51

5.19 Synthesis of Fragment 2C PrP [202-213]

Starting from Wang hydrazide 55 fragment 2 was synthesized using SPPS on the automated peptide synthesizer Liberty Bule™. The first amino acid, i.e. methionine, was coupled using PyBOP and DIPEA. A loading of 0.62 mmol/g was observed using Fmoc-quantification. Thereafter, the peptide was elongated using the conditions optimized for fragment 2. Boc-Asp(OtBu;STmob)-OH 8a was coupled manually in a 3.6 fold excess in a fritted syringe using PyBOP and DIPEA as coupling reagents (Scheme 86). A Kaiser test confirmed
complete coupling and the product was cleaved from the solid support with TFA and scavengers.

Scheme 86: Synthesis of 91: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C 2. 2x 20% piperidine, 90°C; b) 3.6 eq. 8a, 3.6 eq PyBOP, 8 eq. DIPEA, DMF, rt, 16h, c) TFA/water/TIPS (190/5/5, v/v/v)

Crude product 91 was analyzed using LC-MS and MALDI-TOF and confirmed the success of the synthesis. Fortunately, no oxidized methionine residues were found. Thereafter, 91 was purified using RP-HPLC (YMC hydrosphere RP C18).

Figure 58: LC-MS analysis of purified 91 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H₂O (0.1% TFA) in 30 min]

Desired peptide 91 was obtained in 24% yield and used for ligation with fragment 2D (Figure 58).

5.20 Synthesis of fragment 2D using Map protected β-mercapto Aspartic acid 10

Starting from Wang hydrazide 55, the first amino acid (i.e. threonine) was coupled using PyBOP and DIPEA. A loading of 0.59 mmol/g was observed using Fmoc-quantification. The peptide was elongated using the conditions optimized for fragment 2.

Scheme 87: Synthesis of 92: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 90°C 2. 2x 20% piperidine, 90°C
A test cleavage using TFA/water/TIPS (190/5/5, v/v/v) was performed and product 92 was analyzed using LC-MS and showed the desired product in a high crude purity (Figure 59).

Figure 59: LC-MS analysis of 92 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H₂O (0.1% TFA) in 30 min]

Boc-Asp(OrBu,SMap)-OH 11 was coupled manually using PyBOP as coupling reagent and DIPEA as base (Scheme 88). After complete assembly, a test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and the product was analyzed using LC-MS.

Scheme 88: Synthesis of 93: a) 2 eq. 10, 2 eq. PyBOP, 4 eq. DIPEA, DMF, 16 h, b) TFA/water/TIPS (190/5/5, v/v/v)

Surprisingly, desired product 93 was observed only in minor amounts and aspartimide 93a was the main product (Figure 60). Since this side reaction was not seen before when β-mercapto aspartic acid building blocks 8 or 9 were used, it was assumed that the photolabile Map group is responsible for the aspartimide formation. To evaluate, whether this side can be circumvented with a change in coupling conditions, DIC and HOBt were used to couple 10. Unfortunately, as shown in LC-MS analysis, aspartimide formation was not avoided and desired product 93 was observed only in small quantity. Additionally, a different cleavage cocktail was used. All testcleavages were performed using reagent K: TFA/ethane dithiol/thioanisole/phenol/water (85:5:5:2.5:2.5:v:v:v:w:v). Independent of the coupling conditions and cleavage cocktail, aspartimide containing peptide 93a remained the main product and another strategy to obtain fragment 2D with a protected N-terminal β-mercapto aspartic acid was investigated.
5.21 Synthesis of fragment 2D using ligation to thioester 45

To have access to PrP (178-201) bearing a protected thiol function on the N-terminal β-mercapto Asp178, a strategy was evaluated that used ligation of Cys179 from fragment 2D and Boc-Asp(OtBu,STmob) thioester 45.

Therefore, four peptide hydrazides with a free N-terminal cysteine were synthesized, including three glycopeptides.

5.22 Synthesis of PrP [179-201] without glycosylation

Peptide 92 was used and cleaved from the solid support using TFA/water/TIPS (190/5/5, v/v/v) and the product was purified on RP-HPLC (YMC hydrosphere RP C18). The desired product was obtained in 8% yield (Figure 61).
5.23 Synthesis of PrP [179-201] with glycosylation at Asn197 and Asn181

Starting from Wang hydrazide 55 fragment 2 was synthesized using SPPS on the automated peptide synthesizer Liberty Bule™. The first amino acid, *i.e.* threonine, was coupled using PyBOP and DIPEA. A loading of 0.63 mmol/g was observed using Fmoc-quantification. The peptide was elongated using the conditions optimized for fragment 2 (Scheme 90).

A test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and the product was analyzed using LC-MS. Desired intermediate 94 was observed in high crude purity (Figure 62). Thereafter, the free Nα-function was protected with Boc to avoid side reactions during Lansbury coupling. Di-t-butyl dicarbonate (Boc-anhydride) and pyridine in DMF were added to the resin and the slurry was shaken for 20 min. A Kaiser test was performed to ensure complete protection. Glycopeptide 95 was generated using the same conditions introduced for the synthesis of 70.
Thereafter, the allyl ester was cleaved to give a free aspartic acid side chain, using Pd(PPh₃)₄ and phenylsilane as scavenger in DCM. A test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and LC-MS showed the reaction went smoothly and the protecting group was completely removed.

Scheme 91: Synthesis of 95: a) Boc₂O, pyridine, DMF, 20 min b) Pd(PPh₃)₄, phenylsilane, DCM, 2 h, quant. c) 19, PyBOP, DIPEA, DMF/DMSO, 16 h quant d) TFA/water/TIPS (190/5/5, v/v/v)

Afterwards, amino glycan 19 was coupled to the β-carboxylate using PYBOP and DIPEA in DMF/DMSO (Scheme 91). The reaction was complete after 16 hours and 95 was cleaved from the resin with TFA/water/TIPS (190/5/5, v/v/v). Crude peptide 95 was purified using RP-HPLC (YMC hydrosphere RP C18). The desired product was obtained in 7% yield (Figure 63).
5.24 Synthesis of PrP [179-201] with glycosylation at Asn181

Additionally, a second glycopeptide was synthesized using the same strategy shown for 95.

![Scheme 92: Synthesis of 97](image)

Starting from Wang hydrazide Resin 55, the peptide was elongated using the conditions optimized for fragment 2.

![Figure 64: LC-MS analysis of 97](image)

After complete assembly, the free Nα-function was capped using Boc-anhydride. Thereafter, the allyl ester was cleaved using Pd(PPh3)4 and phenylsilane as scavenger in DCM. Amino glycan 19 was coupled to the peptide using PYBOP and DIPEA in DMF/DMSO. Glycopeptide 97 was cleaved from the resin with TFA/water/TIPS (190/5/5, v/v/v) and purified using RP-HPLC (YMC hydrosphere RP C18). The desired product was isolated in 9% yield (Figure 64).

5.25 Synthesis of PrP [179-201] with glycosylation at Asn197

PrP (179-201) with a glycosylation at Asn197 was synthesized using the same strategy that was used for the synthesis of 95 and 97.
The synthesis was started from Wang hydrazide 55 and the optimized conditions for fragment 2 were used. After capping of the free amino with Boc-anhydride, the allyl ester was cleaved with Pd(PPh3)4 and amino glycan 19 was coupled using PyBOP and DIPEA in DMF/DMSO.

After purification using RP-HPLC (YMC hydrosphere RP C18) glycopeptide 99 was obtained in 5% yield (Figure 65).

**5.26 Ligation of PrP II [179-201] 92 with β-mercapto aspartic acid thioester 45**

After the synthesis of the required PrP fragments [179-201] was completed, the ligation to thioester 45 was evaluated to obtain the desired fragments 2C. First, peptide 92 and thioester 45 were reacted using the buffer system optimized for the synthesis of 63. Thioester 45 precipitated in the aqueous buffer and the ligation product 100 did not form. Since, thioester 45 was not soluble in water, thus a 50 mM solution of 45 in THF was added.
The reaction was monitored using HPLC, but the desired product 100 was not observed, which was attributed to low solubility of 45 in the ligation buffer.

### 5.27 Synthesis of fragment 2D using Acm protected β-mercapto aspartic acid 9

To have access to PrP (178-201) bearing a protected thiol function on the N-terminal β-mercapto Asp178, a strategy using orthogonal protected aspartic acid building block 9 was evaluated (Scheme 95). After successful ligation the orthogonal Acm group can be removed and the peptide can be ligation with an expressed protein thioester. Additionally, to have access to homogeneous glycosylated peptides, the β-carboxylate of Asp181 and/or Asp197 was installed having an orthogonal ally protecting group. After complete peptide assembly, the allyl ester can be cleaved to enable Lansbury coupling.

**Scheme 95**: Retrosynthetic analysis of fragment 2D using orthogonal protected aspartic acid 9

### 5.28 Synthesis of PrP [Asp(SAcm)178-201] with glycosylation at Asn197 and Asn181

The synthesis of desired Acm protected glycopeptide hydrazide 103 was started by reacting peptide 94 with building block 9 using PyBOP and DIPEA (Scheme 96).

**Scheme 96**: Synthesis of 101: a) 2 eq. 9, 2 eq. PyBOP, 4 eq. DIPEA, DMF, 16 h

A Kaiser test and a testcleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) to prove complete coupling of Boc-Asp(OrBu,SACm)-OH. LC-MS analysis showed the success of the reaction (Figure 66). Afterwards, the glycopeptide was synthesized using the same conditions described for 70.
The allyl ester was cleaved to give a free aspartic acid side chain using Pd(PPh₃)₄ and phenylsilane as scavenger in DCM (Scheme 97).

A test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and LC-MS showed the protecting group was completely removed (Figure 67).

Having peptide 102 with free aspartic acid 181 and 197 in hand, desired glycopeptide 103 was generated using amino glycan 19, PyBOP and DIPEA in DMF/DMSO (Scheme 98). The reaction was complete after 16 hours and 103 was cleaved from the resin with TFA/water/TIPS (190/5/5, v/v/v). Crude peptide 103 was purified using RP-HPLC (YMC hydrosphere RP C18). The desired product was obtained in 12% yield (Figure 68).
Thereafter, the synthesis of fragment 2D [178-201] bearing two large N-glycans was evaluated. The required amino nonasaccharide 104 was generated starting from 13 using reductive conditions. In a first synthesis, azide 13 was reduced using the same condition established for 19.

Nona-azide 13 was dissolved in anhydrous methanol and the corresponding amine was generated by treatment with hydrogen in the presence of palladium catalyst (Scheme 99).

The resulting Nona-amine 104 was directly coupled to peptide 102 using PYBOP and DIPEA in DMF/DMSO (Scheme 100).
Scheme 100: Synthesis of 105: a) 104, PyBOP, DIPEA, DMF/DMSO, 16 h b) TFA/water/TIPS (190/5/5, v/v/v)

A test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and the reaction was screened using LC-MS. Unfortunately, the desired product was not observed. Analysis of the recovered glycan showed remaining azide 13, indicating that the reduction using palladium under hydrogen atmosphere was incomplete. Therefore, azide 13 was reduced using the conditions introduced by Unverzagt and coworkers. Nona-azide 13 was dissolved in anhydrous methanol and ethanedithiol and DIPEA were added. After 2 hours, the mixture was concentrated and Nona-amine 104 was crushed out in ether (Scheme 99). Dried product 104 was directly coupled to peptide 102 using PYBOP and DIPEA in DMF/DMSO (Scheme 100). LC-MS of a test cleavage showed that no product was observed. Thereafter, HATU and HOBt were used as coupling reagents and DIPEA as base (Scheme 101).

Scheme 101: Synthesis of 105: a) 104, HATU, HOBt, DIPEA, DMF/DMSO, 16 h b) TFA/water/TIPS (190/5/5, v/v/v)

A test cleavage was performed using TFA/water/TIPS (190/5/5, v/v/v) and the reaction was analyzed using LC-MS. Unfortunately, LC-MS analysis showed that the desired product was not formed, but significant aspartimide formation was observed. With this result, it can be assumed that the β-carboxylates of Asp181 and Asp197 are not assessable for coupling to a large glycan.

5.29 Synthesis of PrP [Asp(SAcm)178-201] with glycosylation at Asn181

The synthesis of desired Acm protected glycopeptide hydrazide 106 was performed using the same conditions previously described for the synthesis of 103. Starting from peptide-resin 96, Acm protected β-mercapto aspartic acid was coupled using PyBOP and DIPEA. A Kaiser test and a test cleavage confirmed the success of the coupling. Afterwards, the allyl ester was cleaved using palladium and amino glycan 19 was coupled using PyBOP and DIPEA. Next,
the desired glycopeptide hydrazide was cleaved from the solid support using TFA/water/TIPS (190/5/5, v/v/v) (Scheme 102).

Scheme 102: Synthesis of 106: a) 2 eq. 9, 2 eq. PyBOP, 4 eq. DIMEA, DMF, 16 h b) Pd(PPh3)4, phenylsilane, DCM, 2 h, quant; c) 19, PyBOP, DIPEA, DMF/DMSO, 16 h, quant; d) TFA/water/TIPS (190/5/5, v/v/v)

Crude 106 was purified using RP-HPLC (YMC hydrosphere RP C18). The desired product was obtained in 9% yield (Figure 69).

Figure 69: LC-MS analysis 106 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H2O (0.1% TFA) in 30 min]

5.30 Synthesis of PrP [Asp(SAcm)178-201] with glycosylation at Asn197

Additionally, glycopeptide hydrazide 107 with an orthogonal protected β-mercapto aspartic acid was synthesized using the conditions established for 103.

Scheme 103: Synthesis of 107: a) 9, PyBOP, DIPEA, DMF, 16 h; b) Pd(PPh3)4, phenylsilane, DCM, 2 h, quant; c) 19, PyBOP, DIPEA, DMF/DMSO, 16 h, quant; d) TFA/water/TIPS (190/5/5, v/v/v)

Acm protected β-mercapto aspartic acid was coupled using PyBOP and DIPEA. After a Kaiser test and a testcleavage showed the success of the reaction, the allyl ester was cleaved with palladium and glycan 19 was coupled PyBOP and DIPEA. 107 was cleaved from the solid support using TFA/water/TIPS (190/5/5, v/v/v) (Scheme 103) and purified using RP-HPLC (YMC hydrosphere RP C18). The desired product was obtained in 8% yield (Figure 70).
5.31 Synthesis of PrP [Asp(SAcm)178-201] thioesters with glycosylation at Asn197 and/or Asn181

After the successful synthesis and purification of glycopeptide hydrazides 103, 106 and 107, the products were converted into the corresponding thioester to enable ligation to 91.

Starting with hydrazide 103, thioesterformation was conducted using the conditions introduced for 80. Peptide hydrazide 103 was transformed into a thioester using NaNO₂ at pH 3 as oxidative condition and MMBA at pH 7 (Scheme 104).

Scheme 104: Synthesis of 108: a) 6 M GdmCl, 0.2 M Na₂HPO₄, 0.02 M NaNO₂, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min

Figure 71: LC-MS analysis of 108 and 109 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H₂O (0.1% TFA) in 30 min]
Thioester 108 was purified using gel filtration (Superdex™ peptide 10/300) eluting with 20% ACN in water. The desired thioester 108 was isolated in a mixture with thiolactone 109 and MMBA in 84% yield (Figure 71).

In addition, thioester 110 was synthesized starting from hydrazide 106 by reaction in diazotation buffer containing NaNO₂ at pH 3 and MMBA at pH 7 (Scheme 105).

![Scheme 105: Synthesis of 110](image)

After purification using gel filtration, thioester 110 was obtained in a mixture with thiolactone 111 and MMBA in 79% yield (Figure 72).

Additionally, glycopeptide hydrazide 107 was used to generate thioester 112. Peptide hydrazide 107 was treated with NaNO₂ containing diazotation buffer at pH 3 and MMBA at pH 7 (Scheme 106).

![Scheme 106: Synthesis of 112](image)

The reaction was purified using gel filtration and thioester 112 was obtained in 64% yield (Figure 73).
5.32 Ligation of Fragment 2D thioesters and peptide 91

Afterwards, the ligation between β-mercapto aspartic acid containing peptide 91 and thioester 109 was evaluated. Beforehand, the ligation was conducted using a buffer with 6 M GdmCl, 0.2 M Na₂HPO₄ and 5 eq. TCEP at pH 7 as proposed by Payne and coworkers⁴⁷ to form product 75. Thus, the same buffer system was used to ligate thioester 108 and peptide 91. The reaction mixture was incubated at 37°C and monitored by HPLC. After 12 hours, formation of product was observed. Unfortunately, after additional 24 hours thioester 108 and thiolactonote 109 hydrolyzed completely and the reaction did not proceed any further.

Thus, a thiol containing ligation buffer was used and the ligation was conducted using a buffer with 6 M GdmCl, 0.2 M Na₂HPO₄, 5 eq. MMBA and 30 eq. TCEP at pH 7. The reaction was monitored by HPLC. After 12 hours no formation of the desired product was observed and the ligation reaction was incubated for additional 24 hours. Unfortunately, peptide 91 was only observed as disulfide with MMBA and TCEP was added. The addition of TCEP resulted in desulfurization of the β-mercapto aspartic acid of 91 and the ligation was not observed.

Taking these observations into account, the ligation buffer was further optimized and 6 M GdmCl, 0.2 M Na₂HPO₄, 1 eq. MMBA (or MPAA), 10 eq. TCEP, pH 7, 37°C were used (Scheme 107).

The ligation was incubated at 37°C and monitored by HPLC. After 12 hours the desired product 113 was observed. The reaction was incubated for additional 24 hours. Unfortunately,
the reaction did not proceed, the MMBA-adduct of 91 was observed. Again, the addition of TCEP to reduce the disulfide resulted in desulfurization of 91. Additionally, in this buffer system thioester/thiolactone 108/109 hydrolyzed very fast.

Thereafter, the buffer system was evaluated using MPAA as thiol to form a more reactive thioester and help the reaction to progress faster and result in a higher conversion. Hence, 6 M GdmCl, 0.2 M Na2HPO4, 1 eq. MPAA and 10 eq. TCEP at pH 7 were used. The reaction was kept at 37°C and monitored by HPLC. After 12 hours product 113 was observed, but as already described, the reaction did not proceed because β-mercapto aspartic acid containing peptide 91 formed a disulfide with MPAA and thioester/thiolactone 108/109 hydrolyzed. Unfortunately, purification using size exclusion was not successful and 113 isolated in a mixture with hydrolyzed starting material and the disulfide peptide 91 formed MPAA.

The ligation conditions were also tested for thioester/thiolactone 110/111 and β-mercapto aspartic acid containing peptide 91. 6 M GdmCl, 0.2 M Na2HPO4, 1 eq. MPAA and 10 eq. TCEP at pH 7 were used at 37°C.

The reaction was monitored using HPLC and desired product 114 was observed after 12 hours as disulfide with MMBA. However, as described previously, the conversion was very low, and the reaction was not proceeding due to formed disulfides and hydrolysis of thioester/thiolactone 109/110. Purification of the reaction mixture using size exclusion chromatography was not successful and clean 114 was not separated from the hydrolyzed thioester and the disulfide peptide 91 formed MMBA.

Additionally, thioester 112 was ligated to β-mercapto aspartic acid containing peptide 91 using 6 M GdmCl, 0.2 M Na2HPO4, 1 eq. MPAA and 10 eq. TCEP at pH 7 and 37°C.

After 12 hours ligation product 115 was observed by HPLC. Again, the reaction showed a low conversion and only minor amounts of 115 were found and observed as disulfide. Unfortunately, isolation of product 115 using size exclusion chromatography was not successful and a mixture of the hydrolyzed thioester, the disulfide peptide 91 formed MMBA and the desired product 115 was found.
5.33 Conclusion and outlook for synthesis of Fragment 2 using strategy 4

The synthesis of fragment 2 was evaluated using a ligation-desulfurization approach. The β-mercapto aspartic acid containing PrP (202-213) was synthesized successfully using the optimized conditions for fragment 2. After purification desired peptide 91 was obtained in 25% yield and used for ligation to the thioesters of fragment 2D. Additionally, the fragment 2D was synthesized bearing a Map protected β-mercapto aspartic acid. Unfortunately, desired product 93 was isolated in a mixture with aspartimide 93a. This side reaction was not observed when building block 8 and 9 were used, indicating that protecting the thiol function with Map leads to that side reaction and 10 is not suitable for SPPS.

To evaluate the ligation to thioester 45 four peptides were generated that have a C-terminal cysteine, including three glycopeptides. For the synthesis of glycopeptides Asp181 and/or Asp197 were installed bearing an orthogonal allyl protecting group. After cleavage of the allyl ester with palladium, amino glycan 19 was attached using Lansbury coupling. Unfortunately, synthesis of fragment 2D bearing a protected β-mercapto aspartic acid by ligation to thioester 45 was not possible.

Additionally, three glycopeptides were synthesized bearing an Acm protected β-mercapto aspartic acid. Unfortunately, the installation of Map protected β-mercapto aspartic acid resulted in aspartimide formation, and building block 10 is not suitable for SPPS. Asp181 and/or Asp197 were installed bearing an orthogonal allyl protecting group and the allyl ester was cleaved with palladium. Amino glycan 19 was attached using Lansbury coupling. The purified glycopeptides were successfully converted into the corresponding thioester. As previously described, the thiol function of Cys179 formed a thiolactone in an intramolecular transthioesterification reaction with the C-terminal thioester. Having the desired peptides in hand, the ligation peptide 91 and the thioesters of fragment 2D was evaluated. First, a ligation buffer without additional thiol was used. However, without thiol the thioester/thiolactone hydrolyzed very fast, indicating the lactone is prone to hydrolysis and the addition of a thiol to the buffer is required to avoid this side reaction. Thus, a ligation buffer containing 5 eq. MMBA was investigated. Using these conditions, no product was observed because the thiol function β-mercapto Asp178 immediately formed disulfides. Unfortunately, adding TCEP to reduce the disulfides lead to desulfurization of Asp178. Thus, a ligation buffer using only 1 eq. MMBA or MPAA were used, to help avoiding the hydrolysis of the thioester/thiolactone mixture and reduce the formation of disulfides at the same time. Finally,
the desired ligation product was observable. Unfortunately, the ligation showed only a very low conversion, and after prolonged reaction the thioester/thiolactone mixture hydrolyzed and the thiol function of β-mercapto Asp178 formed disulfides. Although LC-MS analysis showed the formation of the desired product, the yield of the ligation was low and the desired product could not be isolated from the hydrolyzed starting material and the disulfide peptide formed MMBA.

Protection of Cys179 will avoid the formation of thiolactones during thioester formation, and thereby the addition of a thiol during the ligation reaction can be avoided resulting in higher conversion during the ligation reaction.

The synthesis of various glycopeptides bearing a glucosamine at Asn181 and/or Asn197 was established. Various glycopeptide hydrazides and thioesters were synthesized and purified. The peptides were obtained in high crude purity and the connection of amino glycan using Lansbury coupling resulted in quantitative conversion. However, the installation nona-saccharide did not give the desired product, independent of the coupling conditions.

Thus, to generate the desired glycopeptides bearing a large glycan an enzymatic strategy can be applied.
EXPERIMENTAL PART

1. MATERIALS AND METHODS

All chemicals were reagent grade and all solvents anhydrous high-purity grade and used as supplied except where noted otherwise. All reagents and materials were purchased from different commercial suppliers and used without further purification. Reactions were performed in oven-dried glassware under an inert argon atmosphere unless noted otherwise. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F$_{254}$ plates (0.25mm). Compounds were visualized by UV irradiation or heating the plate after dipping in staining solution. Those were cerium sulfate-ammonium molybdate (CAM) solution, basic potassium permanganate solution, acidic ninhydrin-acetone solution or a 3-methoxyphenol-sulfuric acid solution. Flash column chromatography was carried out using a forced flow of the indicated solvent on Fluka silica gel 60 (230-400 mesh, for preparative column chromatography).

$^1$H and $^{13}$C spectra were recorded on a Varian 400 (400 MHz) spectrometer in CDCl$_3$ (7.26 ppm $^1$H, 77.1 ppm $^{13}$C), D$_2$O (4.79 ppm $^1$H), MeOD (4.87 ppm and 3.31 ppm $^1$H, 49.00 ppm $^{13}$C), acetone-d6 (2.05 ppm and 2.84 ppm $^1$H, 206.26 ppm and 29.84 ppm $^{13}$C) unless otherwise stated. Coupling constants are reported in Hertz (Hz). Splitting patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; br, broad singlet; dd, doublet of doublets; m, multiplet; dt, doublet of triplets; h, hextet for $^1$H NMR data.

MALDI-TOF analysis was performed on a Bruker autoflex speed. ESI mass spectral analyses and LC-MS were performed by a Waters Xevo G2-XS QTof with an Acquity H-class UPLC. LC analysis was performed using Agilent 1100 system and LC-MS analysis were performed using Agilent 1100 or Agilent 1200 systems coupled to an ESI-MS. HPLC purification was performed using an Agilent 1200 system or Waters 600 coupled to a waters 2487 dual $\lambda$ absorbance detector. Size exclusion was performed on a Azura-FPLC from Knauer. Incubations were conducted using an eppendorf thermomixer comfort (1.5 ml scale) and a Thermo Scientific MaxQ 420 HP shaker. Products were freeze dried using a Christ Alpha2-4 LDplus lyophilizer. Centrifugation was performed on a VWR Micro Star 17R, VWR Mini Star silverline and thermo scientific Heraeus Multifuge X3R centrifuge with a Thermo TX-100 rotor or Rberlite F15-8x50cy rotor.
2. General Protocols for Peptide Synthesis

Buffer:

HPLC Buffer: Water:ACN (5:1/v:v with 0.1% TFA)

MALDI Buffer: Water:ACN (5:1/v:v with 0.1% TFA)

Diazotation Buffer: 6 M GdmCl, 0.2 M Na₂HPO₄, 0.02 M NaNO₂, pH 3

Ligation Buffer(A): 6 M GdmCl, 0.1 M Na₂HPO₄, 0.05 M MPAA, 5 mM TCEP, pH 7

Ligation Buffer(B): 6 M GdmCl, 0.1 M Na₂HPO₄, 0.05 M MMBA, 5 mM TCEP, pH 7

Ligation Buffer(C): 6 M GdmCl, 0.1 M Na₂HPO₄, 5 equivalents MPAA, 5 equivalents per thiol TCEP, pH 7

Ligation Buffer(D): 6 M GdmCl, 0.1 M Na₂HPO₄, 5 equivalents MMBA, 5 equivalents per thiol TCEP, pH 7

Ligation Buffer(E): 6 M GdmCl, 0.1 M Na₂HPO₄, 5 equivalents per thiol TCEP, pH 7

Test for free amines with Kaisertest

Free amino groups were determined using ninhydrine by the method described by Kaiser.

Solution A: 80% phenol in ethanol (w/v) and KCN in pyridine (2ml of 1mM KCN in 98 ml pyridine) in proportion 1:4 (v/v)

Solution B: 5% ninhydrine in ethanol (w/v)

A few beads washed resin were placed in an 1.5 ml Eppendorf vial, two drops of each solution were added and the mixture was heated to 90°C for 10 min. Free amino groups were indicated by a dark blue color of the resin beads.

Fmoc quantification for determining the resin substitution:

~1 mg of dry resin was placed in a 2 ml Eppendorf vial and 1.5 ml of 20% piperidine in DMF was added. The slurry was shaken for 15 min. After centrifugation, 1 ml of the deprotection solution was diluted with 1 ml 20% piperidine in DMF. The UV-absorption was measured at 290 nm in a 1 ml low volume quartz cuvette against a reference containing the initial solution. The following equation was used to determine the substitution grade:

\[
\text{substitution} \left[ \text{mmol/g} \right] = \frac{[\text{Abs} \ 290 \ nm]}{\text{mg \ resin} \times 1.75}
\]
3. General Synthetic Procedures for Peptide Synthesis

Procedure 1: Loading of Trityl-OH ChemMatrix® resin
In a fritted reaction vessel 200 mg trityl-ChemMatrix® resin (substitution grade 0.62 mmol/g) were swollen in anhydrous DCM for 2 hours. After DCM was drained, a solution of 10% AcBr in anhydrous DCM (14.00 ml) was added to the resin and the slurry was shaken for 4 hours. The resin was washed numerous times with anhydrous DCM and a solution of 350 mg Fmoc-Thr(OtBu)-OH and 400 µl DIPEA in 10 ml anhydrous DCM was added to the resin and shaken for 16 hours. The resin was washed neatly with DCM and the efficiency of the coupling was determined by Fmoc quantification. The resin was capped using a mixture of methanol, DIPEA and DCM (2:1:17) for 10 minutes.

Procedure 2: Formation of Wang hydrazide Resin 55
200 mg of ChemMatrix® Wang resin were swollen in DCM for minimum 2 hour in a 10 ml PP-reactor with PE frit. According to the resin loading (Sigma Aldrich Wang ChemMatrix® resin 0.50-1.20 mmol/g loading or Novabiochem® NovaPEG Wang resin 0.63 mmol/g) 4-nitrophenyl carbonochloridate (242 mg, 1.20 mmol) was dissolved in 2 ml DCM and 4-methylmorpholine (132 µl, 1.20 mmol) was dissolved in 2 ml DCM. The solutions were cooled in the freezer for one hour.

The syringe was placed in the freezer. At the same tome 5 eq. 4-methylmorpholine were added to 2 ml DCM and also placed in the freezer. After both solutions were cooled for approximately 1 hour, 4-methylmrrpholine in DCM was added to the resin and the reaction was shaken for 12 hours. The resin was washed with DCM/DMF/methanol/DCM and again swollen in DCM. 5 eq. hydrazine hydrate were added to 4 ml DCM:DMF (1:1/v:v) and stored in the freezer for one hour. After the solution was cold, it was added to the resin and shaken for 12 hours. The resin was washed with DCM/DMF/methanol/DCM. The resulting Wang hydrazide resin was dried at the high vacuum.

Procedure 3: Loading of Wang hydrazide Resin
200 mg Wang hydrazide of ChemMatrix® 55 was swollen in DCM for 1 hour. According to the resin loading 5 eq. Fmoc-Axx-OH, 5 eq. PyBOP and 10 DIPEA were dissolved in 2 ml DMF. The mixture was added to the resin and the slurry was shaken for 2 hours. The resin was washed with DMF and DCM and the loading was determined by Fmoc-quantification.
**Procedure 4: conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C**

Coupling reagents used are DIC and Oxyma. The coupling reagents are prepared as solutions in DMF with 1 M Oxyma, with 0.1 M DIPEA, and 0.5 M DIC. Amino acids are added as 0.2 M solutions in DMF. All amino acids are coupled twice in five-fold excess. The temperature during coupling is 90°C and the coupling time is 5 minutes, except arginine, cysteine and histidine. Cysteine and histidine were coupled at 50°C for 10 minutes and the first coupling for arginine was carried out at room temperature for 20 minutes and the second coupling was at 90°C for 5 minutes. For all amino acids Fmoc was removed twice using 20% piperidine in DMF at 90°C for 1 minute.

**Procedure 5: conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 50°C**

Coupling reagents used are DIC and Oxyma. The coupling reagents are prepared as solutions in DMF with 1 M Oxyma (with 0.1 M DIPEA) and 0.5 M DIC. Amino acids are added as 0.2 M solutions in DMF. All amino acids are coupled twice in five-fold excess. The temperature during coupling is 50°C and the coupling time is 10 minutes, except arginine. Arginine was carried out at room temperature for 20 minutes and the second coupling was at 50°C for 10 minutes. For all amino acids Fmoc was removed twice using 20% piperidine in DMF at 90°C for 1 minute.

**Procedure 6: conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 50°C and Deprotection without elevated Temperature**

Coupling reagents used are DIC and Oxyma. The coupling reagents are prepared as solutions in DMF with 1 M Oxyma (with 0.1 M DIPEA) and 0.5 M DIC. Amino acids are added as 0.2 M solutions in DMF. All amino acids are coupled twice in five-fold excess. The temperature during coupling is 50°C and the coupling time is 10 minutes, except arginine. Arginine was carried out at room temperature for 20 minutes and the second coupling was at 50°C for 10 minutes. For all amino acids Fmoc was removed three times using 20% piperidine in DMF without microwave for 5 minutes.
Procedure 7: conditions for manual peptide elongation

Before usage, PyBOP was recrystallized from Hexane/DCM.

Building blocks used in manual peptide elongation:

- Fmoc-L-Phe-L-Thr[Ψ(Me,Me)Pro]-OH
- Fmoc-L-Val-L-Thr[Ψ(Me,Me)Pro]-OH
- Fmoc-L-Ile-L-Thr[Ψ(Me,Me)Pro]-OH
- Boc-Asp(OtBu;STmob)-OH \( (8) \)
- Boc-Asp(OtBu;SAc)-OH \( (9) \)
- Boc-Asp(OtBu;SMap)-OH \( (10) \)
- Fmoc-L-Asp(OMap)-OH \( (11) \)

According to the resin loading 2 eq. of the building block, 2 eq. PyBOP and 4 equivalents DIPEA were dissolved in DMF and added to the resin. The resin was shaken for 20 minutes. The building block was coupled twice and washed with DMF/DCM/DMF.

Procedure 8: Removal of Allyl

The peptide was swollen in anhydrous DCM and according to the resin loading 0.2 eq. Pd[P(Ph)\(_3\)]\(_4\) and 20 eq. phenylsilane per allyl protecting group were added in anhydrous DCM. The mixture was shaken for two hours under exclusion of light. The resin was washed with DCM/DMF/DMSO/DCM. To ensure the completeness of the reaction a test cleavage was performed. In case of incomplete allyl removal, the reaction was repeated.

Procedure 9: Lansbury Coupling of amino Glycans

The peptide was swollen in anhydrous DMF. According to the resin loading 2 eq. PyBOP and 4 eq. DIPEA in anhydrous DMF were added to the resin. 2 eq. of amino glycan were dissolved in anhydrous DMSO and added to the resin. The slurry was shaken for 16 hours and was washed with DCM/DMF/DCM. To ensure the completeness of the reaction a test cleavage was performed.

Procedure 10: Reduction of Peptides

The peptide was dissolved in TFA to give a final concentration of 1.5 mmol. The reaction was cooled to 0°C and 50 equivalents DMS and 50 equivalents NH\(_4\)I were added. The mixture was shaken for 20 minutes and quenched with sat. ascorbic acid solution in water. The solution was concentrated under nitrogen and crushed out with ice cold diethyl ether. The precipitate was centrifuged and washed two more times with ice cold diethyl ether. The resulting peptide was dried, dissolved in HPLC Buffer 1 and lyophilized.
Procedure 11: Boc Capping

The peptide was swollen in anhydrous DCM in a fritted syringe. According to the resin loading 20 eq. di-tert-butyl dicarbonate (Boc-anhydride) and 25 eq. pyridine were dissolved in DMF to give a final concentration of 10 mmol. The mixture was added to the resin and the slurry was shaken two hours. A Kaiser test was performed to evaluate the completeness of the reaction. In case of incomplete capping, the reaction was repeated.

Procedure 12: Conversion of peptide hydrazides to thioesters

The peptide hydrazide was dissolved in diazotation buffer at –10°C to give a final concentration of 1 mmol and stirred for 20 min. To that solution 60 eq. MMBA in NaOH (1 molar) were added and the pH was adjusted to 7. The reaction was stirred at rt for 20 min. The product was immediately purified using Superdex™ peptide 10/300 gel filtration column with 20% ACN in water.

Cleavage conditions for peptides

Reagent B: TFA/TIPS/water
(190:5:5/v:v:v)

Reagent K: TFA/ethane dithiol/thioanisole/phenol/water
(85:5:5:2.5:2.5/v:v:v:w:v)

Reagent H: TFA/ethane dithiol/thioanisole/phenol/water/dimethylsulfide/NH₄I

Test cleavages

Depending on the sequence, 200 µl for short sequences, i.e. up to 20 amino acids, and 300 µl for long sequences, i.e. more than 20 amino acids, of reagent B, K or H were added to some bits of dry resin in a 1.5 ml Eppendorf tube and shaken for 60 minutes. The peptide was crushed out in ice cold ether, centrifuged, decanted and washed two more times. The dried product mixed with the resin was dissolved in HPLC Buffer 1, filtered and lyophilized.

Standard cleavage conditions

Depending on the sequence 8 ml of reagent B, K or H was added and the resin (100 mg when synthesis was started) was shaken for 3 hours. The cleavage solution was collected and the resin was washed with another 8 ml TFA. The solution was concentrated under nitrogen and crushed out with ice cold diethyl ether. The precipitate was centrifuged and washed two more times with ice cold diethyl ether. The resulting peptide was dried, dissolved in HPLC Buffer 1 and lyophilized.
4. SYNTHESIS OF GLYCAN BUILDING BLOCKS FOR GLYCOPEPTIDE SYNTHESIS

1,3,4,6-Tri-O-acetyl-2-acetamido-2-deoxy-α/β-D-glucopyranoside 15

20 g 2-amino-2-deoxy-glucose hydrochloride (112 mmol) were suspended in 20 ml pyridine and 105 ml acetic anhydride were added. The mixture was stirred for 16 hours and concentrated under reduced pressure. The resulting oil was dissolved in DCM and washed with sat. NaHCO₃-solution and sat. NaCl-solution. The combined organic phases were dried over NaSO₄. The solvent was removed to give 15 in quantitative yield as yellow oil. ¹H-NMR (400 MHz, CDCl₃): δ = 6.16 (d, J = 3.7 Hz, 1H), 5.57 (d, J = 9.0 Hz, 1H), 5.26 – 5.17 (m, 2H), 4.51 – 4.44 (m, 1H), 4.24 (dd, J = 12.5 Hz, 4.1 Hz, 1H), 4.05 (dd, J = 12.5 Hz, 2.4 Hz, 1H), 4.01 – 3.96 (m, 1H), 2.18 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.93 (s, 3H) ppm.; ESI-MS: Masscalc for C₁₆H₂₃NO₁₀: 390.140 [M+H]+; Massobs: 390.280 [M+H]⁺

2-Acetamido-2-deoxy-α/β-D-glucopyranoside 16

43.5 g of 15 were dissolved in 100 ml methanol and freshly prepared sodium methoxide (by slowly dissolving sodium hydride in anhydrous methanol) was added. The mixture was stirred for 4 hours and neutralized with amberlite resin [H⁺-form]. After filtration the solvent was removed under reduced pressure and the desired product 16 was observed as white foam in 85% yield. ¹H-NMR (400 MHz, Methanol-d₄): δ = 5.10 (d, J = 3.4 Hz, 1H), 3.92 – 3.57 (m, 6H), 1.99 (s, 3H) ppm.

3,4,6-Tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside-1-chloride 17

10 g of 16 were dissolved in 50 ml acetyl chloride and 14 µl conc. HCl were added and the mixture was stirred for 16 hours. The reaction was concentrated under reduced pressure,
dissolved in DCM and poured on ice water. Sat. NaHCO₃-solution was added and the pH was adjusted to 10. The organic layer was separated and washed with water. The combined organic layers were dried over NaSO₄, concentrated and purified using column chromatography to give the desired product 17 in 45% yield as white solid. \( ^1\text{H-NMR (400 MHz, CDCl}_3\): } \delta = 6.17 \ (d, \ J = 3.7 \text{ Hz, } 1H), \ 5.88 \ (d, \ J = 8.7 \text{ Hz, } 1H), \ 5.31 \ (dd, \ J = 10.5 \text{ Hz, } 9.5 \text{ Hz, } 1H), \ 5.22 - 5.17 \ (m, \ 1H), \ 4.52 \ (ddd, \ J = 10.7 \text{ Hz, } 8.7 \text{ Hz, } 3.7 \text{ Hz, } 1H), \ 4.30 - 4.23 \ (m, \ 2H), \ 4.14 - 4.09 \ (m, 1H), \ 2.08 \ (s, \ 3H), \ 2.04 - 2.03 \ (d, \ 6H), \ 1.97 \ (s, \ J = 2.1 \text{ Hz, } 3H) \ \text{ppm; ESI-MS: Mass}_{\text{calc}} \text{ for C}_{14}\text{H}_{20}\text{ClNO}_8: 366.096 \ [\text{M+H}]^{+}; \text{Mass}_{\text{obs}}: 366.080 \ [\text{M+H}]^{+} \)

3,4,6-Tri-O-acetyl-2-acetamido-2-deoxy-\( \beta \)-D-glucopyranoside-1-azide 18

\[
\begin{align*}
\text{AcO} & \text{O} \\
\text{AcNH} & \text{OAcO} \\
& \text{N}_3
\end{align*}
\]

Method 1) 7.45 g 17 were dissolved in 20 ml DMF and 3.30 g (2.5 eq) sodium azide were added. The reaction was stirred for 4 hours, diluted with DCM and washed several times with water. The combined organic layers dried over NaSO₄. The solvent was removed to give 18 in 93% yield as pale yellow solid.

Method 2) 5.10 g 15 were dissolved in anhydrous ethyl acetate and cooled to 0°C. 5.74 ml TMS-azide was added. 2.46 ml SnCl₄ was added dropwise. The reaction was warmed to room temperature and stirred for 14 hours. The mixture was filtered, concentrated under reduced pressure and purified using column chromatography to give the desired product 18 in 63% yield as pale yellow solid. \( ^1\text{H-NMR (400 MHz, CDCl}_3\): } \delta = 6.39 \ (d, \ J = 9.0 \text{ Hz, } 1H), \ 5.26 \ (dd, \ J = 10.5 \text{ Hz, } 9.4 \text{ Hz, } 1H), \ 5.07 - 5.02 \ (m, \ 1H), \ 4.81 \ (d, \ J = 9.3 \text{ Hz, } 1H), \ 4.23 \ (dd, \ J = 12.4 \text{ Hz, } 5.0 \text{ Hz, } 1H), \ 4.15 - 4.11 \ (m, \ 1H), \ 3.89 \ (dt, \ J = 10.5 \text{ Hz, } 9.1 \text{ Hz, } 1H), \ 3.81 \ (ddd, \ J = 10.1 \text{ Hz, } 4.9 \text{ Hz, } 2.4 \text{ Hz, } 1H), \ 2.05 \ (s, \ 3H), \ 2.00 \ (d, \ J = 3.5 \text{ Hz, } 3H), \ 1.98 \ (s, \ 3H), \ 1.94 \ (s, \ 3H) \ \text{ppm; ESI-MS: Mass}_{\text{calc}} \text{ for C}_{14}\text{H}_{20}\text{N}_4\text{O}_8: 373.136 \ [\text{M+H}]^{+}; \text{Mass}_{\text{obs}}: 373.170 \ [\text{M+H}]^{+} \)

2-Acetamido-2-deoxy-\( \beta \)-D-glucopyranoside-1-azide 12

\[
\begin{align*}
\text{OH} & \text{O} \\
\text{AcNH} & \text{O} \\
& \text{N}_3
\end{align*}
\]

7 g of 18 were dissolved in 100 ml methanol and fresh prepared sodium methoxide (by slowly dissolving sodium hydride in anhydrous methanol) was added. The mixture was stirred for 4 hours and neutralized with amberlite resin [H⁺-form]. After filtration the solvent was
removed under reduced pressure and the desired product 12 was observed as white foam in 58% yield. \(^1\)H-NMR (400 MHz, Methanol-\(d_4\)): \(\delta = 4.50\) (d, \(J = 9.3\) Hz, 1H), \(3.90\) (d, \(J = 12.1\) Hz, 1H), \(3.75 - 3.62\) (m, 2H), \(3.50 - 3.34\) (m, 3H), \(1.99\) (d, \(J = 1.0\) Hz, 3H) ppm.

**2-Acetamido-2-deoxy-β-D-glucopyranoside-1-amine 19**

![](image)

12 was dissolved in anhydrous methanol under argon atmosphere to give a 0.1 molar concentration and argon was bubbled through the solution. 0.1 eq. palladium on carbon were added and hydrogen was bubbled through the suspension. Afterwards the suspension was incubated under hydrogen atmosphere for 2 hours. Argon was bubbled through the suspension and the mixture was filtered. The filtrate was concentrated under reduced pressure and 19 was directly used for Lansbury coupling.

**Extraction and purification of glycopeptide (SGP) 20 from egg yolk powder**

L-lysine-L-valine-L-alanine\(\text{N}^4\{-5-N\)-Acetyl-D-neuraminyl-(2\(\rightarrow\)6)-O-β-D-galactopyranosyl-(1\(\rightarrow\)4)-O-2-acetamido-2-deoxy-D-glucopyranosyl-(1\(\rightarrow\)2)-O-α-D-mannopyranosyl-(1\(\rightarrow\)3)-O-[5-N-acetyl-α-D-neuraminyl-(2\(\rightarrow\)6)-O-β-D-galactopyranosyl-(1\(\rightarrow\)4)-O-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1\(\rightarrow\)2)-O-α-D-mannopyranosyl-(1\(\rightarrow\)6)]-O-β-D-mannopyranosyl-(1\(\rightarrow\)4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1\(\rightarrow\)4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)}\}]L-asparagine-L-lysine-L-threonine

H-Lys-Val-Ala-{Asn[(Neu5Ac-Gal-GlcNAc-Man)\(_2\)-Man-GlcNAc-GlcNAc]}Lys-Thr-OH

300 g egg yolk powder were suspended in 900 ml water and stirred for 16 hours. 450 ml MeOH was added. The suspension was separated into 40-ml fractions and centrifuged (4000 rpm for 10 minutes). 10 ml CHCl\(_3\) were added to each fraction and the mixture was
centrifuged again (4000 rpm for 10 minutes). The aqueous phase was decanted and concentrated under reduced pressure. The remainder was suspended in 50 ml 100 mM ammonium acetate buffer, centrifuged (10000 rpm for 10 minutes). The soluble part was filtered and purified using a Sephadex G50 column with 100 mM ammonium acetate and 0.5 ml flow rate. All UV-active fractions were analyzed with MALDI-TOF and the 20 containing fractions were combined and lyophilized to give 600 mg crude 20. ESI-MS: Mass_{calc} for C_{113}H_{191}N_{15}O_{68}: 2863,161 [M-H]^{\cdot}^{-}, 2865,177 [M+H]^{+}; Mass_{obs}: 2864.0779 [M-H]^{\cdot}^{-}, 1431.5789[2M-2H]^{2-}, 2865.0669 [M+H]^{+}

Nona-glycopeptide 21

L-lysine-L-valine-L-alanine[N^4-{D-galactopyranosyl-(1\to4)-O-2-acetamido-2-deoxy-D-glucopyranosyl-(1\to2)-O-\alpha-D-mannopyranosyl-(1\to3)-O-[D-galactopyranosyl-(1\to4)-O-2-acetamido-2-deoxy-\beta-D-glucopyranosyl-(1\to2)-O-\alpha-D-mannopyranosyl-(1\to6)]-O-\beta-D-mannopyranosyl-(1\to4)-O-(2-acetamido-2-deoxy-\beta-D-glucopyranosyl)]-L-asparagine-L-lysine-L-threonine

H-Lys-Val-Ala-{Asn[(Gal-GlcNAc-Man)_{2}-Man-GlcNAc-GlcNAc]}Lys-Thr-OH

Crude 20 was dissolved in 10 mM TFA solution in water. The pH was adjusted to 3 using concentrated TFA. The solution was warmed to 80°C and monitored using MALDI-TOF. After 18 hours both sialic acids were removed and the mixture was lyophilized. Nonasaccharide containing hexapeptide 21 was purified on a superdex 30 size exclusion column with 100 mM ammonium bicarbonate with 0.5 ml/min flow rate. All fractions were analyzed with MALDI-TOF. The 21 containing fractions were combined and lyophilized to give 21 in 38% yield. ¹H-NMR (400 MHz, D₂O): δ = 5.09 (s, 1H), 5.01 (d, J = 9.6 Hz, 1H), 4.90 (s, 1H), 4.74 (s, 2H), 4.66 – 4.61 (m, 1H), 4.56 (dd, J = 12.2 Hz, 7.6 Hz, 3H), 4.44 (dd, J = 7.8 Hz, 2.3 Hz, 2H), 4.37 (dd, J = 8.0 Hz, 6.3 Hz, 1H), 4.27 (q, J = 7.3 Hz, 1H), 4.23 – 4.15 (m, 3H), 4.12 – 4.07 (m, 3H), 3.86 (m, J = 27.8 Hz, 22.2 Hz, 16.4 Hz, 9.5 Hz, 7H), 3.76 – 3.40 (m, 25H), 2.93 – 2.62 (m, 2H), 1.91 – 1.64 (m, 6H), 1.37 (dd, J = 15.2 Hz, 8.8 Hz, 7H),
1.14 (d, \( J = 6.4 \text{ Hz}, \ 3\text{H} \)) ppm; \(^{13}\text{C-NMR} \ (101 \text{ MHz, } \text{D}_2\text{O}) : \delta = 176.31, 174.64, 174.58, 174.56, 174.53, 174.50, 174.28, 172.87, 172.67, 172.19, 171.80, 102.80, 102.77, 101.16, 100.29, 99.42, 99.32, 96.88, 80.28, 79.34, 78.45, 78.30, 78.06, 76.26, 76.11, 76.04, 75.21, 74.56, 74.24, 73.41, 72.70, 72.35, 71.93, 71.80, 70.82, 70.05, 69.26, 68.39, 67.72, 67.19, 67.13, 65.53, 61.49, 60.89, 59.81, 59.22, 54.72, 53.47, 53.16, 49.89, 49.42, 39.03, 38.94, 36.29, 31.78, 30.38, 29.97, 26.31, 26.06, 22.20, 22.09, 21.99, 21.73, 21.35, 19.14, 18.29, 17.67, 16.55 ppm; ESI-MS: Mass\_calc \ for \ C\text{90}H\text{155}N\text{13}O\text{54} : \ 2280,970 \ [M-H]^{-}, 2282,986 \ [M+H]^{+}; \ Mass\_obs: \ 1141.9917 \ [M+2H]^{2+}, \ 2280.9587 \ [M-H]^{-}

Nona-saccharide 22

D-galactopyranosyl-(1\(\rightarrow\)4)-O-2-acetamido-2-deoxy-D-glucopyranosyl-(1\(\rightarrow\)2)-O-\(\alpha\)-D-mannopyranosyl-(1\(\rightarrow\)3)-O-[D-galactopyranosyl-(1\(\rightarrow\)4)-O-2-acetamido-2-deoxy-\(\beta\)-D-glucopyranosyl-(1\(\rightarrow\)2)-O-\(\alpha\)-D-mannopyranosyl-(1\(\rightarrow\)4)]-O-\(\beta\)-D-mannopyranosyl-(1\(\rightarrow\)6)]-O-\(\beta\)-D-mannopyranosyl-(1\(\rightarrow\)4)-O-(2-acetamido-2-deoxy-\(\beta\)-D-glucopyranosyl)-(1\(\rightarrow\)4)-O-(2-acetamido-2-deoxy-\(\beta\)-D-glucopyranosyl

\((\text{Gal-GlcNAc-Man})_2\)-Man-GlcNAc-GlcNAc-OH

210 mg 21 were dissolved in 5 ml 100 mM ammonium acetate buffer. 100 units PNGase were added and the reaction was incubated at 37°C for 24 hours. The completion of the reaction was confirmed using MALDI-TOF. The mixture was concentrated by freeze-drying and purified on a SiliaSep™ C18 cartridge eluting with water. The fractions were lyophilized and gave 22 in 44 % yield. \(^{1}H\text{-NMR} \ (400 \text{ MHz, } \text{D}_2\text{O}) : \delta = 5.04 \ (s, \ 1H), \ 4.98 \ (s, \ 1H), \ 4.78 \ (s, \ 1H), \ 4.44 \ (d, \ J = 8.6 \text{ Hz}, \ 3H), \ 4.31 \ (t, \ J = 10.9 \text{ Hz}, \ 3H), \ 4.20 \ (d, \ J = 13.7 \text{ Hz}, \ 1H), \ 4.11 \ (s, \ 1H), \ 4.05 \ (s, \ 1H), \ 4.03 – 3.91 \ (m, \ 2H), \ 3.88 – 3.69 \ (m, \ 14H), \ 3.69 – 3.31 \ (m, \ 30H), \ 2.85 \ (d, \ J = 7.7 \text{ Hz}, \ 3H), \ 1.90 \ (s, \ 9H) \ ppm; ESI-MS: Mass\_calc \ for \ C\text{62}H\text{104}N\text{4}O\text{46} : \ 1663,582 \ [M+Na]^{+}; \ Mass\_obs: \ 1663.5841 \ [M+Na]^{+}
Nona-saccharide azide 13

D-galactopyranosyl-(1→4)-O-2-acetamido-2-deoxy-D-glucopyranosyl-(1→2)-O-α-D-
mannopyranosyl-(1→3)-O-[D-galactopyranosyl-(1→4)-O-2-acetamido-2-deoxy-β-D-
glucopyranosyl-(1→2)-O-α-D-mannopyranosyl-(1→6)]-O-β-D-mannopyranosyl-(1→4)-O-
(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-O-(2-acetamido-2-deoxy-1-deoxy-β-D-
glucopyranosyl-azide

\[(\text{Gal-GlcNAc-Man})_2\text{-Man-GlcNAc-GlcNAc-N}_3\]

120 mg 22 were dissolved in 1.5 ml water and cooled to 2°C. 1.4 g sodium azide, 170 µl 2,6-lutidine and 124 mg DMC were added. The reaction was stirred at 2°C for one week. 13 was purified Sephadex G25 column eluting with 5% ethanol in water. The fractions were analyzed with MALDI-TOF and 13 containing fractions were lyophilized. 13 was observed in 82% yield. $^1$H-NMR (400 MHz, D$_2$O): $\delta$ = 5.09 (s, 1H), 4.90 (s, 1H), 4.73 (d, $J$ = 9.1 Hz, 2H), 4.57 (t, $J$ = 8.8 Hz, 3H), 4.44 (dd, $J$ = 7.8 Hz, 2.6 Hz, 2H), 4.22 (d, $J$ = 2.5 Hz, 1H), 4.16 (dd, $J$ = 3.3 Hz, 1.6 Hz, 1H), 4.13 – 4.07 (m, 2H), 3.99 – 3.83 (m, 14H), 3.81 – 3.65 (m, 27H), 3.64 – 3.51 (m, 14H), 2.07 – 2.04 (m, 3H), 2.02 (d, $J$ = 2.0 Hz, 9H) ppm; $^{13}$C-NMR (101 MHz, D$_2$O): $\delta$ = 174.66, 174.58, 174.56, 174.51, 102.80, 102.77, 101.20, 100.29, 99.42, 99.31, 96.88, 88.39, 80.30, 79.31, 78.54, 78.31, 76.32, 76.24, 76.13, 75.21, 74.59, 74.55, 74.24, 73.41, 72.70, 72.35, 72.10, 71.94, 71.81, 70.83, 70.15, 70.05, 69.30, 69.24, 68.40, 67.20, 67.15, 65.52, 63.14, 61.58, 61.50, 60.89, 59.82, 59.76, 54.72, 54.35, 52.12, 48.96, 48.93, 44.89, 43.19, 43.12, 39.24, 33.99, 33.79, 33.77, 28.88, 22.21, 22.09, 21.97, 19.09, 18.36, 17.56, 16.43 ppm; ESI-MS: Mass$_{calc}$ for C$_{62}$H$_{103}$N$_{7}$O$_{45}$: 1688.588 [M+Na]$^+$; Mass$_{obs}$: 1688.5905 [M+Na]$^+$
H-Asn-Nona 24

\[N^\alpha-\{D-galactopyranosyl-(1\rightarrow4)-O-2-acetamido-2-deoxy-D-glucopyranosyl-(1\rightarrow2)-O-\alpha-D-mannopyranosyl-(1\rightarrow3)-O-[D-galactopyranosyl-(1\rightarrow4)-O-2-acetamido-2-deoxy-\beta-D-glucopyranosyl-(1\rightarrow2)-O-\alpha-D-mannopyranosyl-(1\rightarrow6)-O-\beta-D-mannopyranosyl-(1\rightarrow4)-O-(2-acetamido-2-deoxy-\beta-D-glucopyranosyl)-(1\rightarrow4)-O-(2-acetamido-2-deoxy-\beta-D-glucopyranosyl)}\}-L-asparagine

H-Asn-[(Gal-GlcNAc-Man)_2-Man-GlcNAc-GlcNAc]-OH

300 mg H-Asn-[(Neu5Ac-Gal-GlcNAc-Man)_2-Man-GlcNAc-GlcNAc]-OH 23 were dissolved in 10 mM TFA solution in water. The pH was adjusted to 3 using concentrated TFA. The solution was warmed to 80°C and monitored using MALDI-TOF. After 36 hours both sialic acids were removed and the mixture was lyophilized. 24 was purified on a G25 size exclusion eluting with 20% ACN in H_2O. The fractions were analyzed with MALDI-TOF and the 24 containing fractions were lyophilized to give 24 in 94% yield. ESI-MS: Mass_{calc} for C_{66}H_{110}N_{6}O_{48}: 1753.627 [M-H]^–, 1752.6333 [M-H]^–.

Fmoc-Asn-Nona 25

Fmoc-[N^\alpha-\{D-galactopyranosyl-(1\rightarrow4)-O-2-acetamido-2-deoxy-D-glucopyranosyl-(1\rightarrow2)-O-\alpha-D-mannopyranosyl-(1\rightarrow3)-O-[D-galactopyranosyl-(1\rightarrow4)-O-2-acetamido-2-deoxy-\beta-D-glucopyranosyl-(1\rightarrow2)-O-\alpha-D-mannopyranosyl-(1\rightarrow6)-O-\beta-D-mannopyranosyl-(1\rightarrow4)-O-(2-acetamido-2-deoxy-\beta-D-glucopyranosyl)-(1\rightarrow4)-O-(2-acetamido-2-deoxy-\beta-D-glucopyranosyl)}\}-L-asparagine

Fmoc-Asn-[(Gal-GlcNAc-Man)_2-Man-GlcNAc-GlcNAc]-OH
210 mg 24 was dissolved in 8 ml water. 81 mg Fmoc-OSu and 100 µl TEA were dissolved in 8 ml DMSO and added. The reaction was stirred for 16 hours. After MALDI-TOF analysis indicated full conversion the reaction was lyophilized and purified Sephadex G25 column eluting with 20% ACN in water. The desired product 25 was observed in 51% yield. \(^1\)H-NMR (400 MHz, D\(_2\)O): \(\delta = 7.78\) (d, \(J = 7.5\) Hz, 2H), 7.59 (d, \(J = 7.6\) Hz, 2H), 7.46 – 7.32 (m, 4H), 5.11 (s, 1H), 4.99 (d, \(J = 9.6\) Hz, 1H), 4.92 (s, 1H), 4.80 (s, 3H), 4.61 – 4.53 (m, 4H), 4.45 (d, \(J = 7.7\) Hz, 4H), 4.24 (s, 1H), 4.18 (s, 2H), 4.10 (s, 1H), 3.93 (q, \(J = 11.8\) Hz, 1H), 3.86 (d, \(J = 14.4\) Hz, 1H), 3.74 (dt, \(J = 17.3\) Hz, 8.4 Hz, 22H), 3.63 – 3.44 (m, 16H), 2.69 (s, 1H), 2.13 – 2.01 (m, 8H), 1.94 (s, 1H), 1.89 (s, 2H) ppm; \(^{13}\)C-NMR (101 MHz, D\(_2\)O): \(\delta = 174.61, 174.49, 140.82, 127.92, 127.34, 120.05, 102.84, 101.20, 99.37, 96.95, 78.43, 76.32, 76.23, 76.11, 75.25, 74.64, 74.30, 73.46, 72.42, 72.00, 71.87, 70.88, 69.37, 68.44, 61.55, 60.92, 59.90, 54.77, 46.83, 22.26, 22.16, 21.87 ppm; ESI-MS: Mass\(_{\text{calc}}\) for C\(_{81}\)H\(_{120}\)N\(_{6}\)O\(_{50}\): 1999.693 [M+Na]\(^+\), 1999.6805 [M+Na]\(^+\)

5. SYNTHESIS OF A PHOTOLABILE PROTECTED ASPARTIC ACID FOR LANSBURY ASPARTYLATION

2,2-dibromo-1-(4-(dimethylamino)phenyl)ethanone 27

1-(4-(dimethylamino)phenyl)ethanone 26 (15 g, 92 mmol) was dissolved in 30 ml sulfuric acid and cooled to 0°C. Bromine (4.73 ml, 92 mmol) was added drop wise. The reaction mixture was stirred for 14 hours and poured on ice. The resulting green solid was separated via suction, dried and gave the desired product 27 in 90% yield (26.5 g, 82 mmol) as a green solid. \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \(\delta = 7.98\) (d, \(J = 9.1\) Hz, 2H), 6.73 – 6.66 (m, 3H), 3.10 (s, 6H) ppm; \(^{13}\)C-NMR (101 MHz, CDCl\(_3\)): \(\delta = 184.20, 154.17, 134.74, 132.20, 110.99, 40.66, 40.18\) ppm; ESI-MS: Mass\(_{\text{calc}}\) for C\(_{10}\)H\(_{11}\)Br\(_2\)NO: 319.929 [M+H]\(^+\); Mass\(_{\text{obs}}\): 320.030 [M+H]\(^+\)
2-bromo-1-(4-(dimethylamino)phenyl)ethanone 28

27 (24.45 g, 76 mmol) was dissolved in 200 ml THF and the mixture was cooled to 0°C. After adding diethyl phosphite (9.83 ml, 76 mmol) dropwise, TEA (15.60 ml, 76 mmol) in 50 ml THF was added. The mixture was stirred for 14 hours and poured on ice. The resulting solid was separated via suction. Purification via silica gel column (hexane/ethyl acetate; 1:1) gave the desired product 28 in 42.8% yield (7.90 g, 32.6 mmol) as a yellow solid. ¹H-NMR (400 MHz, CDCl₃): δ = 7.89 (d, J = 9.1 Hz, 2H), 6.66 (d, J = 9.1 Hz, 2H), 4.36 (s, 2H), 3.08 (s, 6H) ppm; ¹³C-NMR (101 MHz, CDCl₃): δ = 189.51, 153.92, 132.21, 131.43, 110.88, 40.18, 30.92 ppm; ESI-MS: Mass calc for C₁₀H₁₂BrNO: 242.018 [M+H]⁺; Mass obs: 243.006 [M+H]⁺

L-Aspartic Acid Copper(II) complex 30

L-Aspartic acid (5.0 g, 37.6 mmol) was dissolved in 100 ml water and heated to 70 °C. Diacetoxycopper hydrate (7.5 g, 37.6 mmol) in 100 ml water was added dropwise over one hour. The resulting mixture was stirred for 48 hours, until the product crushed out. The product was collected by suction, washed with water, ethanol and ether and dried at high vacuum. The desired product 30 was obtained as blue solid in 90% yield (13.1 g, 33.8 mmol).

Fmoc-L-Asp(Map)-OH 11

L-Aspartic acid copper (II) complex 30 (3.16 g, 8.16 mmol) and L-aspartic acid (2.17 g, 16.32 mmol) were dissolved in DMF/water (10:1, 100 ml). N,N,N’,N’-Tetramethylguanidin (4.1 ml, 32.6 mmol) was added slowly and the mixture was stirred until all solids were
dissolved. The resulting L-aspartic acid copper (II) complex \( N,N,N',N' - \)Tetramethylguanidinium salt was directly used for the next reaction step.

\( 28 \) (7.9 g, 32.6 mmol) was added to the L-aspartic acid copper (II) complex \( N,N,N',N' - \)Tetramethylguanidinium salt in DMF/water and stirred for 18 hours. Acetone was added and the solid was separated via suction and dried. The resulting 4-(dimethylamino)phenacyl (Map) ester copper complex was added in small portions to a freshly prepared EDTA solution (5.8 g NaHCO\(_3\) were dissolved in 70 ml water and 10 g EDTA disodium salt dihydrate was added slowly). The mixture was stirred until the solid was completely dissolved. The mixture was cooled to approximately 0°C and the resulting solid was separated via suction, dried and gave the desired \( \text{NH}_2\text{-Asp(Map)}\text{-OH} \) as a brown solid in 60.1% yield over three steps (5.77 g, 19.6 mmol). \( \text{NH}_2\text{-Asp(Map)}\text{-OH} \) (5.77 g, 19.6 mmol) and sodium carbonate (4.16 g, 39.2 mmol) were dissolved in 75 ml water. Fmoc-OSu (6.6 g, 19.6 mmol) was dissolved in 75 ml THF and added to the amino acid containing water solution. The reaction was stirred at room temperature for 14 hours. The reaction was diluted with ether and the resulting solid was separated via suction, washed with water to give the desired compound \( 11 \) as a brownish yellow solid in 70.1 % yield (7.1 g, 13.75 mmol). \(^1\text{H}-\text{NMR} \) (400 MHz, DMSO): \( \delta = 7.89 \) (d, \( J = 7.5 \) Hz, 2H), 7.78 (d, \( J = 9.1 \) Hz, 2H), 7.70 (d, \( J = 7.4 \) Hz, 2H), 7.41 (t, \( J = 7.5 \) Hz, 2H), 7.36 – 7.29 (m, 2H), 6.72 (d, \( J = 9.1 \) Hz, 2H), 5.24 (s, 2H), 4.27 – 4.18 (m, 3H), 3.95 (dd, \( J = 12.7 \) Hz, 6.5 Hz, 1H), 3.01 (s, 6H), 2.85 – 2.76 (m, 1H), 2.67 (dd, \( J = 15.1 \) Hz, 6.9 Hz, 1H) ppm; \(^{13}\text{C}-\text{NMR} \) (101 MHz, DMSO): \( \delta = 190.03, 171.06, 153.60, 144.02, 140.71, 129.75, 127.63, 127.14, 125.33, 121.41, 120.13, 110.76, 65.60, 46.73, 39.62 \) ppm; ESI-MS: Mass\(_{\text{calc}}\) for \( \text{C}_{29}\text{H}_{28}\text{N}_{2}\text{O}_{7} \) 539.179 \([\text{M+Na}]^{+}\); Mass\(_{\text{obs}}\): 539.174 \([\text{M+Na}]^{+}\)

**Fmoc-Thr(OtBu)-Thr(OtBu)-Gly-Glu(OtBu)-Asp(OMap)-Phe-Thr(psiMe,Mepro)-Glu(OtBu)-Thr(OtBu)-OH 36**

The resin was loaded using Procedure 1 (loading of Trityl-OH ChemMatrix\textsuperscript{®} resin). The substitution grade was 0.35 mmol/g determined by Fmoc quantification. For the following coupling HBTU, HOBt and DIPEA were used and each amino acid was coupled twice in a fivefold excess, except for Fmoc-Phe-Thr(psiMe,Mepro)-OH, which was coupled in a twofold excess. All amino acids were double coupled for 20 minutes, only for the pseudoproline building block and for the Fmoc-Asp(Map)-OH amino acid the coupling time was prolonged to two times 60 minutes. Using Reagent B a test cleavage was performed to ensure the success of the synthesis. ESI-MS: Mass\(_{\text{calc}}\) for \( \text{C}_{72}\text{H}_{96}\text{N}_{13}\text{O}_{24} \) 1511.583 \([\text{M+H}]^{+}\); Mass\(_{\text{obs}}\): 756.6 \([2\text{M+H}]^{2+}\)
Fmoc-Thr(OtBu)-Thr(OtBu)-Gly-Glu(OtBu)-Asp(OH)-Phe-Thr(psiMe,Mepro)-Glu(OtBu)-Thr(OtBu)-OH 37

The 36 containing resin was swollen in a 1:1 mixture of ethanol and DCM. Photolysis of Map was performed in a continuous flow system using a high-pressure Hg-lamp (hv>300 nm) in flow (flow rate 1 ml/min, 18 ml loop, radiation time 18 min). A test cleavage was performed to confirm completion of the reaction. ESI-MS: Mass$_{\text{calc}}$ for C$_{62}$H$_{83}$N$_{11}$O$_{23}$ 1350.382 [M+H]$^+$; Mass$_{\text{obs}}$: 1350.4 [M+H]$^+$, 675.9 [2M+H]$^{2+}$

Fmoc-Thr(OtBu)-Thr(OtBu)-Gly-Glu(OtBu)-Asp(NH GlcNAc)-Phe-Thr(psiMe,Mepro)-Glu(OtBu)-Thr(OtBu)-OH 38

27 mg resin (according to 9 mg unprotected peptide) containing 37 were taken and reacted with 19 according to Procedure 7 (Lansbury Coupling of amino Glycans). Using 2 ml of Reagent B a test cleavage was performed. ESI-MS: Mass$_{\text{calc}}$ for C$_{70}$H$_{98}$N$_{14}$O$_{26}$ 1552.613 [M+H]$^+$; Mass$_{\text{obs}}$: 1552.5 [M+H]$^+$, 777.0 [2M+H]$^{2+}$

6. SYNTHESIS OF ASPARTIC ACID BUILDING BLOCKS FOR LIGATION-DESULFURIZATION STRATEGIES

(S)-1-Allyl-4-tert-butyl 2-((tert-butoxycarbonyl)amino)succinate 40

5 g (S)-4-(tert-Butoxy)-2-((tert-butoxycarbonyl)amino)-4-oxobutanoic acid were dissolved in 50 ml DMF and 4 ml DIPEA were added. To the mixture 2 ml allyl bromide were added and the reaction was stirred for 16 hours. The reaction was concentrated under reduced pressure, dissolved in DCM and washed with water. The combined organic were dried over NaSO$_4$ and concentrated to give the desired product 40 in quantitative yield. $^1$H-NMR (400 MHz, CDCl$_3$): δ = 5.87 (ddt, J = 16.5 Hz, 10.9 Hz, 5.7 Hz, 1H), 5.47 (d, J = 8.9 Hz, 1H), 5.30 (dd, J = 17.2 Hz, 1.6 Hz, 1H), 5.21 (dd, J = 10.4 Hz, 1.5 Hz, 1H), 4.68 – 4.55 (m, 2H), 4.52 (dt, J = 9.2 Hz, 4.7 Hz, 1H), 2.92 – 2.83 (m, 1H), 2.70 (dd, J = 16.7 Hz, 4.7 Hz, 1H), 1.42 (s, 9H), 1.40 (s, 9H) ppm; ESI-MS: Mass$_{\text{calc}}$ for C$_{16}$H$_{27}$NO$_6$: 352,174 [M+Na]$^+$; Mass$_{\text{obs}}$: 352.280 [M+Na]$^+$
**S-(2,4,6-trimethoxybenzyl)-4-methylbenzenesulfonothioate 43**

1 g (2,4,6-Trimethoxyphenyl)methanol was dissolved in 10 ml DCM, 0.8 ml TEA were added and the mixture was cooled to 0°C. TFAA (0.72 ml) was added dropwise and the mixture was stirred for 15 minutes. 1.15 g Potassium 4-methylbenzenesulfonothioate was dissolved in 10 ml acetone and added to the reaction. The reaction was stirred for 16 hours and concentrated under reduced pressure, dissolved in ethyl acetate and washed with water. The combined organic were dried over NaSO₄, concentrated and purified using column chromatography to give the desired product **43** in 72% yield as yellow solid. ¹H-NMR (400 MHz, CDCl₃): δ = 7.85 (d, J = 8.1 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 6.01 (s, 2H), 4.29 (d, J = 0.9 Hz, 2H), 3.77 (d, J = 1.0 Hz, 3H), 3.69 (d, J = 1.0 Hz, 6H), 2.45 (s, 3H) ppm; ¹³C-NMR (101 MHz, CDCl₃): δ = 161.57, 159.24, 144.07, 142.58, 129.56, 127.27, 102.65, 90.50, 55.80, 55.48, 29.25, 21.74 ppm.

**(2R)-1-Allyl-4-tert-butyl 2-((tert-butoxycarbonyl)amino)-3-((2,4,6-trimethoxybenzyl)thio)succinate 44**

1.18 g **40** were dissolved in 20 ml anhydrous THF under argon atmosphere and cooled to -78°C. 7.9 ml 1 molar LiHMDS in THF was added dropwise over 30 minutes. The mixture was stirred at -78°C for 90 minutes and 1.45 g **43** in 20 ml anhydrous THF was added. The reaction was stirred for 2 hours and quenched with sat. NH₄Cl-solution. THF was removed under reduced pressure and the mixture was dissolved in ethyl acetate and washed with sat. NH₄Cl-solution. The combined organic were dried over NaSO₄, concentrated and purified using column chromatography to give the desired product **44** as mixture of enantiomers in 76% yield as yellow oil. ¹H-NMR (400 MHz, CDCl₃): δ = 6.83 (s, 2H), 5.96 – 5.84 (m, 1H), 5.66 (d, J = 10.3 Hz, 1H), 5.34 (dt, J = 17.2 Hz, 1.5 Hz, 1H), 5.28 – 5.21 (m, 1H), 4.79 (dd, J = 10.3 Hz, 5.5 Hz, 1H), 4.71 – 4.57 (m, 2H), 4.08 (d, J = 10.7 Hz, 1H), 4.01 – 3.85 (m, 2H), 1.50 (s, 9H), 1.45 (s, 9H) ppm; ¹³C-NMR (101 MHz, CDCl₃): δ = 160.74, 159.13, 159.07, 131.85, 131.78, 118.56, 90.78, 90.73, 82.08, 77.36, 66.28, 66.14, 60.53, 55.91, 55.89, 55.47,
55.29, 50.59, 49.19, 28.46, 28.42, 28.15, 28.04, 28.02, 25.11, 24.59, 21.20, 14.35 ppm; ESI-MS: Mass$_{calc}$ for C$_{16}$H$_{27}$NO$_{6}$: 564.224 [M+Na]$^+$; Mass$_{obs}$: 564.226 [M+Na]$^+$

(2R)-4-(tert-Butoxy)-2-((tert-butoxycarbonyl)amino)-4-oxo-3-((2,4,6-trimethoxybenzyl)thio)butanoic acid 8a and 8b

1.47 g 44 were dissolved in 20 ml DCM. 0.7 ml phenylsilane and 320 mg Pd(PPh$_3$)$_4$ were added. The mixture was stirred for 2 hours, concentrated and purified using column chromatography to give the desired product 8 as mixture of stereo isomers (34:66) in 80% yield as yellow oil. The stereo isomers were separated using RP-HPLC using a gradient 50% ACN to 75% ACN in water (0.1% TFA) in 20 min on Macherey-Nagel Nucleodur C18 Pyramid 5 µm, 250x21 mm.

Peak 1 eluting at 21.1 minutes:
$^1$H-NMR (400 MHz, CDCl$_3$): δ = 6.09 (s, 2H), 5.42 (d, $J$ = 7.7 Hz, 1H), 4.71 (t, $J$ = 7.0 Hz, 1H), 3.88 (s, 2H), 3.80 (s, 6H), 3.78 (s, 3H), 1.45 (s, 9H), 1.41 (s, 9H) ppm; $^{13}$C-NMR (101 MHz, CDCl$_3$): δ = 174.21, 169.15, 161.03, 160.80, 158.97, 156.13, 126.55, 106.39, 90.91, 90.75, 90.69, 82.95, 82.25, 80.65, 77.36, 56.07, 55.86, 55.44, 55.41, 54.80, 53.55, 49.74, 45.97, 29.79, 28.35, 27.94, 27.91, 27.86, 27.05, 24.63 ppm; ESI-MS: Mass$_{calc}$ for C$_{23}$H$_{35}$NO$_9$S: 524,193 [M+Na]$^+$; Mass$_{obs}$: 524.193 [M+Na]$^+$

Peak 2 eluting at 23.6 minutes:
$^1$H-NMR (400 MHz, CDCl$_3$): δ = 6.08 (s, 2H), 5.75 (d, $J$ = 9.6 Hz, 1H), 4.72 (dd, $J$ = 9.6 Hz, 4.4 Hz, 1H), 4.01 – 3.94 (m, 2H), 3.77 (s, 6H), 3.76 (s, 3H), 1.42 (s, 9H), 1.40 (s, 9H) ppm; $^{13}$C-NMR (101 MHz, CDCl$_3$): δ = 174.57, 171.46, 169.15, 160.69, 160.60, 158.94, 158.93, 158.87, 156.11, 155.76, 106.76, 106.36, 90.65, 90.58, 82.70, 82.06, 81.01, 80.35, 80.09, 56.12, 55.75, 55.72, 55.30, 55.29, 54.87, 50.28, 49.81, 49.31, 49.25, 48.73, 28.27, 27.82, 27.80, 27.67, 25.09, 24.87, 24.51 ppm; ESI-MS: Mass$_{calc}$ for C$_{23}$H$_{35}$NO$_9$S: 524,193 [M+Na]$^+$; Mass$_{obs}$: 524.193 [M+Na]$^+$
(3S)-**tert-Butyl 4-(benzylthio)-3-((tert-butoxycarbonyl)amino)-4-oxo-2-((2,4,6-trimethoxybenzyl)thio)butanoate 45**

66 mg 8a were dissolved in 2 ml anhydrous DMF and the mixture was cooled to 0°C. 137 mg PyBOP and 92 µl DIPEA were added and stirred for 15 minutes. To the mixture 31 µl benzyl mercaptan were added and the reaction was stirred for 1 hour at 0°C. The reaction was concentrated under reduced pressure and purified using column chromatography to give the desired product 45 in quantitative yield. \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \(\delta = 7.23\) (d, \(J = 4.4\) Hz, 5H), 6.08 (s, 2H), 5.58 (d, \(J = 9.1\) Hz, 1H), 4.87 (dd, \(J = 9.1\) Hz, 5.2 Hz, 1H), 4.11 - 3.92 (m, 4H), 1.42 (d, \(J = 7.9\) Hz, 18H) ppm; \(^{13}\)C-NMR (101 MHz, CDCl\(_3\)): \(\delta = 199.71, 169.03, 160.77, 159.00, 155.24, 137.09, 129.53, 129.04, 128.63, 128.60, 127.31, 106.53, 90.73, 82.29, 80.47, 77.36, 61.46, 55.89, 55.45, 50.64, 33.76, 32.05, 29.83, 28.43, 27.95, 26.46, 26.38, 24.73, 22.82 ppm; ESI-MS: Mass\(_{calc}\) for C\(_{30}\)H\(_{40}\)NO\(_8\)S\(_2\): 630.217 [M+H]^+; Mass\(_{obs}\): 630.200[M+Na]^+

**S-(Acetamidomethyl) 4-methylbenzenesulfonothioate 47**

3 g \(N\)-(hydroxymethyl)acetamide were dissolved in 40 ml DCM and cooled to 0°C. To the mixture 6.7 ml DIPEA were added and 4.8 ml TFAA were added dropwise. The mixture was stirred for 15 minutes. 7.6 g Potassium 4-methylbenzenesulfonothioate were dissolved in 80 ml acetone and added to the reaction. The reaction was stirred for 16 hours and concentrated under reduced pressure, dissolved in ethyl acetate and washed with water. The combined organic were dried over NaSO\(_4\), concentrated and purified using column chromatography to give the desired product 47 in 52% yield as yellow solid. \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \(\delta = 7.78\) (d, \(J = 8.3\) Hz, 2H), 7.37 – 7.34 (d, \(J = 7.9\) Hz, 2H), 4.68 (d, \(J = 6.7\) Hz, 2H), 2.44 (s, 3H), 1.92 (s, 3H) ppm; \(^{13}\)C-NMR (101 MHz, CDCl\(_3\)): \(\delta = 168.39, 144.69, 132.94, 129.11, 127.84, 109.15, 59.50, 21.86, 20.90 ppm.\)
(3R)-4-Allyl-1-tert-butyl 2-((acetamidomethyl)thio)-3-((tert-butoxycarbonyl)amino)succinate 48

1.8 g 40 were dissolved in 25 ml anhydrous THF under argon atmosphere and cooled to -78°C. 12 ml 1 M LiHMDS in THF were added dropwise over a period of 30 minutes. The mixture was stirred at -78°C for 90 minutes and 1.3 g 47 in 12 ml anhydrous THF was added. The reaction was stirred for 2 hours and quenched with sat. NH₄Cl-solution. THF was removed under reduced pressure and the mixture was dissolved in ethyl acetate and washed with sat. NH₄Cl-solution. The combined organic phases were dried over NaSO₄, concentrated and purified using column chromatography to give the desired product 48 as mixture of stereo isomers in 14% yield (55% yield brsm) as brownish oil. ¹H-NMR (400 MHz, Acetone-₆): \( \delta = 6.04 - 5.87 \) (m, 1H), 5.46 - 5.24 (m, 1H), 5.22 (m, 1H), 4.81 - 4.71 (m, 1H), 4.65 - 4.61 (m, 2H), 4.54 (s, 1H), 4.11 (m, 1H), 3.37 - 3.10 (m, 1H), 2.03 - 1.81 (m, 3H), 1.49 - 1.38 (m, 18H) ppm; ¹³C-NMR (101 MHz, Acetone-₆): \( \delta = 171.47, 170.52, 170.26, 154.30, 133.31, 133.07, 118.48, 118.26, 83.00, 81.81, 80.00, 79.75, 66.51, 66.46, 55.88, 53.91, 49.14, 49.11, 48.39, 42.36, 42.23, 37.48, 30.39, 30.20, 30.00, 28.60, 28.57, 28.26, 28.19, 28.14, 28.09, 23.04, 23.00, 22.88 ppm; ESI-MS: Mass calc for C₁₉H₃₂N₂O₇S: 455.183 [M+Na]⁺; Mass obs: 455.181 [M+Na]⁺

(2R)-3-((Acetamidomethyl)thio)-4-((tert-butoxy)-2-((tert-butoxycarbonyl)amino)-4-oxobutanoic acid 9a and 9b

335 mg 48 were dissolved in 2 ml DCM. 0.48 ml phenylsilane and 45 mg Pd(PPh₃)₄ were added. The mixture was stirred for 2 hours, concentrated and purified using column chromatography to give the desired product 9 as mixture of stereo isomers in 57% yield as brownish oil. The stereo isomers were separated using RP-HPLC using a gradient 20% ACN to 55% ACN in water (0.1% TFA) in 32.2 min on Macherey-Nagel Nucleodur C18 Pyramid 5 µm, 250x21 mm.
Peak 1 eluting at 30.8 minutes:

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 4.78$ (s, 1H), 4.54 (d, $J = 13.2$ Hz, 1H), 4.48 – 4.39 (m, 1H), 3.87 (s, 1H), 2.05 (s, 3H), 1.46 (d, $J = 9.0$ Hz, 18H) ppm; ESI-MS: Mass$_{calc}$ for C$_{16}$H$_{28}$N$_2$O$_7$S: 391,154 [M-H]$^-$; Mass$_{obs}$: 391.0 [M-H]$^-$, 415,151 [M+Na]$^+$

Peak 2 eluting at 33.4 minutes:

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 5.68$ (d, $J = 9.8$ Hz, 1H), 4.74 (s, 1H), 4.62 – 4.54 (m, 1H), 4.51 – 4.40 (m, 1H), 4.02 (d, $J = 4.6$ Hz, 1H), 1.48 – 1.43 (d, 18H) ppm; $^{13}$C-NMR (101 MHz, CDCl$_3$): $\delta = 171.86, 170.16, 156.37, 83.81, 81.00, 54.82, 53.37, 49.90, 42.29, 28.23, 27.79, 27.72, 22.84$ ppm; ESI-MS: Mass$_{calc}$ for C$_{16}$H$_{28}$N$_2$O$_7$S: 391,154 [M-H]$^-$; Mass$_{obs}$: 391.0 [M-H]$^-$, 415,151 [M+Na]$^+$

S-(4-(Dimethylamino)phenyl)-4-methylbenzenesulfonothioate 49

6.2 g 28 and 5.8 g Potassium 4-methylbenzenesulfonothioate were dissolved in anhydrous DMF. The mixture was stirred for 16 hours. The reaction mixture diluted with DCM, washed with water and concentrated under reduced pressure. Purification by column chromatography afforded pure 49 in 38% yield. $^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 7.81$ (d, $J = 8.4$ Hz, 2H), 7.74 (d, $J = 9.1$ Hz, 2H), 7.31 (d, $J = 8.2$ Hz, 2H), 6.62 – 6.58 (m, 2H), 4.48 (s, 2H), 3.05 (s, 9H), 2.42 (s, 3H) ppm; $^{13}$C-NMR (101 MHz, CDCl$_3$): $\delta = 188.96, 153.90, 144.87, 141.54, 132.12, 130.87, 130.77, 130.48, 129.83, 128.11, 127.07, 122.46, 110.83, 110.79, 110.72, 43.12, 40.01, 21.63$ ppm.

(3R)-4-Allyl-1-tert-buty 2-((4-(dimethylamino)phenyl)thio)-3-((tert-butoxycarbonyl)amino) succinate 50

1.3 g 40 were dissolved in 20 ml anhydrous THF under argon atmosphere and cooled to -78°C. 12 ml 1 M LiHMDS in THF were added dropwise over a period of 30 minutes. The mixture was stirred at -78°C for 90 minutes and 1.4 g 49 in 20 ml anhydrous THF was added.
The reaction was stirred for 2 hours and quenched with sat. NH₄Cl-solution. THF was removed under reduced pressure and the mixture was dissolved in ethyl acetate and washed with sat. NH₄Cl-solution. The combined organic were dried over Na₂SO₄, concentrated and purified using column chromatography to give the desired product 50 as mixture of stereo isomers in 62% yield as yellow oil. ¹H-NMR (400 MHz, CDCl₃): δ = 7.82 (d, J = 9.0 Hz, 6.8 Hz, 2H), 6.60 (d, J = 9.2 Hz, 2.6 Hz, 2H), 5.84 (ddq, J = 15.9 Hz, 10.6 Hz, 5.3 Hz, 1H), 5.64 (d, J = 10.2 Hz, 1H), 5.36 – 5.13 (m, 2H), 4.79 (dd, J = 10.3 Hz, 4.9 Hz, 1H), 4.59 (ddt, J = 13.2 Hz, 8.7 Hz, 6.4 Hz, 2H), 3.03 (s, 6H), 1.50 – 1.28 (m, 18H) ppm; ¹³C-NMR (101 MHz, CDCl₃): δ = 191.97, 171.05, 169.97, 169.80, 169.70, 155.72, 153.62, 153.60, 131.47, 131.38, 130.80, 130.78, 123.12, 123.09, 118.64, 118.61, 110.63, 110.61, 82.86, 82.40, 66.25, 66.16, 60.31, 54.64, 48.72, 39.95, 37.77, 28.23, 28.18, 27.82, 27.78, 20.99, 14.15 ppm; ESI-MS: Masscalc for C₂₆H₃₈N₂O₇S: 523.248 [M+H]+; Massobs: 523.2 [M+H]+, 546.0 [M+Na]+

(3R)-1-tert-Butyl 2-((4-(dimethylamino)phenyl)thio)-3-((tert-butoxycarbonyl)amino)succinate 10a and 10b

1.3 g 50 were dissolved in 20 ml DCM. 0.6 ml phenylsilane and 280 mg Pd(PPh₃)₄ were added. The mixture was stirred for 2 hours, concentrated and purified using column chromatography to give the desired product 10 as mixture of stereo isomers (29:71) in 72% yield as yellow oil. The stereo isomers were separated using RP-HPLC using a gradient 50% ACN to 75% ACN in water (0.1% TFA) in 20 min on Macherey-Nagel Nucleodur C18 Pyramid 5 µm, 250x21 mm.

Peak 2 eluting at 37 minutes:
¹H-NMR (400 MHz, CDCl₃): δ = 9.83 (bs, 9H), 7.86 (d, J = 8.7 Hz, 2H), 6.71 (d, J = 8.7 Hz, 2H), 5.76 (d, J = 9.6 Hz, 1H), 4.78 (dd, J = 9.7 Hz, 5.0 Hz, 1H), 4.09 (s, 2H), 3.96 (d, J = 5.0 Hz, 1H), 3.07 (s, 6H), 1.43 (s, 18H) ppm; ¹³C-NMR (101 MHz, CDCl₃): δ = 208.15, 192.67, 173.22, 170.20, 156.05, 153.31, 131.04, 130.97, 123.83, 111.52, 83.45, 80.62, 54.58, 48.50, 40.46, 37.80, 30.91, 28.23, 27.79, 27.72 ppm; ESI-MS: Masscalc for C₂₃H₃₄N₂O₇S: 505.198 [M+H]+; Massobs: 505.1994 [M+H]⁺
7. SYNTHESIS OF PRP FRAGMENT 1 (PRP I [214-231])

**PrP I [214-213] Dbz 52**

H-Thz-Val-Thr-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-AlaTyr-Tyr-Glu-Gly-Arg-Ser-Ser-Dbz

Starting from a commercially available Dawson Dbz NovaSyn® TGR resin the desired peptide was synthesized using Procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). A test cleavage was performed using reagent B. ESI-MS: Mass\text{calc} for $C_{98}H_{144}N_{28}O_{34}S$: 2271.001 [M+H]$^+$; Mass\text{obs}: 568.9 [4M+4H]$^{4+}$, 783.3 [3M+3H]$^{3+}$, 1136.9 [2M+2H]$^{2+}$

**PrP I [214-213] Nbz 53**

H-Thz-Val-Thr-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-AlaTyr-Tyr-Glu-Gly-Arg-Ser-Ser-Dbz

To the resin a p-nitrochloroformate solution (50 mg, 0.25 mmol, 50 mM in DCM) was added. The resin was bubbled with nitrogen for 40 min and washed with DCM and treated with 0.5 M DIPEA in DMF for 15 min. The peptide was cleaved from the solid support using reagent B. ESI-MS: Mass\text{calc} for $C_{99}H_{140}N_{28}O_{34}S$: 2296.981 [M+H]$^+$; Mass\text{obs}: 573.2 [4M+4H]$^{4+}$, 766.9 [3M+3H]$^{3+}$, 1149.4 [2M+2H]$^{2+}$

**PrP I [214-231] hydrazide 56**


Starting from Wang ChemMatrix® hydrazide 55 the peptide was loaded according to procedure 3 (Loading of Wang hydrazide resin). The substitution grade was 0.63 mmol/g determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). The peptide was cleaved from the solid support using reagent B and purified on Synergi Hydro RP C18 [250x10 mm; 4 m] 5% ACN to 35% ACN in H$_2$O (0.1% TFA) in 38 min or YMC hydrosphere RP C18 [250x10 mm; 5 m] 5% ACN to 25% ACN in H$_2$O (0.1% TFA) in 25 min. The pure product 56 was obtained in 31% yield. ESI-MS: Mass\text{calc} for $C_{97}H_{132}N_{23}O_{26}S$: 2139.964 [M+H]$^+$; Mass\text{obs}: 536.2 [4M+4H]$^{4+}$, 714.5 [3M+3H]$^{3+}$, 1071.3 [2M+2H]$^{2+}$
PrP I [214-231] hydrazide 57

H-Cys(SAcm)-Val-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-Ala-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-NHNH₂

Starting from Wang ChemMatrix® hydrazide 55 the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.63 mmol/g determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). The peptide was cleaved from the solid support using reagent B and crude product 57 was obtained in 60% yield. ESI-MS: Masscalc for C₉₀H₁₃₇N₂₇O₃₁S: 2123.969 [M+H⁺]; Massobs: 554.0 [4M+4H⁺]⁺, 738.3 [3M+3H⁺]³⁺, 1106.4 [2M+2H⁺]²⁺

Synthesis of MMBA 60

Methyl 4-(chloromethyl)benzoate and thiourea were suspended in water and refluxed for one hour to give Methyl 4-(isothiuroniummethyl)benzoate. Hydrolysis of Methyl 4-(isothiuroniummethyl)benzoate was performed with 10 N NaOH and refluxing the mixture for one hour. The mixture was acidified to pH 1 with concentrated HCl. The precipitate was separated via suction to give pure product 60 in 98% yield. ¹H-NMR (400 MHz, DMSO-d₆): δ = 12.92 (bs, 1H) 7.90 – 7.86 (d, 2H), 7.45 (d, J = 8.1 Hz, 2H), 3.78 (d, J = 7.8 Hz, 2H), 3.00 (t, J = 7.9 Hz, 1H) ppm; ¹³C-NMR (101 MHz, DMSO-d₆): δ = 167.14, 146.84, 129.52, 129.12, 128.39, 27.44 ppm.

PrP I [214-231] thioester 58

H-Cys(SAcm)-Val-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-Ala-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-MMBA

3 mg of 57 were reacted according to procedure 12. The desired product was observed in 25% yield. ESI-MS: Masscalc for C₈₇H₁₃₂N₂₃O₂₆S: 2139.964 [M+H⁺]; Massobs: 783.7 [3M+3H⁺]³⁺, 1174.6 [2M+2H⁺]²⁺

PrP I (Acm) [214-231] DiMan 63

H-Cys(SAcm)-Val-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-Ala-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-Cys-DiMan

0.7 mg 61 and 0.3 mg Cys(DiMan) 62 were dissolved in ligation buffer D. The reaction was incubated for 24 hours and purified using Superdex™ peptide 10/300 gel filtration column with 20% ACN in water. The desired product was observed as mixture with disulfide 63b in
84% yield. ESI-MS: Mass_{calc} for C_{113}H_{178}N_{28}O_{48}PS_{2}: 2790.153 [M+H]^+; Mass_{obs}: 932.0064 [3M+3H]^3+, 1397.4285 [2M+2H]^2+; ESI-MS: Mass_{calc} for C_{121}H_{184}N_{28}O_{50}PS_{3}: 2956.162 [M+H]^+; Mass_{obs}: 986.3483 [3M+3H]^3+, 1478.9326 [2M+2H]^2+

**PrP I [214-231] DiMan 64**


a) To a mixture of 63 and 63a in 0.2 ml water, 0.5 mg PdCl₂ were added and the reaction was incubated at 37°C for 4 hours. 1.5 mg DTT in 0.1 ml water were added, the reaction was centrifuged and immediately purified using Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC using a YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 25% ACN in H₂O (0.1% TFA) in 25 min).

b) 0.7 mg 61 and 0.3 mg Cys(DiMan) 62 were dissolved in ligation buffer D. The reaction was incubated for 24 hours. 0.5 mg PdCl₂ were added and the reaction was incubated at 37°C for 1 hour. 1.5 mg DTT in 0.1 ml water were added, the reaction was centrifuged and immediately purified using Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC using a YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 25% ACN in H₂O (0.1% TFA) in 25 min). MALDI-TOF: Mass_{calc} for C_{110}H_{172}N_{27}O_{47}PS_{2}: 2717.100 [M-H]⁻; 2717.148 [M-H]⁻

**8. SYNTHESIS OF PRP FRAGMENT 2 (PRP II [178-213])**

**PrP II [Fmoc-198-213] 66**

Fmoc-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-NHNH₂

Starting from Wang ChemMatrix® hydrazide 55 the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.58 mmol/g determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C) and Fmoc-L-Phe-L-Thr[Ψ(Me,Me)Pro]-OH was coupled according to procedure 7 (conditions for manual peptide elongation). A test cleavage was performed using reagent B. ESI-MS: Mass_{calc} for C_{98}H_{149}N_{23}O_{29}S_{3}: 2209.013 [M+H]^+; Mass_{obs}: 737.3 [3M+3H]^3+, 1005.4 [2M+2H]^2+
PrP II [179-213] 67

NH₂-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Glu-Gln-Met-NHNH₂

Starting from 66 the peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). The building blocks Fmoc-L-Val-L-Thr[Ψ(Me,Me)Pro]-OH and Fmoc-L-Ile-L-Thr[Ψ(Me,Me)Pro]-OH were coupled according to procedure 7 (conditions for manual peptide elongation). A test cleavage was performed using reagent B. ESI-MS: Masscalc for C₁₇₀H₂₈₇N₄₉O₅₇S₄: 4056.003 [M+H]⁺; Massobs: 677.0393 [6M+6H]⁶⁺, 812.0470 [5M+5H]⁵⁺, 1015.0582 [4M+4H]⁴⁺

PrP II [178Asp(SH)-213] 68

NH₂-Asp(SH)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Glu-Gln-Met-NHNH₂

Starting from 0.1 mmol 67, Boc-Asp(OtBu;STmob)-OH 8 was coupled according to procedure 7 (conditions for manual peptide elongation). The peptide was cleaved from the solid support using reagent B and reduced using procedure 10 (reduction of peptides) to give the crude product in 9% yield. The peptide was purified using a Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H₂O (0.1% TFA) in 35 min). After reduction and purification 68 was observed in 5% yield. ESI-MS: Masscalc for C₁₇₄H₂₉₂N₅₀O₆₀S₅: [M+H]⁺; Massobs: 701.3738 [6M+6H]⁶⁺, 841.4468 [5M+5H]⁵⁺, 1051.5573 [4M+4H]⁴⁺

PrP II [178Asp(SH)-213] 181GlcNAc and 197GlcNAc 70

NH₂-Asp(SH)-Cys-Val-Asn(GlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Lys-Gly-Glu-Asn(GlcNAc)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Glu-Gln-Met-NHNH₂

Starting from 66, the peptide was elongated using procedure 6 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 50°C and deprotection without elevated temperate). The building blocks Fmoc-L-Val-L-Thr[Ψ(Me,Me)Pro]-OH and Fmoc-L-Ile-L-Thr[Ψ(Me,Me)Pro]-OH were coupled according to procedure 7 (conditions for manual peptide elongation). 0.03 mmol of the resin bound peptide were used and Boc-Asp(OtBu;STmob)-OH 8 was coupled according to procedure 7 (conditions for manual peptide elongation). The allyl ester was cleaved
according to procedure 8 and glycosly amine 19 was coupled using procedure 9. The peptide was cleaved from the solid support using reagent B to give the crude product in 31% yield and reduced using procedure 10 (reduction of peptides). The peptide was purified using a Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H2O (0.1% TFA) in 35 min). After reduction and purification desulfurized product 70a was observed. ESI-MS: Masscalc for C_{190}H_{318}N_{52}O_{70}S_{5}: 4608.153 [M+H]^+; Massobs (desulfurized product): 764.0804 [6M+6H]^6+, 916.6892 [5M+5H]^5+, 1145.6129 [4M+4H]^4+

PrP II [178Asp(SH)-213] 181GlcNAc 71

NH2-Asp(SH)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asn(GlcNAc)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-NHNH2

Starting from 66, the peptide was elongated using procedure 6 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 50°C and deprotection without elevated temperate). The building blocks Fmoc-L-Val-L-Thr[Ψ(Me,Me)Pro]-OH and Fmoc-L-Ile-L-Thr[Ψ(Me,Me)Pro]-OH were coupled according to procedure 7 (conditions for manual peptide elongation). 0.03 mmol of the resin bound peptide were used and Boc-Asp(OtBu;STmob)-OH 8 was coupled according to procedure 7 (conditions for manual peptide elongation). The allyl ester was cleaved according to procedure 8 and glycosly amine 19 was coupled using procedure 9. The peptide was cleaved from the solid support using reagent B to give the crude product in 31% yield and reduced using procedure 10 (reduction of peptides). The peptide was purified using a Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H2O (0.1% TFA) in 35 min). After reduction and purification desulfurized product 71a was observed. ESI-MS: Masscalc for C_{183}H_{303}N_{51}O_{65}S_{5}: 4405.073 [M+H]^+; Massobs (desulfurized product): 701.3738 [6M+6H]^6+, 841.4468 [5M+5H]^5+, 1051.5573 [4M+4H]^4+
PrP II [178Asp(SH)-213] 197GlcNAc 72

NH2-Asp(SH)-Cys-Val-Asn(GlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-NHNH2

Starting from 66, the peptide was elongated using procedure 6 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 50°C and deprotection without elevated temperate). The building blocks Fmoc-L-Val-L-Thr[Ψ(Me,Me)Pro]-OH and Fmoc-L-Ile-L-Thr[Ψ(Me,Me)Pro]-OH were coupled according to procedure 7 (conditions for manual peptide elongation). 0.03 mmol of the resin bound peptide were used and Boc-Asp(OtBu;STmob)-OH was coupled according to procedure 7 (conditions for manual peptide elongation). The allyl ester was cleaved according to procedure 8 and glycosly amine 19 was coupled using procedure 9. The peptide was cleaved from the solid support using reagent B to give the crude product in 31% yield and reduced using procedure 10 (reduction of peptides). The peptide was purified using a Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H2O (0.1% TFA) in 35 min). After reduction and purification desulfurized product 72a was observed. ESI-MS: Masscalc for C185H305N51O65S5: 4405.073 [M+H]+; Massobs(desulfurized product): 701.3738 [6M+6H]6+, 841.4568 [5M+5H]5+, 1051.5573 [4M+4H]4+

PrP III [161-177] hydrazide 73

NH2-Val-Tyr-Tyr-Arg-Pro-Met-Asp-Glu-Tyr-Ser-Asn-Gln-Asn-Asn-Phe-Val-His-NHNH2

Starting from Wang ChemMatrix® hydrazide 55 the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.63 mmol/g determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H2O (0.1% TFA) in 35 min). Product 73 was obtained in 25% yield. ESI-MS: Masscalc for C97H136N28O29S: 2189.983 [M+H]+; Massobs: 548.5 [4M+4H]4+, 776.3 [3M+3H]3+, 1163.6 [2M+2H]2+
PrP III [161-177] thioester 74
NH₂-Val-Tyr-Tyr-Arg-Pro-Met-Asp-Glu-Tyr-Ser-Asn-Asn-Asn-Phe-Val-His-MMBA
25 mg of 73 were reacted according to procedure 12. The desired product was isolated in 10% yield. ESI-MS: Mass_{calc} for C_{105}H_{140}N_{26}O_{31}S_{2}: 2325,970 [M+H]^+; Mass_{obs}: 582.5 [4M+4H]^4+, 776.3[3M+3H]^3+, 1163.6 [2M+2H]^2+

PrP III-PrP II [178Asp(SH)-213] 75
0.4 mg 68 and 0.4 mg 74 were dissolved in ligation buffer E. The mixture was incubated at 37°C for 48 hours. The product 75 was purified using Superdex™ peptide 10/300 gel filtration column with 20% ACN in water. ESI-MS: Mass_{calc} for C_{97}H_{136}N_{28}O_{29}S: 6359.939 [M+H]^+; Mass_{obs}: 909.8 [7M+7H]^7+, 1061.0 [6M+6H]^6+

PrP II [178Asp(SAcm)-213] 76
NH₂-Asp(SAcm)-Cys-Val-Asn(GlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Glu-Gln-Met-NHNH₂
Starting from 0.012 mmol 67, Boc-Asp(OtBu;SAcm)-OH 9 was coupled according to procedure 7 (conditions for manual peptide elongation). The peptide was cleaved from the solid support using reagent B and reduced using procedure 10 (reduction of peptides) to give the crude product in 9% yield. The peptide was purified using a Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H₂O (0.1% TFA) in 35 min). After reduction and purification 76 was observed in 5% yield. ESI-MS: Mass_{calc} for C_{177}H_{297}N_{51}O_{61}S₅: 4274,039 [M+H]^+; Mass_{obs}: 611.5 [7M+7H]^7+, 713.5[6M+6H]^6+, 855.9 [5M+5H]^5+, 1096.8 [4M+4H]^4+.
PrP II [178Asp(SAcm)-213] 181Allyl 78

NH₂-Asp(SAcm)-Cys-Val-Asp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-NHNH₂

Starting from 66, the peptide was elongated using procedure 6 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 50°C and deprotection without elevated temperate). The building blocks Fmoc-L-Val-L-Thr[Ψ(Me,Me)Pro]-OH and Fmoc-L-Ile-L-Thr[Ψ(Me,Me)Pro]-OH were coupled according to procedure 7 (conditions for manual peptide elongation). 0.017 mmol of the resin bound peptide were used and Boc-Asp(OtBu;SAcm)-OH 9 was coupled according to procedure 7 (conditions for manual peptide elongation). A test cleavage was performed using reagent B. ESI-MS: Masscalc for C₁₈₀H₃₀₀N₅₀O₆₂S₅: 4315.054 [M+H]⁺; Mass obs: 720.3679 [6M+6H]⁶⁺, 864.2437 [5M+5H]⁵⁺, 1080.0426 [4M+4H]⁴⁺

PrP II [178Asp(SAcm)-213] 181GlcNAc 79

NH₂-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-NHNH₂

Starting from 0.017 mmol resin bound 78 anhydrous DCM was used for swelling and the allyl ester was cleaved according to procedure 8 (removal of allyl). Afterwards 19 was coupled according to procedure 9 (Lansbury coupling of amino glycans). The peptide was cleaved from the solid support using reagent B and reduced using procedure 10 (reduction of peptides) to give the crude product in 55% yield. The peptide was purified using a Superdex™ peptide 10/300 gel filtration column with 20% ACN in water and RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H₂O (0.1% TFA) in 35 min). After reduction and purification 79 was observed in 5% yield. ESI-MS: Masscalc for C₁₈₅H₃₁₀N₅₂O₆₆S₅: 4477.118 [M+H]⁺; Massobs: 747.3612 [6M+6H]⁶⁺, 896.6094 [5M+5H]⁵⁺, 1120.4611 [4M+4H]⁴⁺
PrP II [178Asp(SAcm)-213] 181GlcNAc thioester 80

NH₂-Asp(SAcm)-Cys-Val-Asn(NH-GlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-
Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-
Met-MMBA

0.5 mg of 79 were reacted according to procedure 12. The desired product was isolated in
80% yield as a mixture of thioester 80 and thiolactone 81. ESI-MS: Mass\textsubscript{calc} for
C\textsubscript{193}H\textsubscript{314}N\textsubscript{50}O\textsubscript{68}S\textsubscript{5}: 4580.125 [M+H]\textsuperscript{+}; Mass\textsubscript{obs}: 769.6926 [6M+6H]\textsuperscript{6+}, 923.3942 [5M+5H]\textsuperscript{5+},
1154.2042 [4M+4H]\textsuperscript{4+}; ESI-MS: Mass\textsubscript{calc} for C\textsubscript{185}H\textsubscript{306}N\textsubscript{50}O\textsubscript{66}S\textsubscript{5}: 4445.081 [M+H]\textsuperscript{+}; Mass\textsubscript{obs}: 744.6919 [6M+6H]\textsuperscript{6+}, 893.4043 [5M+5H]\textsuperscript{5+}, 1116.4611 [4M+4H]\textsuperscript{4+}

PrPII [196-213] 83

NH₂-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-
NHNH₂

Starting from peptide 66, the peptide was elongated procedure 7 (conditions for manual
peptide elongation). A testcleavage was performed using reagent B. ESI-MS: Mass\textsubscript{calc} for
C\textsubscript{92}H\textsubscript{152}N\textsubscript{26}O\textsubscript{32}S\textsubscript{3}: 2230.031 [M+H]\textsuperscript{+}; Mass\textsubscript{obs}: 558.2712 [4M+4H]\textsuperscript{4+}, 744.0281 [3M+3H]\textsuperscript{3+},
1115.5304 [2M+2H]\textsuperscript{2+}

PrPII [196-213] Asp197Allyl 84

NH₂-Glu-Asp(Allyl)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-
Met-NHNH₂

Starting from peptide 66, the peptide was elongated procedure 7 (conditions for manual
peptide elongation). A testcleavage was performed using reagent B. ESI-MS: Mass\textsubscript{calc} for
C\textsubscript{95}H\textsubscript{155}N\textsubscript{25}O\textsubscript{33}S\textsubscript{3}: 2271.046 [M+H]\textsuperscript{+}; Mass\textsubscript{obs}: 832.1 [3M+3H]\textsuperscript{3+}, 1247.3 [2M+2H]\textsuperscript{2+}

PrPII [196-213] Asp197Map 85

NH₂-Glu-Asp(Map)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-
Met-NHNH₂

Starting from peptide 66, the peptide was elongated procedure 7 (conditions for manual
peptide elongation). A testcleavage was performed using reagent B. ESI-MS: Mass\textsubscript{calc} for
C\textsubscript{102}H\textsubscript{162}N\textsubscript{26}O\textsubscript{34}S\textsubscript{3}: 2392.099 [M+H]\textsuperscript{+}; Mass\textsubscript{obs}: 654.5 [4M+4H]\textsuperscript{4+}, 872.5 [3M+3H]\textsuperscript{3+}, 1308.1 [2M+2H]\textsuperscript{2+}
PrPPI [Fmoc179-195] 87
NHFmoc-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-OH
The resin was loaded using procedure 1 (Loading of Trityl-OH ChemMatrix® resin). The substitution grade was 0.65 mmol/g determined by Fmoc quantification. The peptide was elongated according to procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). A test cleavage was performed using reagent B. ESI-MS: Mass calc for C_{93}H_{147}N_{23}O_{28}S: 2067.058 [M+H]^+; Mass obs: 689.7433 [3M+3H]^3+, 1034.0999 [2M+2H]^2+

PrPPI [Fmoc179-195] Asp181Allyl 88
NHFmoc-Cys-Val-Asp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-OH
The resin was loaded using procedure 1 (Loading of Trityl-OH ChemMatrix® resin). The substitution grade was 0.65 mmol/g determined by Fmoc quantification. The peptide was elongated according to procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). A test cleavage was performed using reagent B. ESI-MS: Mass calc for C_{96}H_{150}N_{22}O_{29}S: 2108.074 [M+H]^+; Mass obs: 703.7601 [3M+3H]^3+, 1054.6174 [2M+2H]^2+

PrPPI [Fmoc179-195] 89
NHFmoc-Cys(STrt)-Val-Asn(NHTrt)-Ile-Thr(OtBu)-Ile-Lys(NHBoc)-Gln(NHTrt)-His(NBoc)-Thr(OtBu)-Val-Thr(OtBu)-Thr(OtBu)-Thr(OtBu)-Thr(OtBu)-Lys(NHBoc)-Gly-OH
Fully protected peptide 89 was cleaved from the resin using 1% TFA in DCM (3 times 10 ml for 3 min). The mixture was directly dissolved in DIPEA containing DCM. The mixture was concentrated under reduced pressure, dissolved in DCM and extracted with water. The organic phase was evaporated to give 89 in 38% yield. ESI-MS: Mass calc for C_{189}H_{261}N_{23}O_{34}S: 3429.920 [M+H]^+; Mass obs: 3429.6062 [M+H]^+; 3329.5928 [M(-Boc)+H]^+;
PrPII [Fmoc179-195] Asp181Allyl 90

NHFmoc-Cys(STrt)-Val-Asn(NHTrt)-Ile-Thr(OtBu)-Ile-Lys(NHBoc)-Gln(NHTrt)-His(NBoc)-Thr(OtBu)-Val-Thr(OtBu)-Thr(OtBu)-Thr(OtBu)-Lys(NHBoc)-Gly-OH

Fully protected peptide 90 was cleaved from the resin using 1% TFA in DCM (3 times 10 ml for 3 min). The mixture was directly dissolved in DIPEA containing DCM. The mixture was concentrated under reduced pressure, dissolved in DCM and extracted with water. The organic phase was evaporated to give 90 in 68% yield. ESI-MS: Masscalc for $C_{173}H_{250}N_{22}O_{35}S$: 3228.826 [M+H]$^+$; Massobs: 3121.5361 [M(-Boc)+H]$^+$

PrPII [179-201] 92

NH$_2$-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Val-Thr-Lys-Gly-Glu-Asn-Phe-Thr-NHNH$_2$

Starting from Wang ChemMatrix® hydrazide 55 the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.59 mmol/g determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 35% ACN in H$_2$O (0.1% TFA) in 35 min). Product 92 was obtained in 8% yield. ESI-MS: Masscalc for $C_{109}H_{182}N_{32}O_{38}S$: 2579.301 [M+H]$^+$; Massobs: 860.6010 [3M+3H]$^3+$, 1290.1495 [2M+2H]$^2+$

PrPII [178Asp(SMap)-201] 93

NH$_2$-Asp(SMap)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Val-Thr-Lys-Gly-Glu-Asn-Phe-Thr-NHNH$_2$

Starting from 0.035 mmol 92, Boc-Asp(OtBu;SMap)-OH 10 was coupled according to procedure 7 (conditions for manual peptide elongation). The peptide was cleaved from the solid support using reagent B. The desired product 93 was obtained as a mixture with the corresponding aspartimide 93a. ESI-MS: Masscalc for $C_{123}H_{198}N_{34}O_{42}S_2$: 2888,392 [M+H]$^+$; Massobs: 578.5 [5M+3H]$^5+$; 723.1 [4M+4H]$^4+$; 963.7 [3M+3H]$^3+$; ESI-MS: Masscalc for $C_{123}H_{197}N_{33}O_{41}S_2$: 2870,382 [M+H]$^+$; Massobs: 575.2 [5M+3H]$^5+$; 718.6 [4M+4H]$^4+$; 957.8 [3M+3H]$^3+$

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PrP II [179-201] I97Asp(OAllyl) and I81Asp(OAllyl) 94

NH₂-Cys-Val-AspP(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-
Glu-Asp(OAllyl)-Phe-Thr-NH₂

Starting from Wang ChemMatrix® hydrazide 55 the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.59 mmol/g determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). A test cleavage was performed using reagent B. ESI-MS: Masscalc for C₁₁₅H₁₈₈N₃₀O₄₀S: 2661.332 [M+H]⁺; Massobs: 533.2872 [5M+5H]⁵⁺, 666.3553 [4M+4H]⁴⁺, 888.1390 [3M+3H]³⁺

PrP II [179-201] I97Asp(OH) and I81Asp(OH) 94a

NH₂-Cys-Val-Asp(OH)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-
Glu-Asp(OH)-Phe-Thr-NH₂

Starting from 0.028 mmol 94, the peptide was capped according to procedure 11 (Boc capping). The allyl ester was cleaved according to procedure 8 (removal of allyl). A test cleavage was performed using reagent B. ESI-MS: Masscalc for C₁₀₉H₁₈₀N₃₀O₄₀S: 2581.269 [M+H]⁺; Massobs: 517.2756 [5M+5H]⁵⁺, 646.3410 [4M+4H]⁴⁺, 861.4529 [3M+3H]³⁺

PrP 179-204 I97Asn(NHGlcNAc) and I81Asn(NHGlcNAc) 95

NH₂-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-
Glu-Asn(NHGlcNAc)-Phe-Thr-NH₂

Starting from 0.028 mmol 94a, amino glycan 19 was coupled according to procedure 9 (Lansbury coupling of amino glycans). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 20% ACN in H₂O (0.1% TFA) in 25 min). Product 95 was obtained in 9% yield. ESI-MS: Masscalc for C₁₂₅H₂₀₈N₃₄O₄₈S: 2985.460 [M+H]⁺; 597.0926 [5M+5H]⁵⁺, 747.6163 [4M+4H]⁴⁺, 996.1514 [3M+3H]³⁺

PrP II [179-201] I81Asp(OAllyl) 96

NH₂-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-
Glu-Asp(OAllyl)-Phe-Thr-NH₂

Starting from Wang ChemMatrix® hydrazide 55, the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.59 mmol/g
determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). A test cleavage was performed using reagent B. ESI-MS: Mass$_{calc}$ for C$_{112}$H$_{185}$N$_{31}$O$_{39}$S: 2620,317 [M+H]$^+$; Mass$_{obs}$: 656.0979 [4M+4H]$^{4+}$, 874.3007 [3M+3H]$^{3+}$, 1311.4462 [2M+2H]$^{2+}$

PrPPI [179-201] I81Asp(OH) 96a

NH$_2$-Cys-Val-Asp(OH)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-NHNH$_2$

Starting from 0.028 mmol 96, the peptide was capped according to procedure 11 (Boc capping). The allyl ester was cleaved according to procedure 8 (removal of allyl). A test cleavage was performed using reagent B. ESI-MS: Mass$_{calc}$ for C$_{109}$H$_{181}$N$_{31}$O$_{39}$S: 2580,285 [M+H]$^+$; Mass$_{obs}$: 517.0803 [5M+5H]$^{5+}$, 646.0967 [4M+4H]$^{4+}$, 861.1288 [3M+3H]$^{3+}$

PrPPI [179-201] 181Asn(NHGlcNAc) 97

NH$_2$-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-NHNH$_2$

Starting from 0.028 mmol 96a, amino glycan 19 was coupled according to procedure 9 (Lansbury coupling of amino glycans). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 20% ACN in H$_2$O (0.1% TFA) in 25 min). Product 97 was obtained in 9% yield. ESI-MS: Mass$_{calc}$ for C$_{117}$H$_{195}$N$_{33}$O$_{43}$S: 2782,381 [M+H]$^+$; 557.489 [5M+5H]$^{5+}$, 696.6116 [4M+4H]$^{4+}$, 928.8100 [3M+3H]$^{3+}$

PrPPI [179-201] I97Asp(OAllyl) 98

NH$_2$-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asp(OAllyl)-Phe-Thr-NHNH$_2$

Starting from Wang ChemMatrix® hydrazide 55, the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.59 mmol/g determined by Fmoc quantification. The peptide was elongated using procedure 4 (conditions for peptide elongation using CEM microwave assisted peptide synthesizer Liberty Blue® at 90°C). A test cleavage was performed using reagent B. ESI-MS: Mass$_{calc}$ for C$_{112}$H$_{185}$N$_{31}$O$_{39}$S: 2620,317 [M+H]$^+$; Mass$_{obs}$: 656.2236 [4M+4H]$^{4+}$, 874.3007 [3M+3H]$^{3+}$, 1311.4462 [2M+2H]$^{2+}$
PrPII [179-201] 197Asp(OH) 98a

\[ \text{NH}_2\text{-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Lys-Gly-Glu-Asp(OH)-Phe-Thr-NHNH}_2 \]

Starting from 0.028 mmol 98, the peptide was capped according to procedure 11 (Boc capping). The allyl ester was cleaved according to procedure 8 (removal of allyl). A test cleavage was performed using reagent B. ESI-MS: Mass\text{calc} for C\text{109H}_{181}\text{N}_{31}\text{O}_{39}\text{S}: 2580.285 [M+H]\text{+}; Mass\text{obs}: 517.0803 [5M+5H]\text{5+}, 646.0967 [4M+4H]\text{4+}, 861.1349 [3M+3H]\text{3+}

PrPII [179-201] 197Asn(NHGlcNAc) 99

\[ \text{NH}_2\text{-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn(NHGlcNAc)-Phe-Thr-NHNH}_2 \]

Starting from 0.028 mmol 98a, amino glycan 19 was coupled according to procedure 9 (Lansbury coupling of amino glycans). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 20% ACN in H\text{2}O (0.1% TFA) in 25 min). Product 99 was obtained in 5% yield. ESI-MS: Mass\text{calc} for C\text{117H}_{195}\text{N}_{33}\text{O}_{43}\text{S}: 2782.381 [M+H]\text{+}; Mass\text{obs}: 557.6782 [5M+5H]\text{5+}, 696.1077 [4M+4H]\text{4+}, 928.1411 [3M+3H]\text{3+}

PrPII [179Asp(SAcm)-201] 197Asp(OAllyl) 81Asp(OAllyl) 101

\[ \text{NH}_2\text{-Asp(SAcm)-Cys-Val-Asp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asp(OAllyl)-Phe-Thr-NHNH}_2 \]

Starting from 8 µmol 94, Boc-Asp(OtBu;SAcm)-OH 9 was coupled according to procedure 7 (conditions for manual peptide elongation). A test cleavage was performed using reagent B. ESI-MS: Mass\text{calc} for C\text{122H}_{198}\text{N}_{32}\text{O}_{44}\text{S}_2: 2879.368 [M+H]\text{+}; 577.0433 [5M+5H]\text{5+}, 721.0547 [4M+4H]\text{4+}, 961.0687 [3M+3H]\text{3+}

PrPII [179Asp(SAcm)-201] 197Asp(OH) 81Asp(OH) 102

\[ \text{NH}_2\text{-Asp(SAcm)-Cys-Val-Asp(OH)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asp(OH)-Phe-Thr-NHNH}_2 \]

Starting from 8 µmol 101, the allyl ester was cleaved according to procedure 8 (removal of Allyl). A test cleavage was performed using reagent B. ESI-MS: Mass\text{calc} for C\text{116H}_{190}\text{N}_{32}\text{O}_{44}\text{S}_2: 2799.306 [M+H]\text{+}; 561.0348 [5M+5H]\text{5+}, 700.7952 [4M+4H]\text{4+}, 934.0596 [3M+3H]\text{3+}
PrPII [179Asp(SAcm)-201] 197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 103
NH2-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn(NHGlcNAc)-Phe-Thr-NHNH2
Starting from 8 µmol 102, amino glycan 19 was coupled according to procedure 9 (Lansbury coupling of amino glycans). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 20% ACN in H2O (0.1% TFA) in 25 min). Product 103 was obtained in 12% yield. ESI-MS: Masscalc for C132H218N36O52S2: 3203.496 [M+H] +; 641.6708 [5M+5H] 5+, 801.8330 [4M+4H] 4+, 1068.7792 [3M+3H] 3+

PrPII [179Asp(SAcm)-201] 181Asp(OAllyl) 96a
NH2-Asp(SAcm)-Cys-Val-Asp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-NHNH2
Starting from 8 µmol 96, Boc-Asp(OtBu;SAcm)-OH 9 was coupled according to procedure 7 (conditions for manual peptide elongation). A test cleavage was performed using reagent B. ESI-MS: Masscalc for C119H195N33O43S2: 2838.353 [M+H] +; 568.6403[5M+5H]5+, 710.5454 [4M+4H] 4+, 947.0598 [3M+3H] 3+

PrPII [179Asp(SAcm)-201] 181Asp(OH) 96b
NH2-Asp(SAcm)-Cys-Val-Asp(OH)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-NHNH2
Starting from 8 µmol 96a, the allyl ester was cleaved according to procedure 8 (removal of Allyl). A test cleavage was performed using reagent B. ESI-MS: Masscalc for C116H191N33O43S2: 2798.321 [M+H] +; 560.6376 [5M+5H] 5+, 700.5515 [4M+4H] 4+, 933.7285 [3M+3H] 3+

PrPII [179Asp(SAcm)-201] 181Asn(NHGlcNAc) 106
NH2-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-NHNH2
Starting from 8 µmol 96b, amino glycan 19 was coupled according to procedure 9 (Lansbury coupling of amino glycans). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 20% ACN in H2O (0.1% TFA) in 25 min). Product 106 was obtained in 9% yield. ESI-MS:
Mass\textsubscript{calc} for $C_{124}H_{205}N_{35}O_{47}S_2$: 3000.417 [M+H]$^+$; 601.0521 [5M+5H]$^{5+}$, 751.0661 [4M+4H]$^{4+}$, 1001.084 [3M+3H]$^{3+}$

\textbf{PrPII [179Asp(SAcm)-201] 197Asp(OAllyl) 98a}

NH\textsubscript{2}-Asp(SAcm)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asp(OAllyl)-Phe-Thr-NHNH\textsubscript{2}

Starting from 8 µmol 98, Boc-Asp(OtBu;SAcm)-OH 9 was coupled according to procedure 7 (conditions for manual peptide elongation). A test cleavage was performed using reagent B. ESI-MS: Mass\textsubscript{calc} for $C_{119}H_{195}N_{33}O_{43}S_2$: 2838.353 [M+H]$^+$; Mass\textsubscript{obs}: 568.8401 [5M+5H]$^{5+}$, 710.5454 [4M+4H]$^{4+}$, 947.0598 [3M+3H]$^{3+}$

\textbf{PrPII [179Asp(SAcm)-201] 197Asp(OH) 98b}

NH\textsubscript{2}-Asp(SAcm)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asp(OH)-Phe-Thr-NHNH\textsubscript{2}

Starting from 8 µmol 98, the allyl ester was cleaved according to procedure 8 (removal of Allyl). A test cleavage was performed using reagent B. ESI-MS: Mass\textsubscript{calc} for $C_{116}H_{191}N_{33}O_{43}S_2$: 2798.321 [M+H]$^+$; Mass\textsubscript{obs}: 560.6376 [5M+5H]$^{5+}$, 700.5461 [4M+4H]$^{4+}$, 933.7285 [3M+3H]$^{3+}$

\textbf{PrPII [179Asp(SAcm)-201] 197Asn(NHGlcNAc) 107}

NH\textsubscript{2}-Asp(SAcm)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asn(NHGlcNAc)-Phe-Thr-NHNH\textsubscript{2}

Starting from 8 µmol 98b, amino glycan 19 was coupled according to procedure 9 (Lansbury coupling of amino glycans). The peptide was cleaved from the solid support using reagent B and purified using RP-HPLC (YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 20% ACN in H\textsubscript{2}O (0.1% TFA) in 25 min). Product 107 was obtained in 8% yield. ESI-MS: Mass\textsubscript{calc} for $C_{124}H_{205}N_{35}O_{47}S_2$: 3000.417 [M+H]$^+$; 601.0521 [5M+5H]$^{5+}$, 751.0717 [4M+4H]$^{4+}$, 1001.084 [3M+3H]$^{3+}$

\textbf{PrPII [179Asp(SAcm)-201] 197Asn(NHGlcNAc) thioester 108}

NH\textsubscript{2}-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Lys-Gly-Glu-Asn(NHGlcNAc)-Phe-Thr-MMBA

1.7 mg of 103 were reacted according to procedure 12. The desired product was observed in 84% yield as a mixture of desired thioester 108 and thiolactone 109. ESI-MS: Mass\textsubscript{calc} for
C\textsubscript{140}H\textsubscript{222}N\textsubscript{34}O\textsubscript{54}S\textsubscript{3}: 3340,491 [M+H]\textsuperscript{+}; 678.8711 [5M+5H]\textsuperscript{5+}, 836.0992 [4M+4H]\textsuperscript{4+}, 1114.8026 [3M+3H]\textsuperscript{3+}; ESI-MS: Mass\textsubscript{calc} for C\textsubscript{133}H\textsubscript{214}N\textsubscript{34}O\textsubscript{52}S\textsubscript{2}: 3171,459 [M+H]\textsuperscript{+}; 643.0701 [5M+5H]\textsuperscript{5+}, 794.3464 [4M+4H]\textsuperscript{4+}, 1058.4524 [3M+3H]\textsuperscript{3+}

PrPII [179Asp(SAcm)-201] 181Asn(NHGlcNAc) thioester 110
NH\textsubscript{2}-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-MMBA
1.5 mg of 106 were reacted according to procedure 12. The desired product was observed in 79% yield as a mixture of desired thioester 110 and thiolactone 111. ESI-MS: Mass\textsubscript{calc} for C\textsubscript{132}H\textsubscript{209}N\textsubscript{33}O\textsubscript{49}S\textsubscript{3}: 3137,412 [M+H]\textsuperscript{+}; 636.5219 [5M+5H]\textsuperscript{5+}, 785.0719 [4M+4H]\textsuperscript{4+}, 1046.4288 [3M+3H]\textsuperscript{3+}; ESI-MS: Mass\textsubscript{calc} for C\textsubscript{132}H\textsubscript{209}N\textsubscript{33}O\textsubscript{47}S\textsubscript{2}: 2968,379 [M+H]\textsuperscript{+}; 602.5219 [5M+5H]\textsuperscript{5+}, 743.1570 [4M+4H]\textsuperscript{4+}, 990.5410 [3M+3H]\textsuperscript{3+}

PrPII [179Asp(SAcm)-201] 197Asn(NHGlcNAc) thioester 112
NH\textsubscript{2}-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-MMBA
1.2 mg of 107 were reacted according to procedure 12. The desired product 112 was observed in 64% yield. ESI-MS: Mass\textsubscript{calc} for C\textsubscript{132}H\textsubscript{209}N\textsubscript{33}O\textsubscript{49}S\textsubscript{3}: 3137,412 [M+H]\textsuperscript{+}; 636.0476 [5M+5H]\textsuperscript{5+}, 785.3240[4M+4H]\textsuperscript{4+}, 1047.0970 [3M+3H]\textsuperscript{3+}

PrPII [179Asp(SAcm)-213] 202Asp(SH)197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 113
NH\textsubscript{2}-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn(NHGlcNAc)-Phe-Thr-Glu-Thr-Asp(SH)-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-NHNH\textsubscript{2}
1.3 mg 108/109 and 0.7 mg 91 were dissolved in ligation buffer C or ligation buffer D. The reaction was incubated at 37°C and monitored using HPLC. ESI-MS: Mass\textsubscript{calc} for C\textsubscript{193}H\textsubscript{323}N\textsubscript{53}O\textsubscript{71}S\textsubscript{6}: 4712,170 [M+H]\textsuperscript{+}; 786.5 [6M+6H]\textsuperscript{6+}, 943.4 [5M+5H]\textsuperscript{5+}, 1179.4 [4M+4H]\textsuperscript{4+}, 1571.2 [3M+3H]\textsuperscript{3+}
PrPII [179Asp(SAcm)-213] 202Asp(SH)197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 114
NH₂-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-
Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp(SH)-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-
Gln-Met-NHNH₂

1.1 mg \textbf{110/111} and 0.7 mg \textbf{91} were dissolved in ligation buffer C or ligation buffer D. The
reaction was incubated at 37°C and monitored using HPLC. ESI-MS: Mass\textsubscript{calc} for
\text{C}_{193}\text{H}_{316}\text{N}_{52}\text{O}_{68}\text{S}_{7}: 4675.099 [M+H]\textsuperscript{+}; 780.2 [6M+6H]\textsuperscript{6+}, 936.1 [5M+5H]\textsuperscript{5+}, 1170.1
[4M+4H]\textsuperscript{4+}, 1559.6 [3M+3H]\textsuperscript{3+}

PrPII [179Asp(SAcm)-213] 202Asp(SH)197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 114
NH₂-Asp(SAcm)-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-
Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp(SH)-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-
Gln-Met-NHNH₂

0.8 mg \textbf{112} and 0.4 mg \textbf{91} were dissolved in ligation buffer C or ligation buffer D. The
reaction was incubated at 37°C and monitored using HPLC. ESI-MS: Mass\textsubscript{calc} for
\text{C}_{193}\text{H}_{316}\text{N}_{52}\text{O}_{68}\text{S}_{7}: 4675.099 \text{[M+H]}\textsuperscript{+}; 780.3 \text{[6M+6H]}\textsuperscript{6+}, 936.2 \text{[5M+5H]}\textsuperscript{5+}, 1170.0
\text{[4M+4H]}\textsuperscript{4+}, 1559.5 \text{[3M+3H]}\textsuperscript{3+}
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