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

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TABLE OF CONTENTS

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 cid
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
Dmnb	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
Et_3N	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 flourescent protein
GlcN	glucoseamine
GlcNAc	*N*-acetylglucosamine
Glu	glutamine
Gly	glycine
GPI	glycosylphosphatidylinositol
h	hour
h	hextet
H_2	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 benzotriazol

HPLC	high performance liquid chromatography
Hz	Hertz
I_2	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
NaN_3	sodium azide
$NaNO_2$	sodium nitrite
NaOMe	sodium methoxide
Nbz	*N*-acyl-benzimidazolinone
NCL	native chemical ligation
NH_4I	ammonium iodide
nm	nanometer
NMP	*N*-methyl-2-pyrrolidone
Ox	oxidation

Oxyma	pthyl cyanohydroxyiminoacetate
Pd	palladium
PEG	poly-ethylene glycol
PG	protecting groups
pH	-log $[H]^+$, more precisely a
PhAcm	Phenylacetamidomethyl
Phe	phenylalanine
PNGaseF	Peptide:N-Glycosidase F
ppm	parts per million
Pro	proline
PrP^C	prion protein cellular form
PrP^{Sc}	prion protein scrapie form
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphate
q	quartet
Qtof	quadrupole TOF
quant	quantitative
R	rectus
RP-HPLC	reverse phase HPLC
rpm	rounds per minute
rPrP	recombinant prion protein
rt	room temperature
S	sinister
s	singulett
sat.	saturated
Ser	serine
SGP	sialglycopeptide
SPFC	solid phase fragment condensation
SPPS	solid phase peptide synthesis
t	triplett
tBu	tert-butyl
TCEP	Tris(2-carboxyethyl)phosphin
TEA	Triethylamine
TFA	trifluoroacetic acid
TFAA	riflic acid anhydride

THF	tetrahydrofuran
Thr	threonine
Thz	Thiazolidine
TIPS	triisopropylsilane
Tmob	trimethoxybenzy
TSE	transmissible spongiform encephalitis
UPLC	Ultra-Performance Liquid Chromatography
v	volume
Val	Valine

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^{Sc}), an insoluble, partially protease-resistant isoform. PrP^{C} is a cell surface glycoprotein of 208-209 amino acids of unknown function. [1] 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.

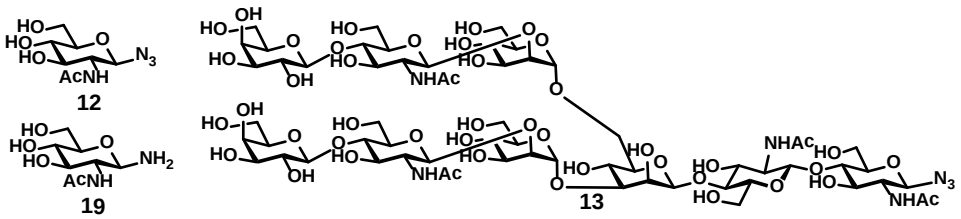


Figure 1: Glycan azides and amine for glycopeptide synthesis using Lansbury aspartylation

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 (hv>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.

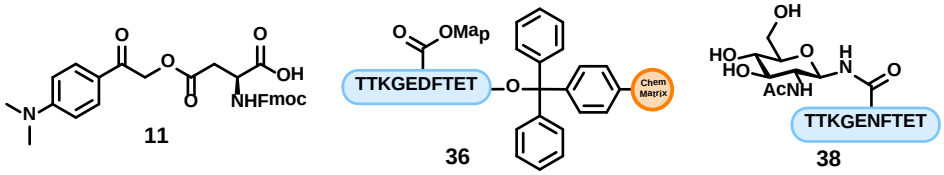


Figure 2: Orthogonal protected aspartate building block and model peptides **36** and **38**

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(O*t*Bu,STmob)-OH **8** was synthesized by reaction of a fully protected aspartic acid and STmob-tosyalte 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 separartion 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.

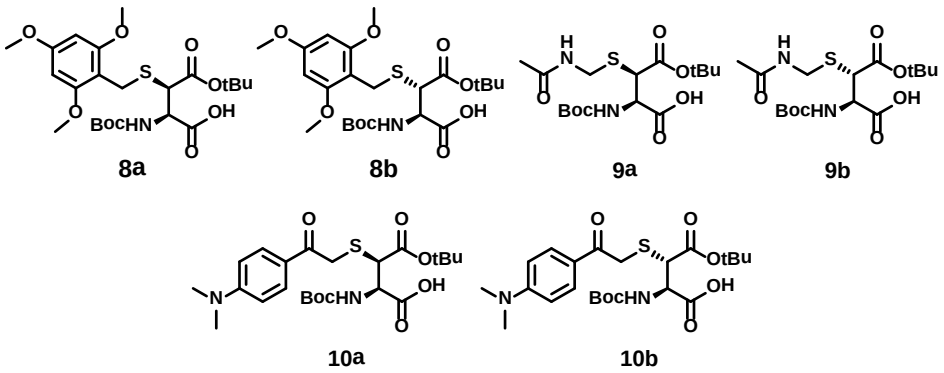


Figure 3: β-mercapto aspartic acid building blocks for ligation-desulfurization strategy

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.

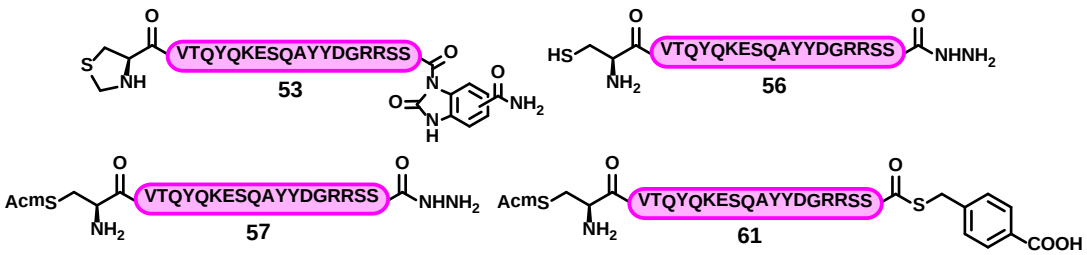


Figure 4: PrP peptide fragment 1 [PrP I (214-231)] thioester precursors (**53, 56** and **57**) and thioester **61**

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

desired ligation product was obtained with an optimized ligation buffer using 6 M GdmCl, 0.1 M Na_2HPO_4, 5 eq MMBA and 30 eq TCEP. After successful ligation, the orthogonal Acm protecting on cysteine of **63** was removed with $PdCl_2$ and the desired product PrP I [Cys-DiMan] **64** was isolated.

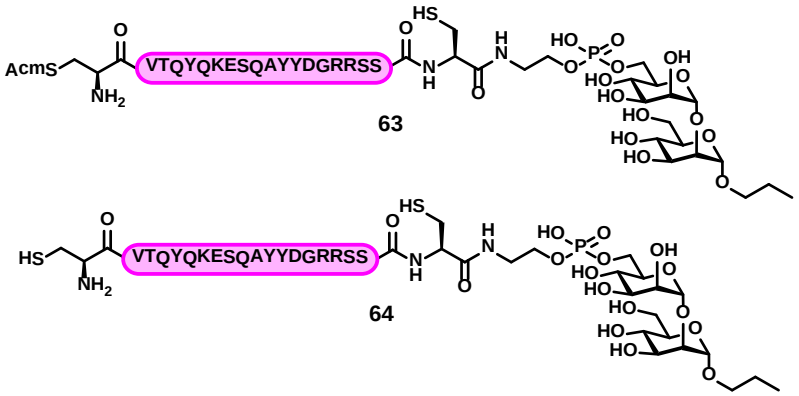


Figure 5: DiMan anchored PrP I peptides

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.

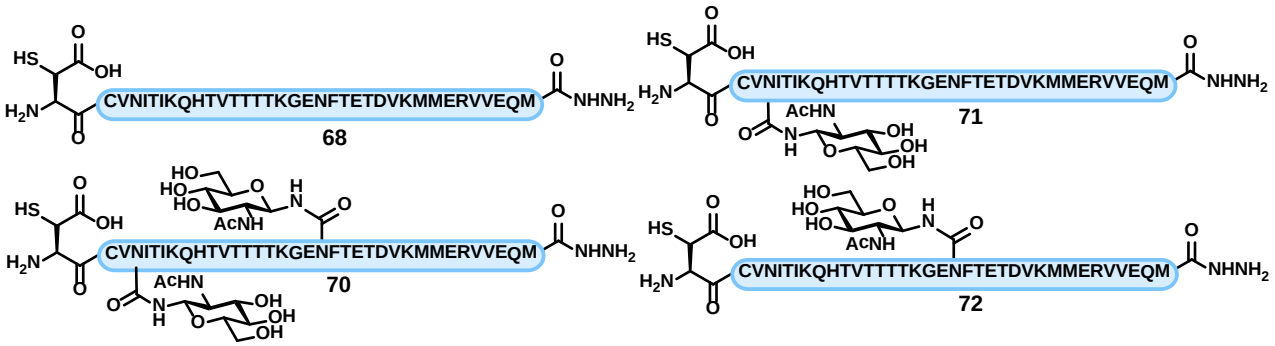


Figure 6: PrP II (178-213) peptides and glycopeptides using β-mercapto aspartic acid **8**

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 modelpeptide **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**.

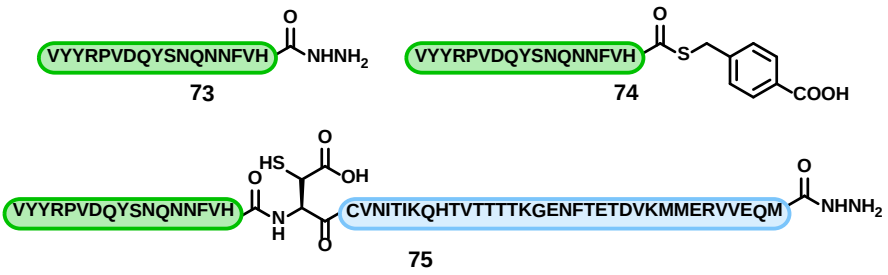


Figure 7: Hydrazide **73**, thioester **74** and ligation product **75**

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.

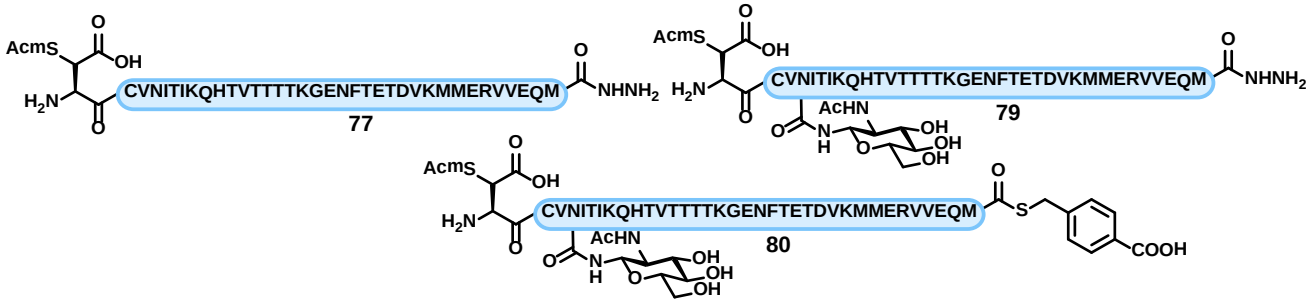


Figure 8: Hydrazide **77** and glycopeptide hydrazide **79** using β-mercapto aspartic acid **9** and glycopeptide thioester **80**

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 thioesterformation 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.

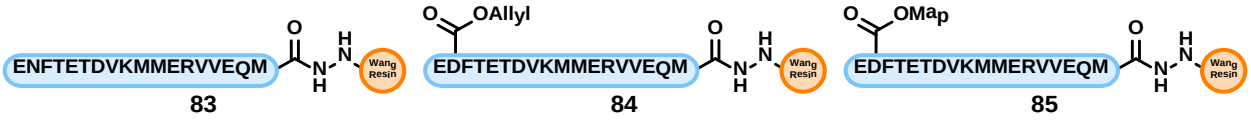


Figure 9: Fragment 2A for SPFC

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.

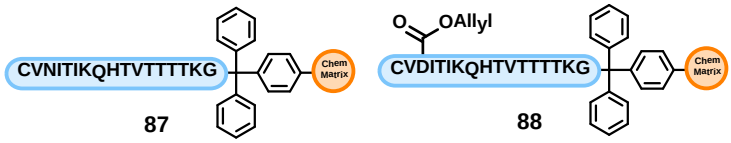


Figure 10: Fragment 2B for SPFC

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.

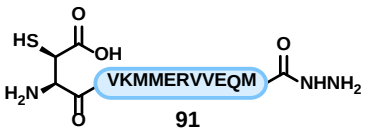


Figure 11: Fragment 2C (202-213) 91

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.

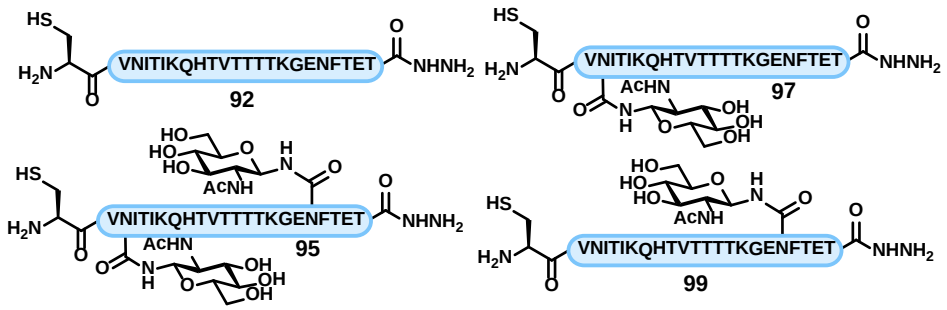


Figure 12: Peptide- and glycopeptide hydrazides for ligation to thioester **45**

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.

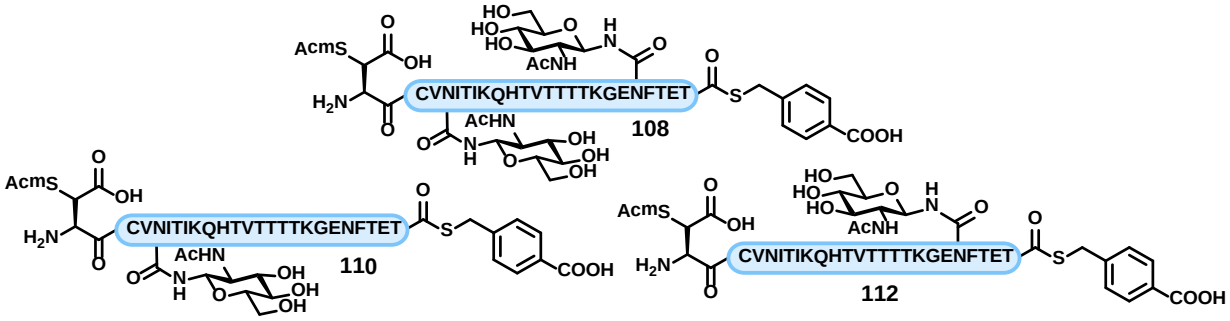


Figure 13: Acm protected thioesters for ligation to **91**

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.

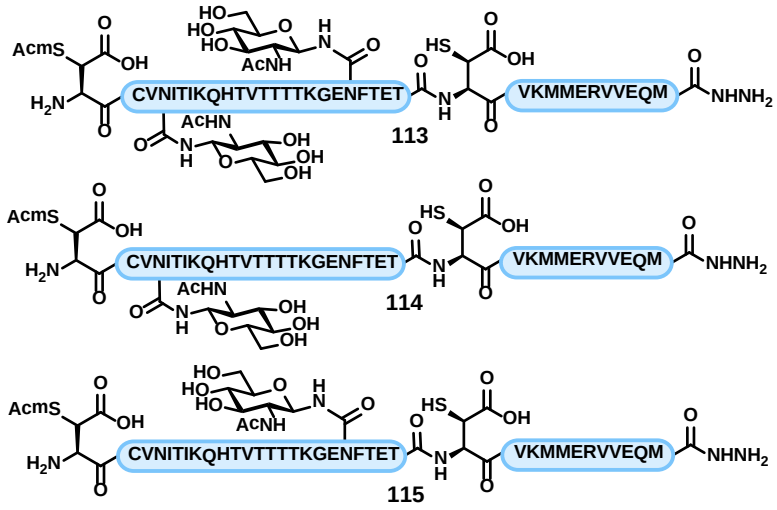


Figure 14: Ligation products for the synthesis of fragment 2

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^{C}) der Prion Proteins in die sogenannte Scrapie Form (PrP^{Sc}) des Proteins assoziiert. PrP^{Sc} ist unlöslich und teilweise resistent gegenüber Proteasen. PrP^{C} 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.

Um die Aufgabe dieser Kohlenhydrate und des GPI-Ankers genau zu studieren, ist es von hoher Wichtigkeit homogene Formen des Prion Glykoproteins zu haben. Mit heutigen Methoden ist es sehr kompliziert homogene Glykoproteine aus natürlichen Proben zu gewinnen. Außerdem ist die chemische Synthese von Glykopeptiden und Glykoproteinen eine sehr komplexe Aufgabe.

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.

Die geeignetste Methode für die Synthese von Glykopeptiden ist das Koppeln eines Zuckers an ein geschütztes synthetisches Peptid, dessen Schutzgruppen sich später leicht entfernen lassen, unter Nutzung der so genannten Lansbury Aspartlierung. Für die Synthese von Glykopeptiden ist es essentiell leichten Zugang zu Glykanaziden zu haben, die, nach der Reduktion des Azids zum Amin, direkt an das Peptid gekoppelt werden. Aus diesem Grund wurde, beginnend von kommerziell erhältlichem Glucosamin Hydrochlorid, *N*-Acetylglucosamin Azid **12** auf zwei verschiedenen synthetischen Wegen erzeugt. Durch Reduktion wurde *N*-Acetylglucosamin Amin **19** hergestellt und für die Synthese von verschiedenen Glykopeptiden genutzt. Zusätzlich wurde Nonasaccharid Azid **13** durch chemisch enzymatische Modifizierung eines aus Eigelb extrahierten *N*-Acetylneuraminsäure besitzenden Glykopeptids synthetisiert. Im Anschluss daran wurden die *N*-Acetylneuraminsäuren unter sauren Bedingungen abgespalten und das Glykan durch

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

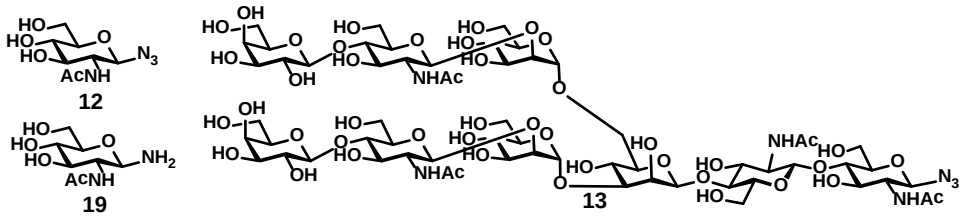


Abbildung 1: Glykan Azid und Amin für die Synthese von Glykopeptiden

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

Eine zweite essentielle Voraussetzung für die Synthese von glykosylierten Peptiden unter Nutzung der Aspartylierungsbedingungen nach Lansbury ist das orthogonale Schützen der Säureseitenkettenfunktion von Asparaginsäure. Nach dessen Entfernung bleiben alle anderen Schutzgruppen intakt und der Zucker kann selektiv gekoppelt werden. Das Prion Protein besitzt zwei Glykosylierungsstellen nah beieinander an Position Asn181 und Asn197. Dadurch werden zwei unterschiedliche orthogonal geschützte Asparaginsäuren benötigt und eine lichtsensitiv geschützte Asparaginsäure wurde etabliert um die weitläufig genutzte Allyl Schutzgruppe zu komplementieren. Die Synthese begann mit kommerziell erhältlichen 4-(Dimethylamin)-Acetophenon und L-Asparaginsäure und der benötigte Fmoc-Asp(OMap)-OH **11** Baustein wurde in 7 Schritten hergestellt. Anschließend wurde Aminosäure **11** genutzt und in Modelpeptid **36** durch Peptidfestphasensynthese eingebaut. Baustein **11** konnte ohne das Auftreten von Nebenreaktionen eingebaut werden und lichtsensitive Map-Schutzgruppe war stabil während das Peptid weiter verlängert wurde. Anschließend konnte gezeigt werden, dass sich die Map-Schutzgruppe selektiv durch Photolyse mit einer Quecksilberlampe (hν>300 nm) entfernen lässt. An die freie Säurefunktion von Asparaginsäure wurde *N*-Acetylglucosamin Amin **19** gekoppelt und damit Glycopeptid **38** hergestellt. Mit der erfolgreichen Synthese von Glykopeptid **38** wurde die Gebrauchstauglichkeit von Fmoc-Asp(OMap)-OH **11** für die Synthese von homogenen Glykopeptiden bewiesen.

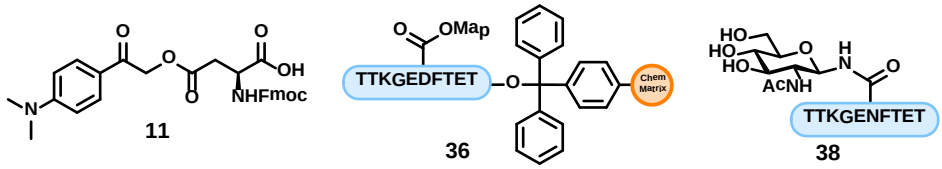


Abbildung 2: Orthogonal geschützte Asparaginsäure **11** und Modelpeptide **36** und **38**

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.

Die benötigten β-Mercaptyl Asparaginsäurebausteine für die Installation an Asp178 sind nicht kommerziell erhältlich. Daher wurden, beginnend aus einer voll geschützten Asparaginsäure und einem Sulfunierungsreagenz, drei β-Mercaptyl Asparaginsäuren synthetisiert.

Zuerst wurde mit dieser Methode Boc-Asp(O*t*Bu,STmob)-OH **8** erzeugt. Die Reaktion einer voll geschützten Asparaginsäure mit einem STmob-Tosylat bei tiefen Temperaturen und unter stark basischen Bedingungen ergab eine β-Mercaptyl funktionalisierte Asparaginsäure als Mischung von Diastereomeren. Nachdem der Allylester gespalten wurde, konnten die Isomere **8a** und **8b** mit Hilfe von RP-HPLC getrennt werden und beide Isomere wurden für die Peptidfestphasensynthese genutzt.

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 Sulfunierungsreagenz. Die resultierenden Diastereomere wurden nach Allylesterspaltung durch RP-HPLC getrennt und beide Isomere, **9a** und **9b**, wurden für die Synthese von Prion Peptidfragementen genutzt die eine orthogonal geschützte *N*-terminale β-Mercaptyl Asparaginsäure besitzen.

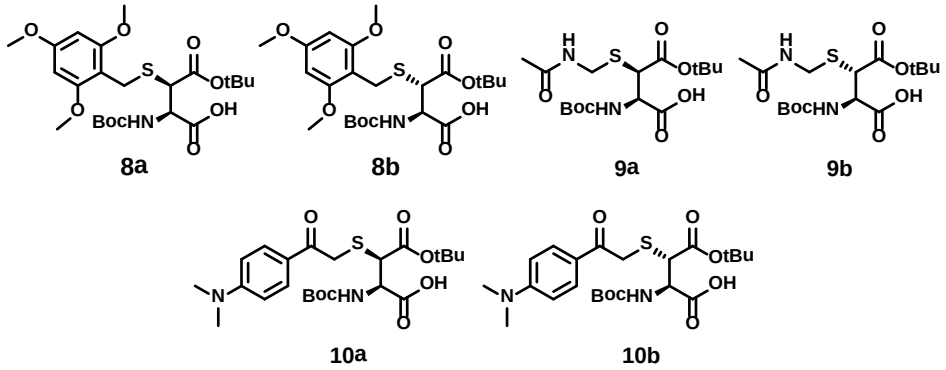


Abbildung 3: β-Mercaptyl Asparaginsäurebausteine für Ligations- und Entschwefelungsstrategien

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 Sulfunierungsreagenz verwendet. Nach der Allylestersplatung 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.

Das Prionproteinfragment 1 [PrP I (214-231)] wurde durch Peptidfestphasensynthese synthetisiert. Um die Peptide als Thioester zu generieren und diese für native chemische Ligation zu nutzen, wurden sie als Peptidhydrazid und als Nbz-Peptid synthetisiert. Nbz-Peptid **53** war keine geeignete Thioestervorstufe und aus diesem Grund wurden Peptidhydrazide genutzt um Thioester für NCL herzustellen. Für eine Ligationsstrategie in C nach N Richtung wurde Peptidhydrazid **56** mit einem ungeschützten *N*-terminalen Cystein synthetisiert. Außerdem wurde ein Prionproteinfragment 1 mit einem orthogonal geschützten Cystein synthetisiert um sequentielle Ligation in N nach C Richtung zu ermöglichen.

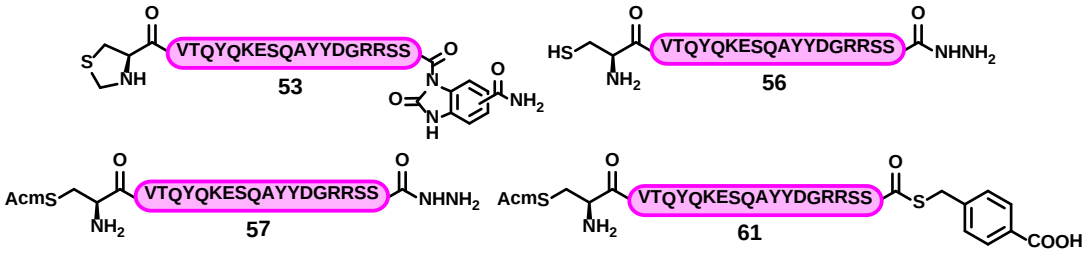


Abbildung 4: Prionproteinfragmente [PrP I (214-231)]

Nach der erfolgreichen Synthese von Prionproteinhydrazid **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 Na_2HPO_4, 5 eq MMBA und 30 eq TCEP wurde das gewünschte Ligationsprodukt **63** hergestellt. Anschließend wurde die Acm-Schutzgruppe mit Hilfe von $PdCl_2$ entfernt und PrP I [Cys-DiMan] **64** wurde isoliert.

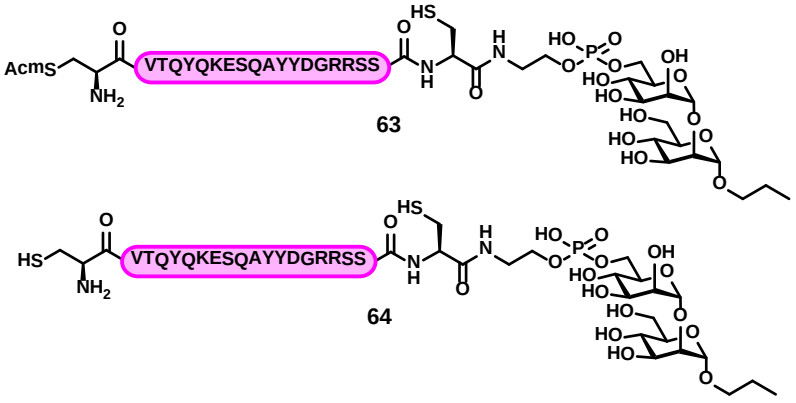


Abbildung 5: Prionproteinfragmente gekoppelt mit einer Cystein modifizierten GPI-Dimannose

Für die Synthese des Prionproteinfragments 2 [PrP II (178-213)] wurden vier verschiedene Strategien untersucht. Die gewünschten Peptide wurden durch Peptidfestphasensynthese als Peptidhydrazide synthetisiert um sie anschließend in Thioester umzuwandeln. Während der Synthese von Prionproteinfragment 2 wurde die Oxidation von Methionin Met205, Met206

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.

Als erste Strategie wurde eine Ligations- und Entschwefelungsstrategie in N nach C Richtung untersucht. Aus diesem Grund wurde die säurelabil geschützte β-Mercaptyl Asparaginsäure **8** als *N*-terminale Aminosäure installiert. Ein Peptidhydrazid und drei Glykopeptidhydrazide mit einer β-Mercaptyl Asparaginsäure an Position Asp178 wurden synthetisiert. Für die Synthese von Glykopeptiden wurden Asp181 und/oder Asp197 mit einer orthogonalen Allylschutzgruppe in die Peptidsequenz eingebaut. Anschließend wurde der Allylester gespalten und *N*-Acetylglucosamin Amin **19** wurde gekoppelt.

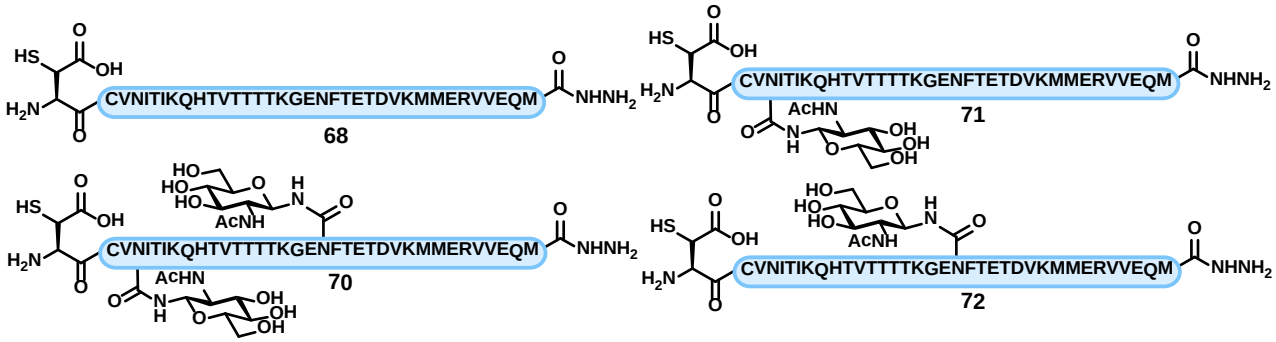


Abbildung 6: PrP II (178-213) Peptide und Glycopeptide mit β-Mercaptyl Asparaginsäure **8**

Da die freie Thiolgruppe von Cys179 und β-Mercaptyl Asp178 inter- und intramolekulare Disulfide formten, wurden die Peptide vor der Reinigung mit RP-HPLC mit TCEP reduziert. Leider führte das zu einer Entschwefelung von β-Mercaptyl Asparaginsäure der Glykopeptide **70**, **71** und **72**. Zusätzlich wurde eine Peptidfragment (160-177) des Proteinthioesters [PrP (23-177)] als Peptidhydrazid **73** synthetisiert und in Thioester **74** umgewandelt. Peptidthioester **74** und Peptid **68** wurden chemoselektiv ligiert und das resultierende Produkt **75** wurde mit LC-MS Analyse nachgewiesen.

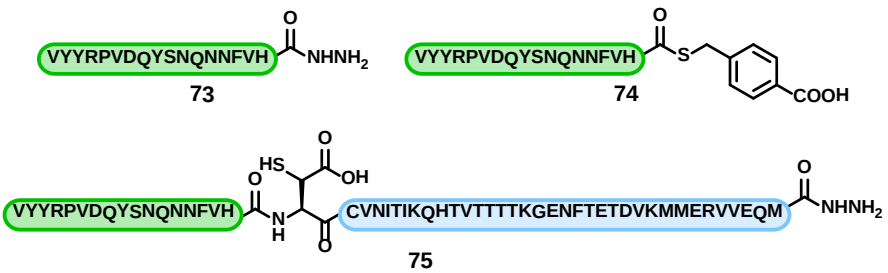


Abbildung 7: Hydrazid **73**, Thioester **74** und Ligationsprodukt **75**

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

Schützung der Thiolfunktion mit der Acm Schutzgruppe sequentielle Ligation in C nach N Richtung. Diesem Ansatz folgend wurden das Peptidhydrazid **77** und das Glykopeptid Hydrazid **79** erfolgreich synthetisiert. Zur Installation der Glykosylierung in **79** wurde eine Allylester geschützte Asparaginsäure an Position 181 eingeführt. Nach erfolgter Esterspaltung wurde die resultierende Säurefunktion mit Glykan Amin Baustein **19** in einer Lansbury Reaktion gekoppelt. Hinsichtlich der im Anschluss erfolgenden Thioesterformierung wurde festgestellt, dass außerordentliche Aufreinigung des Peptidhydrazids von Nöten ist, um eine erfolgreiche Umsetzung zu gewährleisten. Der resultierende Glykopeptidthioester **80** wurde in Mischung mit dem entsprechenden Thiolacton, welches zwischen Cystein 179 und dem *C*-Terminus geformt wird, isoliert. Der Glykopeptidthioester **80** wurde in Ligationsreaktionen mit Fragment 1 gestestet.

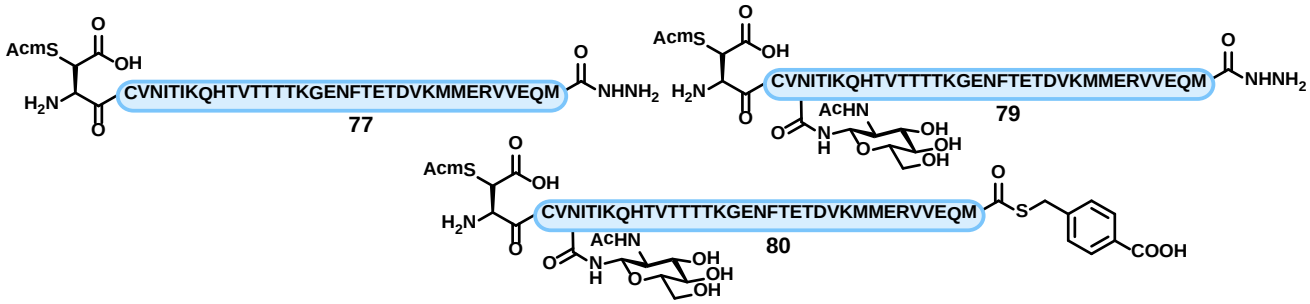


Abbildung 8: *N*-Terminus β-Mercaptyl Asparaginsäure **9** modifiziertes Hydrazid **77** und Glykopeptid Hydrazid **79** und Glykopeptidthioester **80**

Die dritte untersuchte Strategie zur Synthese von Fragment 2 bediente sich der Festphasenfragmentkondensation. Fragment 2A PrP (196-213) und Fragment 2B PrP (178-195), wurden als zwei kleinere Fragmente von PrP II (179-213) synthetisiert. Um Racemisierung während der Kopplung zu vermeiden, wurde die für dieses Vorhaben geeignete Aminosäure Gly195 am *C*-Terminus von Fragment 2B gewählt.

Um die anvisierte Thioesterformierung und Ligation zu ermöglichen, wurde Fragment 2A als Peptidhydrazid synthetisiert. Zum Zwecke der optionalen Lansbury Kupplung von Glykan Aminen nach erfolgreicher Peptidfestphasensynthese und Kondensation, wurden in zwei zusätzlichen Synthesen die enthaltene Asparaginsäure 197 mit den orthogonalen Allyl- und Map-Schutzgruppen synthetisiert. Die entsprechenden Peptide **83**, **84** und **85** wurden in hoher roher Sauberkeit synthetisiert.

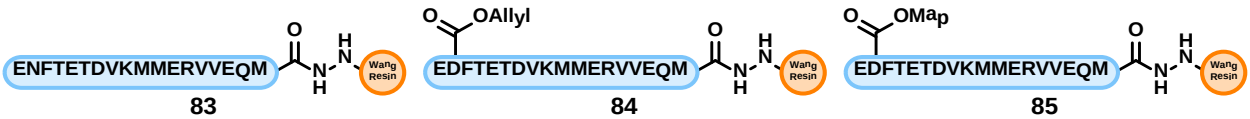


Abbildung 9: Synthetisierte Fragmente 2A für die SPFC mit orthogonalen Schutzgruppen zur möglichen Installation von Glykosylierungen

Die Glykosylierungsstelle an Asparaginsäure 181 im Fragment 2B wurde zusätzlich mit einer orthogonalen Allyl-Schutzgruppe ausgestattet.

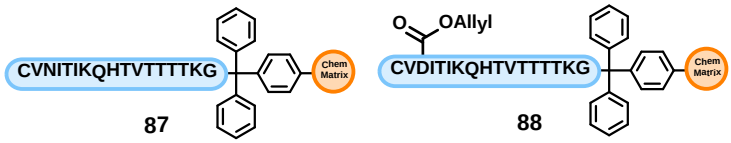


Abbildung 10: Synthetisierte resingebundene Fragmente 2B für die SPFC

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.

Für die Synthese von Fragment 2 (178-213) wurde weiterhin eine Ligation-Entschwefelungsstrategie studiert. Für die Synthese des gewünschten Fragments wurden zwei Peptidhyrazide dargestellt. Während Fragment 2C (202-213) unter Verwendung der *N*-terminalen Aminosäure **8** synthetisiert wurde, erforderte die Synthese von Fragment D (178-201) die Verwendung einer geschützten β-Mercaptyl Asparaginsäure an Position Asp178 zur späteren Durchführung einer sequentiellen Ligation in C nach N Richtung.

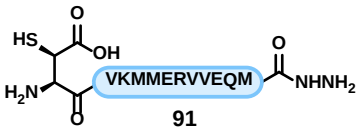


Abbildung 11: Fragment 2C (202-213) 91

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.

Die Synthese von Fragment 2D (178-201) wurde auf zwei Wegen untersucht. Zunächst wurde ein *N*-terminales Cystein an den dargestellten Peptiden installiert. Wiederum, wurden dabei die Asparaginsäuren 181 und 197 als mögliche Glykosylierungsstellen mit der orthogonalen Allyl-Schutzgruppe installiert. Nach erfolgter Esterspaltung wurde das Glykan Amin **19** mit Hilfe der Lansbury Kopplung angebracht.

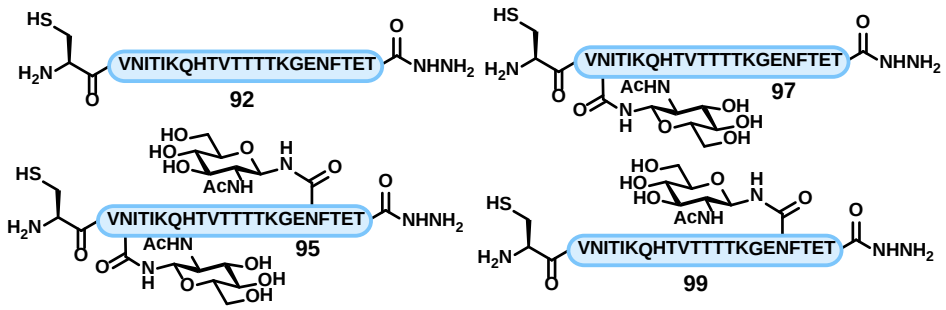


Abbildung 12: Dargestellte Peptid- und Glycopeptid-hydrazide für die spätere Ligation mit geschützter β-mercaptyl Asparaginsäure

Die entsprechend aufgereinigten Peptid- und Glykopeptid-hydrazide **92**, **95**, **97** und **99** wurden im Anschluss auf ihre Verwendung in der Ligation mit β-Mercaptyl Asparaginsäure hin untersucht. Unglücklicherweise, wurden hierbei aufgrund der geringen Löslichkeit des voll geschützten Asparaginsärethioesters keine Ligationsprodukte geformt.

Der zweite Ansatz zur Synthese von Fragment 2D machte zur Darstellung von Acm geschützten Glykopeptid-hydraziden von Baustein **9** gebrauch. Die installierten Allyl-Schutzgruppen der Asparaginsäuren 181 und/oder 197 wurden entfernt und die resultierenden Säurefunktionen mit Glykan Amine **19** in einer Lansbury Kopplung ausgestattet. Die resultierenden Glykopeptide wurden hochrein abgespalten und leicht aufgereinigt. Die isolierten Hydrazide wurden daraufhin erfolgreich in Thioester überführt und nach erfolgter Größenausschlusschromatographie als Mischung mit den entsprechenden, sich im Gleichgewicht befindlichen, Thiolactonen isoliert.

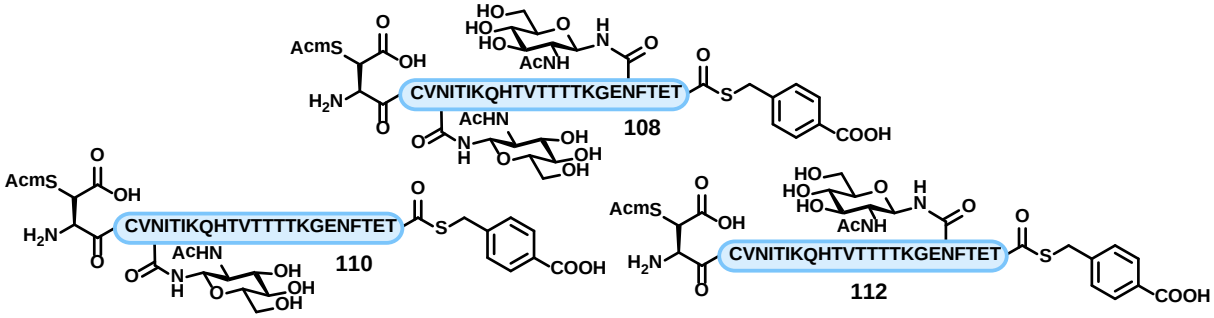


Abbildung 13: Synthetisierte Acm geschützte Thioester für die Ligation mit Fragment 2C **91**

Daraufhin wurde die Ligation der Peptidthioester mit β-mercaptyl Asparaginsäure enthaltenen Peptid **91** optimiert. Vorherig verwendete Ligationspuffer ohne zusätzlich zugesetztes Thiol resultierten in schneller Hydrolyse des Thioesters und des Thiolactons und somit nicht zur Formierung des Ligationsproduktes.

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 reaktivere 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.

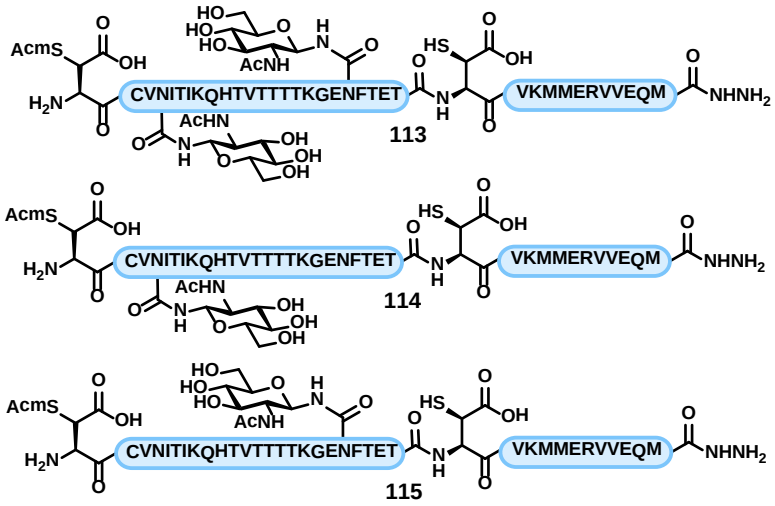


Abbildung 14: Ligationsprodukte zur Darstellung von Fragment 2

Zusammenfassend wurde die Synthese homogener *N*-glykosylierter GPI-verankerter Prion Proteine mit Hilfe einer Strategie, die sequenzielle native chemische Ligation von Protein-, Peptid- und Glykopeptidthioester verwendet, untersucht. Nach erfolgreicher Darstellung der benötigten Bausteine für die Glykopeptidsynthese und die Ligation-Entschwefelungsstrategie wurden diese benutzt um die nötigen Peptid- und Glykopeptidthioester zu generieren. Die Ligation des *C*-terminalen PrP Fragments 1 (214-231) mit einer cysteine-modifizierten DiMan Struktur war nach Optimierung des Puffersystems effektiv und belastbar. Diese Bedingungen führten jedoch nicht zum gewünschten Produkt, wenn ein komplexer lipidierter GPI genutzt wurde und weitere Optimierung ist hier von Nöten. Zusätzlich wurde PrP Fragment 2 (178-213) in nicht-, mono- und diglykosylierten Formen mit Hilfe einer SPPS und Ligation-Entschwefelungsstrategie dargestellt. Die dargestellten Thioester wurden erfolgreich in verschiedenen Ligationen verwendet. Um die geringe Umsetzung der Thioester in zukünftigen Anwendungen zu verbessern, ist die permanente Schützung des Cysteins 179 von höchster Bedeutung. Während der Darstellung der Glykopeptide zeigte sich die Installierung eines Glukosamins effektiv, während ein eigens isoliertes Nonasaccharid nicht mit Hife der Lansbury Kopplung installiert werden konnte. Für die Zukunft erscheint hier die enzymatische Verlängerung der synthetisierten Glykopeptide mit Hilfe eines Glykanoxazolidin geeignet.

INTRODUCTION

1. GLYCOPROTEINS

The majority of proteins are post translationally modified[2] including phosphorylation, lipidation, or glycosylation,[3-4] whereby the attachment of carbohydrates is the most abundant alteration in nature.[2] 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.[5-7] 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.[8-12] The importance of understanding the functions of glycoproteins is also motivated by their role played in many human diseases like autoimmune infections and cancer.[5, 13-14] Furthermore, it has been suggested that the naturally occurring mixtures of glycoforms provide a spectrum of activities that can be seen as fine-tuning.[15-16] *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.[17] 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.[9] 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*.[9] Carbohydrate modifications can change properties of the attached protein by altering the stability, protease resistance, or quaternary structure.[8] 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.[18]

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, *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 6.4×10^7 "words", carbohydrates can create more than 1.05×10^{12} variations.[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.[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.[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.[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".[24]

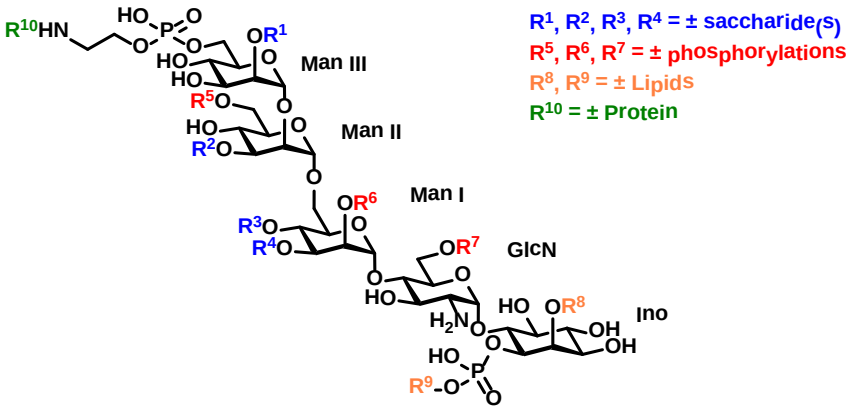
1.3 Types of Oligosaccharide linkages

Among the known carbohydrate-protein linkages (*C*-, *N*-, *O*-, and *S*-linkages), most common are *N*- and *O*-linked glycoproteins. Regarding the *O*-linked type, small oligosaccharides are attached to either serine or threonine usually having an *N*-acetyl-α-D-galactosamine as first unit. These sequences of small oligosaccharides are classified into 8 groups.[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 $Man_3GlcNAc_2$-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 GPIs 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]



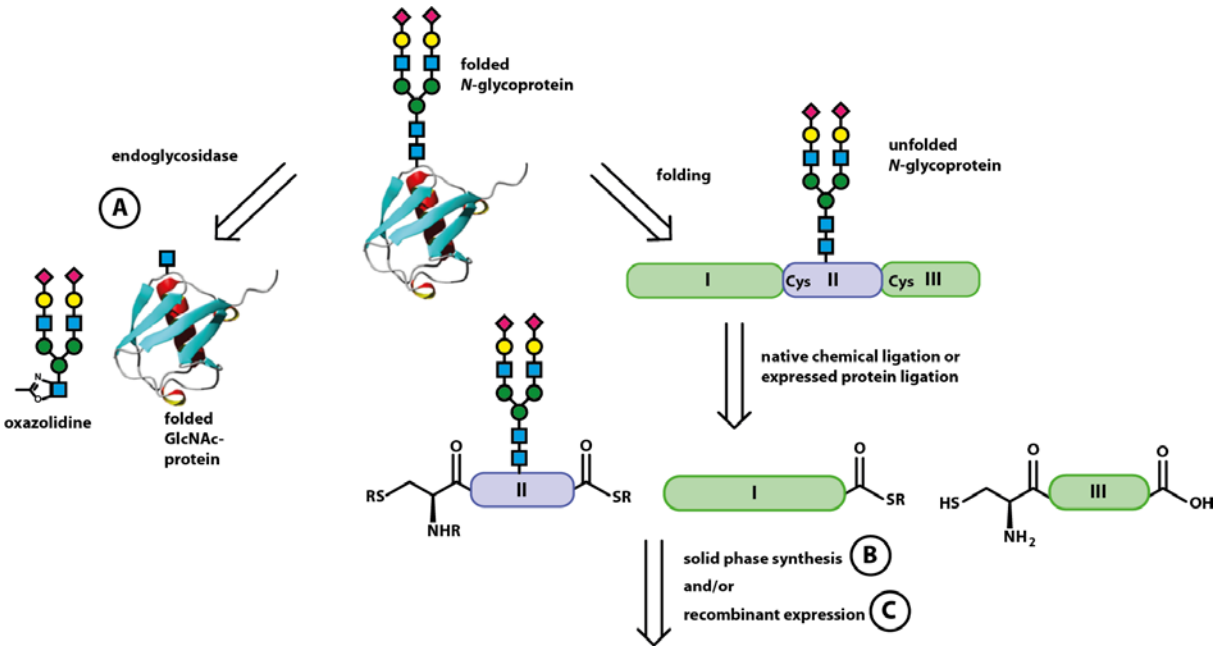
Scheme 1: General structure of GPIs: the black core can bear different appendages depending on species, tissue and cell-type origin

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.[29] 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.



Scheme 2: Retrosynthetic pathways for the synthesis of *N*-glycoproteins

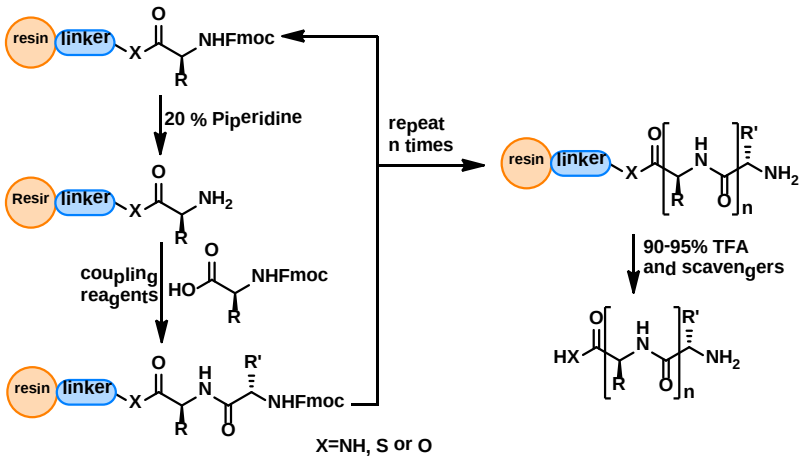
Transglycosylation (A, Scheme 2) involves the enzymatic manipulation of an expressed glycoprotein with a glycosidase followed by an enzymatic coupling of the desired oligosaccharides. A homogeneous glycoprotein can as well be obtained by sole chemical synthesis. Small peptides are synthesized by solid 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)

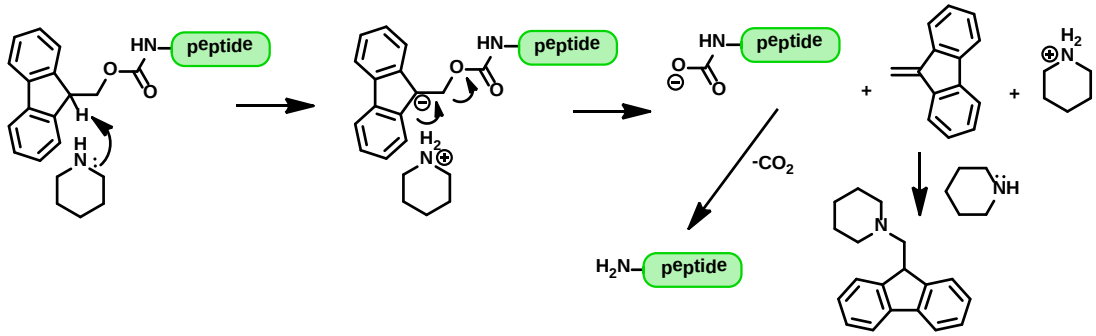
Synthetic peptides are used ubiquitously for studies in biology, biochemistry, chemical biology, medicinal chemistry, and many other areas of research and industry.[34] Solid phase peptide synthesis (SPPS) is the predominant method for preparation of peptides. It was introduced by Merrifield *et al.*[35] 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 N^{α}-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 N^{α}-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 triflouroacetic acid (TFA) or TFA in dichlormethane (DCM).[34] 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 triflouromethanesulfonic acid for the deprotection of the side-chain protecting groups, which can lead not only to several byproducts but are also dangerous to handle.[34] 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-Fluorenylmethoxycarbonyl (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.[34] 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.



Scheme 3: Fmoc-SPPS

During Fmoc-deprotection the Fmoc-group undergoes an $E1_{cb}$ 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]

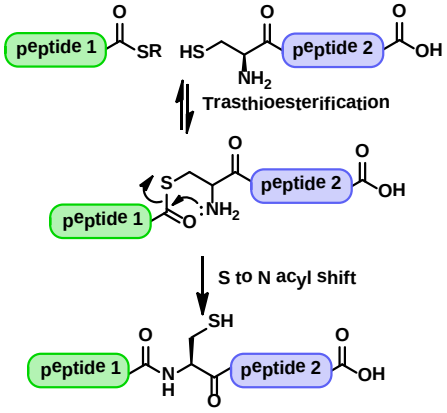


Scheme 4: Fmoc deprotection

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.[38] 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.



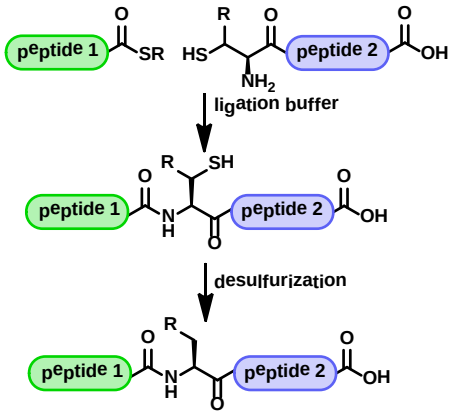
Scheme 5: Native Chemical Ligation

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[38] 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.[39] The main focus thereby lies on the development of new *N*-terminal cysteine surrogates and of new *C*-terminal acyl donors.[39-40] 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.[41-42] 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).



Scheme 6: Ligation-desulfurization

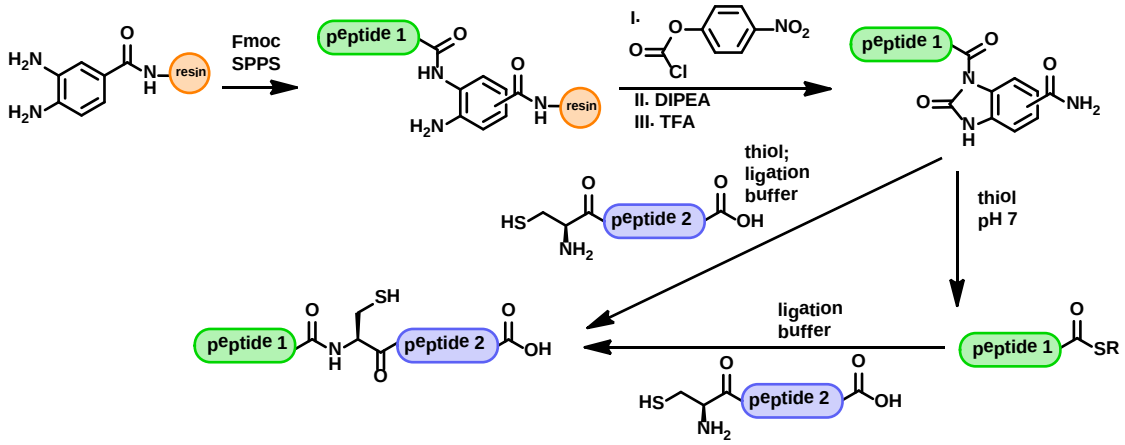
A great number of thiol-derived amino acids for the ligation-desulfurization strategy have been reported including: phenylalanine, valine, lysine, threonine, leucine, proline, glutamine, arginine, aspartic acid, glutamic acid and tryptophan.[39, 43] Ligation-desulfurization chemistry using these β- or 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.[44] However, to date only γ-selenoproline[45] and β-selenophenylalanine[46] 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.[47] 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.[47]

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]



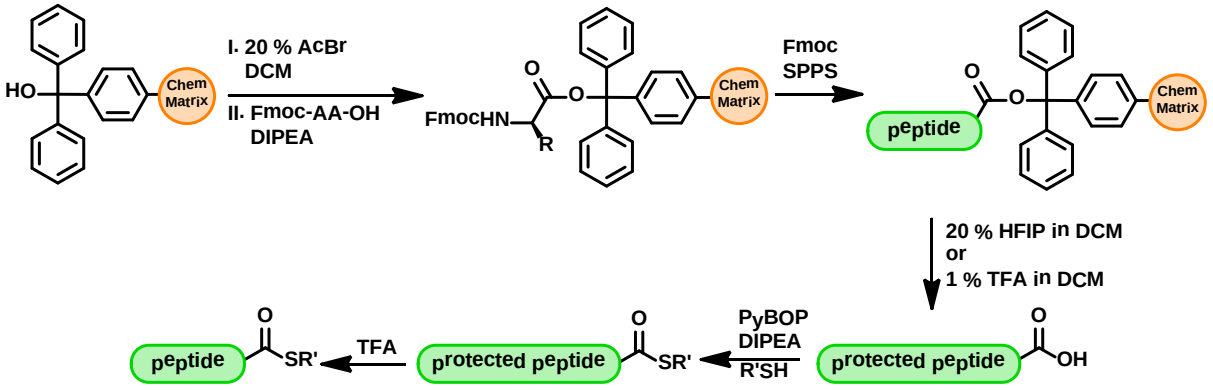
Scheme 7: Synthesis of a peptide thioester precursor using Dawson Dbz linker

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.

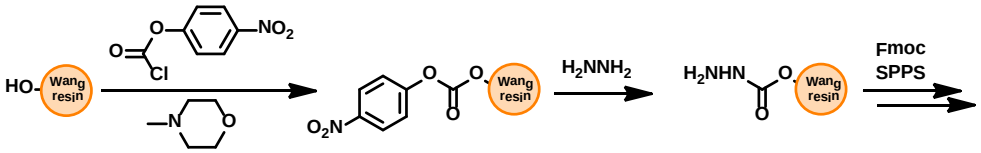


Scheme 8: Synthesis of peptides thioester from fully protected peptide fragments

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

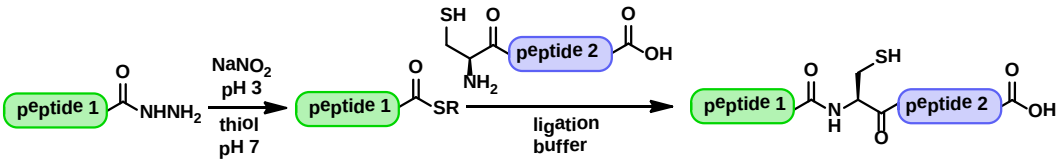
A different strategy to generate a peptide thioester precursor directly from the solid support is the synthesis of peptide hydrazides. To prepare the required hydrazide carboxylate the hydroxyl group of a Wang resin is activated with *p*-nitrophenyl chloroformate and *N*-methyl morpholine and subsequently reacted with hydrazinium hydroxide (Scheme 9).[51]



Scheme 9: Synthesis of Wang hydrazide resin

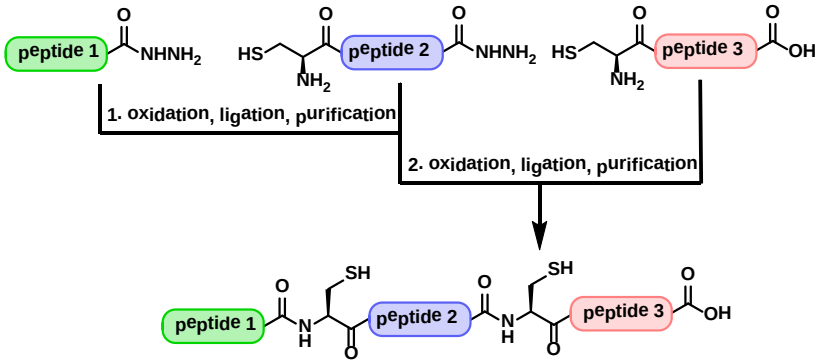
After complete elongation, the peptide is cleaved from the solid support with TFA. The unprotected peptide is further reacted to the desired thioester using an oxidation with $NaNO_2$ and followed by a reaction with a thiol. Mechanistic studies showed that during the oxidation step the peptide hydrazide is converted into an azide using $NaNO_2$ 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).



Scheme 10: Diazotation and ligation using peptide hydrazides

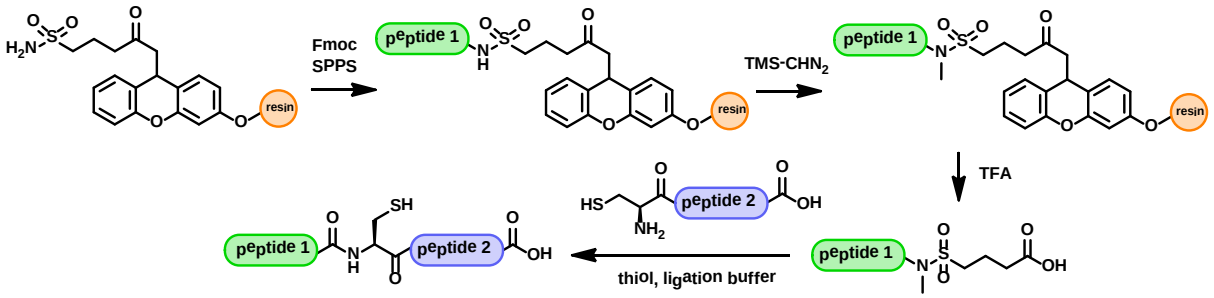
An advantage of using peptide hydrazides as thioester precursors is the applicability in sequential native chemical ligation. Previously, sequential NCL of multiple peptide fragments was performed in C to N direction, while the *N*-terminal cysteine group was protected. A new approach, conducting sequential NCL in N to C direction, using peptide hydrazides was introduced by Liu *et al.* in 2011.[51] 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.



Scheme 11: Sequential native chemical ligation 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.



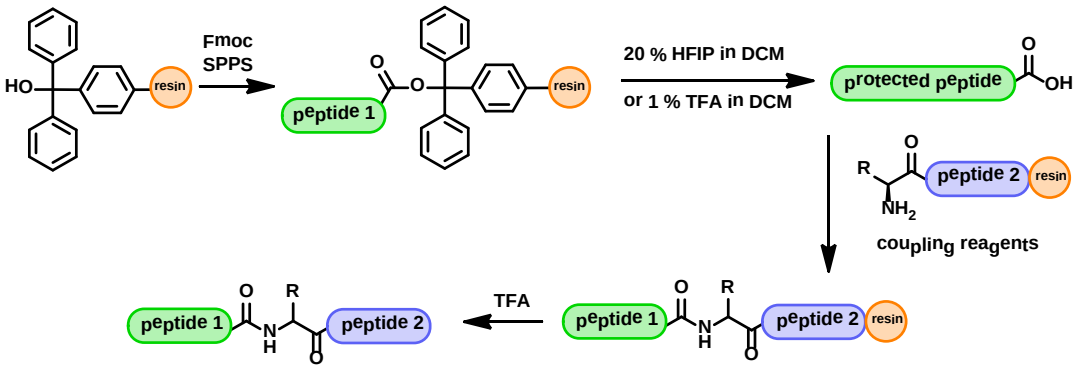
Scheme 12: Synthesis of peptide thioester precursor using sulfonamides

This method was improved by Burlina *et al.*,[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, *e.g.* rapid reaction, high yield and easy removal of excess reagent, are combined.[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.[36]



Scheme 13: Synthesis of peptides via SPFC

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.[54-56] 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[36] 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.[57] 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.[57] 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.[58] Installing the *N*-terminal cysteine as Thiazolidine (Thz) (Figure 15) has been widely used for this purpose.[58-61] The required Boc-L-Thioproline 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.[58]

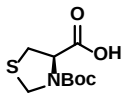


Figure 15: L-Boc-Thioproline

Other commonly used orthogonal protecting groups for cysteine are Acetamidomethyl (Acm) and Phenylacetamidomethyl (PhAcm) (Figure 16).[56, 62-65] The required amino acid building blocks are commercially available and they can be coupled to the peptide chain using standard SPPS conditions.

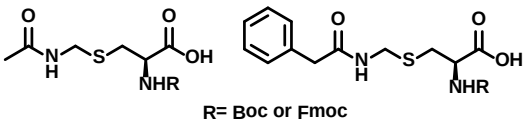


Figure 16: Acetamidomethly (Acm) and Phenylacetamidomethyl (PhAcm) protected cysteine

Both protecting groups (Acm and PhAcm) can be removed with mercury salts (Hg[II]) to give the free thiol and with iodine (I_2) 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.[57] Recently, it was shown by Brik *et al.*[66] 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.*[67] 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).[68] 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.[69]

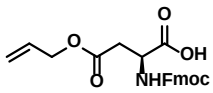


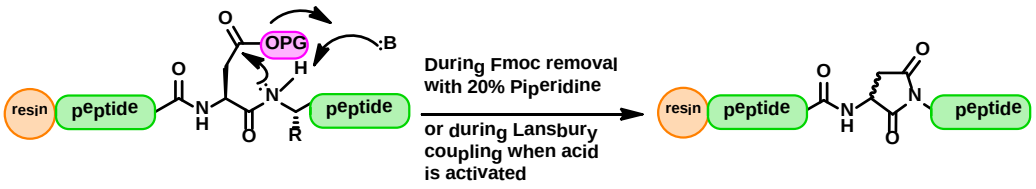
Figure 17: Allyl protected aspartic acid

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(O*t*Bu)-Xaa motif, where Xaa=Asn(Trt), Gly, Ser, Thr.[34, 70] 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.[70-71] 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.[72-73] Yet, for residues other than glycine this approach is tedious and causes racemization when coupling backbone protected dipeptides.[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.[69] The method takes advantage of the glycosylation consensus-sequence (Asn-Xaa-Ser/Thr; Xaa≠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).

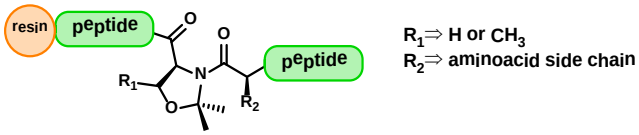
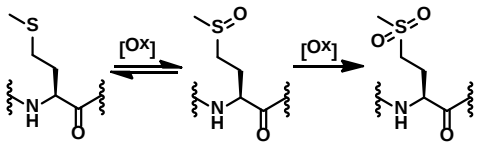


Figure 18: *Cis*-conformation of pseudoproline containing peptides

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

Oxidation of Methionine

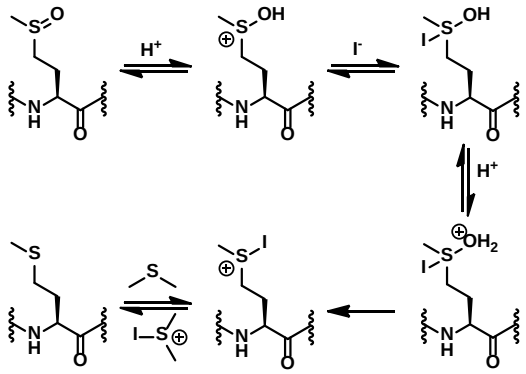
A major problem associated with peptide synthesis is the oxidative degradation of oxidation sensitive amino acids. Together with cysteine, methionine is one of the easily oxidized amino acids.[76] The thioether side chain function of methionine has a low oxidation potential and is particularly sensitive 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

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.[77]

Unfortunately, only the number of oxidized methionine can be determined using mass spectrometry but the exact position usually remains unclear.[78] The amino acid sequence surrounding the methionine and/or conformational changes possibly play a role in the oxidation process.[76] However, a consensus sequence for methionine oxidation has yet to be identified. Reduction of Met(O) *in vivo* is performed by two enzymes, *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 *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.[79-81]



Scheme 16: Reduction of methionine with NH_4I and DMS in TFA

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_2 generated during sulfoxide reduction. To circumvent this reaction, addition of dimethylsulfide (DMS) is necessary (Scheme 16).[79, 81]

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.[37, 82-83] Intermolecular hydrophobic aggregation of the protected peptide chains and/or formation of secondary structures such as β-sheets are usually the reason for these undesired physicochemical problems.[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.

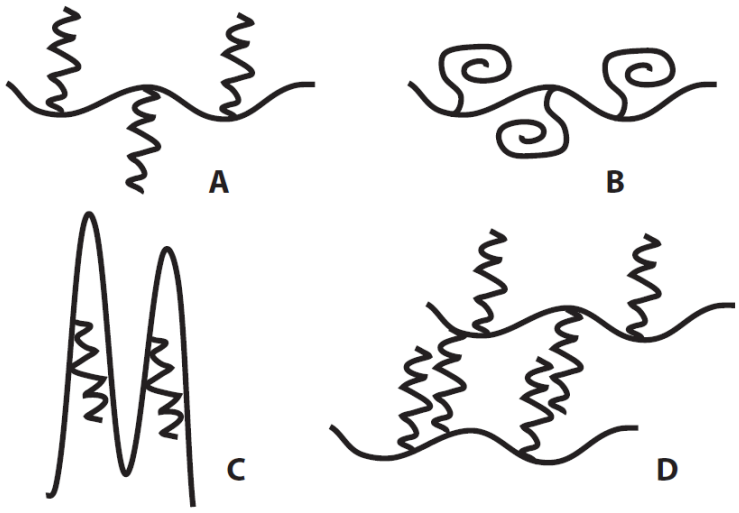
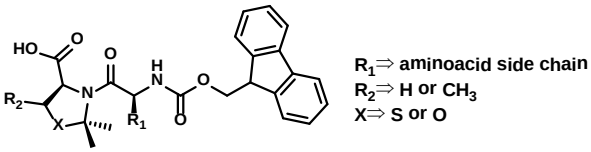


Figure 19: Scenarios depicting the possible solvation or aggregation of a polymer bound peptide sequence.

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.[86-87] The introduction of an amide protecting group within the peptide chain has shown to prevent associations involving hydrogen-bonding.[88] The earlier introduced pseudoprolines (Ψ-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.

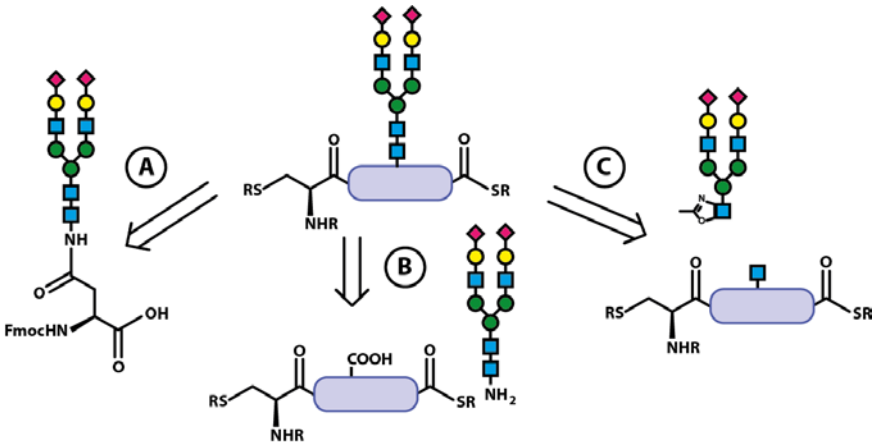


Scheme 17: Fmoc-Ψ-Proline Dipeptide

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.[82] 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.[89]

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.[69] However, after incorporation of larger glycans, further elongation of the peptide is complicated, since side reactions, involving free hydroxyl groups, can occur.[90] Moreover, solubility and reactivity of the peptide are affected in a negative manner.



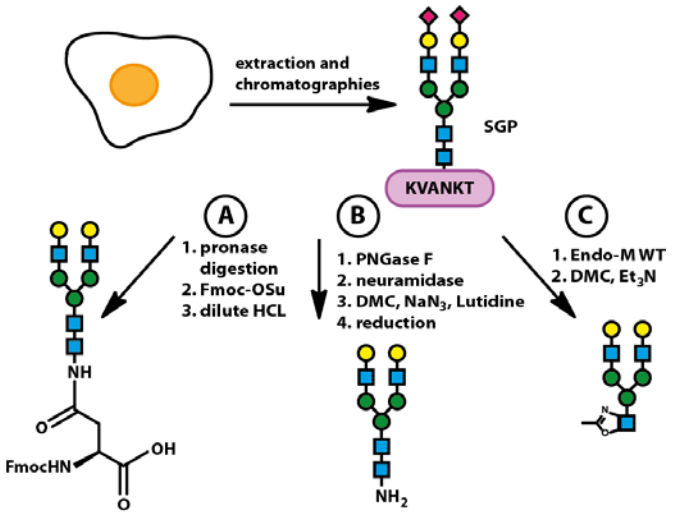
Scheme 18: Retrosynthetic pathways for the synthesis of *N*-glycopeptides

Sequential elongation of glycopeptides is more demanding compared to peptides without modifications, especially when longer or difficult sequences are targeted.[91] Therefore, installation of glycans in the last step of the synthesis is often employed as a better alternative.[67] 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).[69]

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*-acetylglucosamine residue.[92-93] 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).[94] This disialylated undecasaccharide can be degraded to valuable Fmoc-Asn building blocks, to a glycan amine and to a glycan oxazoline.



Scheme 19: Synthesis of complex *N*-Glycan-Asn and *N*-Glycans form egg yolk

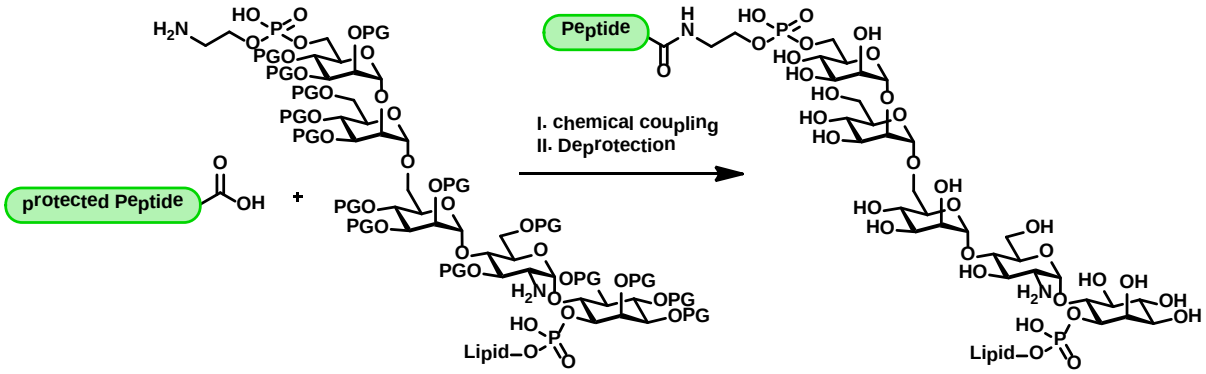
The generation of *N*-glycan-Asn building blocks for a stepwise synthesis of glycopeptides using egg yolk extracted material starts with pronase digestion of the isolated SGP. Afterwards, the free amine of the released asparagine is protected with Fmoc and the sialic acid residues are hydrolyzed under acidic conditions using diluted hydrochloric acid (A, Scheme 19).[91, 94-95] For the synthesis of complex glycan-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

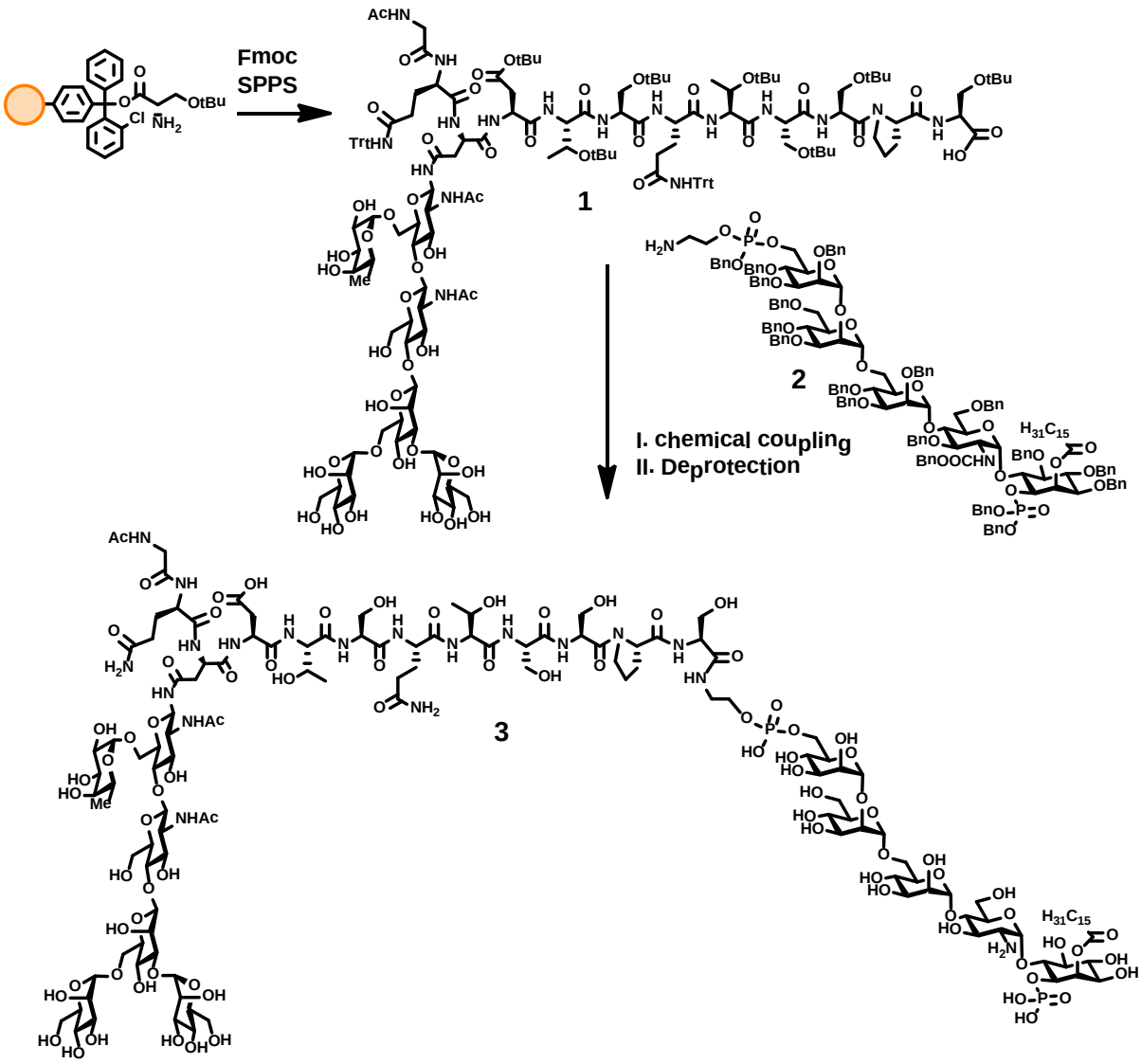
One strategy to generate homogeneous GPI-anchored peptides and glycopeptide is based on a conventional, regioselective condensation between a properly protected GPI-anchor and a fully protected peptide or glycopeptide depicted in Scheme 20.



Scheme 20: Chemical total synthesis of GPI-anchored peptides and glycopeptides

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).



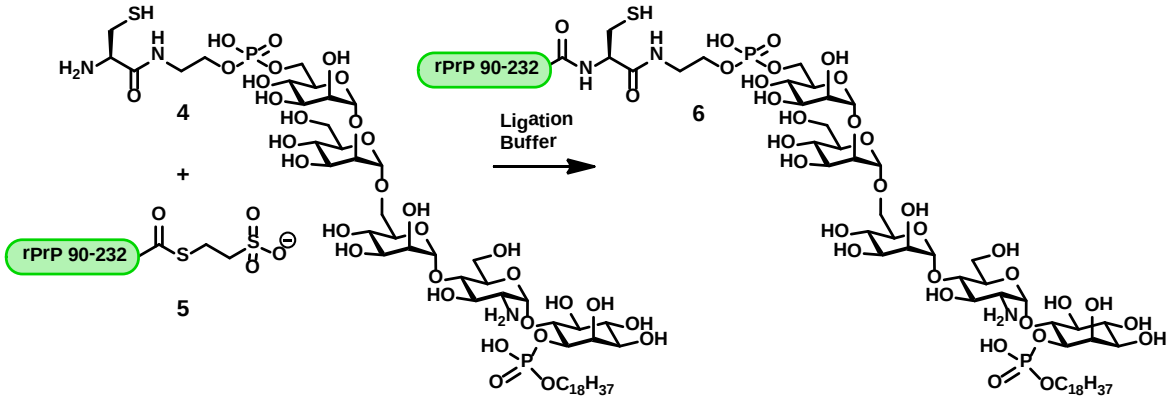
Scheme 21: Synthesis of a skeleton structure of sperm antigen CD52

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.[37, 48] 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.[101] 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.[102] 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

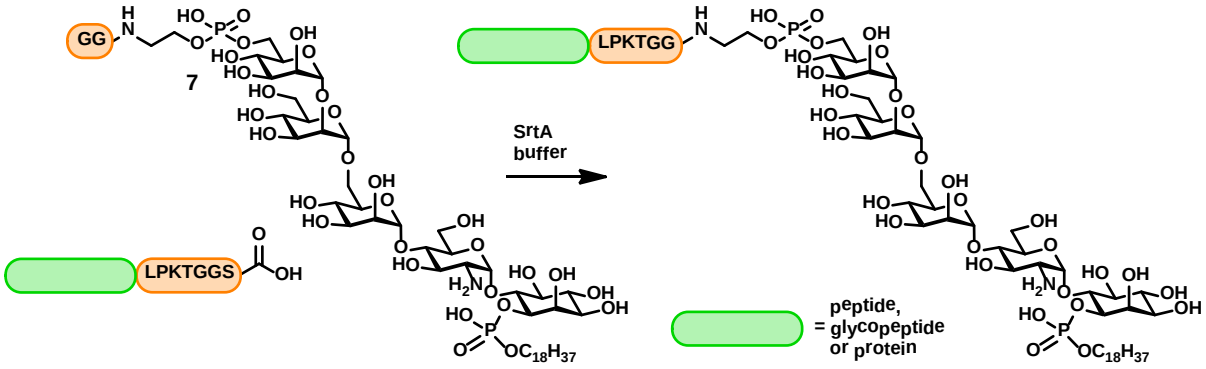
reaction.[38] Following this approach, the first protein anchored to a complete GPI was synthesized.



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.[98, 103-106] 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.[98, 107-109] 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.[104-105] Additionally, this enzyme-catalyzed ligation was used to generate a GPI-anchored glycopeptide with a partial sequence of MUC1 and a GPI-anchored GFP.[104, 106]



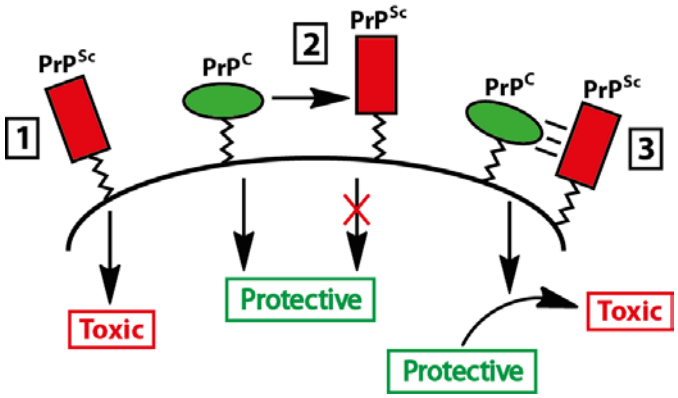
Scheme 23: Synthesis of GPI-anchored peptides, glycopeptide and proteins using SrtA

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.[110] According to the hypothesis, an abnormal form of one of the body's own proteins cellular prion protein (PrP^{C}) is transformed to an alternative form, PrP^{SC}, the so called scrapie form of the protein. It is suggested that PrP^{Sc} operates as a template which forwards the conversion of PrP^{C} to the scrapie form.[1, 111] The two isoforms differ in their conformation and aggregation, whereby the new formed PrP^{Sc} is the infectious aggregate causing neurodegenerative diseases.[111-112] The cellular prion protein is a GPI anchored membrane protein and contains two *N*-linked glycosylation sites, which are variable in their carbohydrate structure.[1, 112]

4.1 Prion Diseases

Prion diseases are infectious, fatal neurodegenerative diseases that include BSE and scrapie in animals and CJD in humans.[110, 113] 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.[114] Although prion diseases are rare neurodegenerative disorders, due to the unique biology of the transmissible agent prion, remarkable attention is given to this illness.[113] The classes of prion diseases can be grouped in genetic, infectious, or sporadic disorders, all of which involve modification of the prion protein.[115] The prion concept explains how a single disease can have genetic or infectious etiology.[111] Prions are defined transmissible particles that are devoid of nucleic acid and composed exclusively of a modified prion protein (PrP^{Sc}).[116-117] The term "prion" is derived from **pro**teinaceous and **in**fectious.[118] Prions are composed entirely of a protein that adopts an abnormal conformation and is therefore defined as infectious protein. In a posttranslational process, PrP^{C} is converted into the β-sheet rich PrP^{Sc}, thereby introducing profound changes in the physicochemical construct of PrP. Prions encipher their strain-specific in the tertiary structure of PrP.[60, 62]

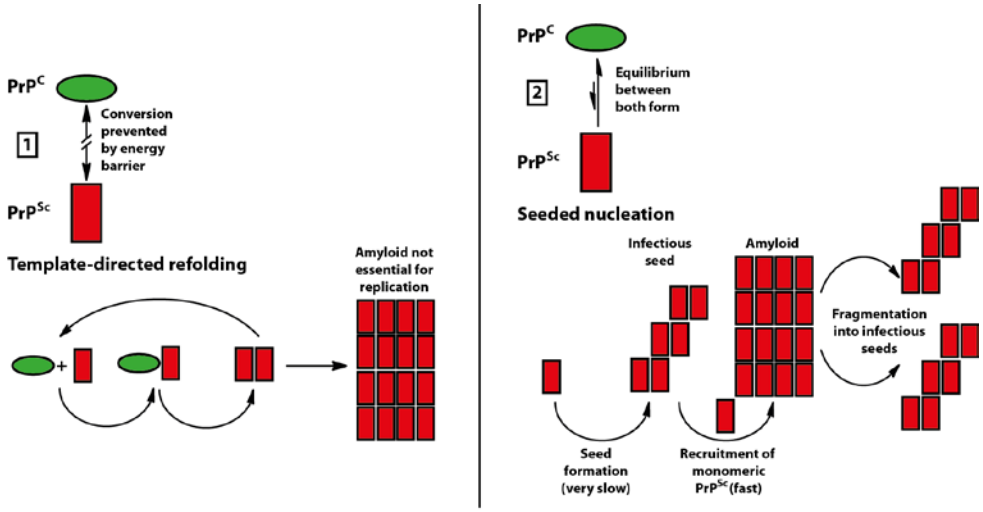


Scheme 24: Hypothesis for CNS pathology caused by the structural change of PrP^{C} to PrP^{Sc}

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 PrP^{Sc} causes the central nervous system (CNS) pathology. Frequently discussed are three hypotheses explaining how changes in PrP^{C} 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 PrP^{Sc} possesses a novel toxic property that is not related to the normal, physiological function of PrP^{C}. An alternative hypothesis, the "loss-of-function" hypothesis (2, Scheme 24), suggests that PrP^{C} holds biological activity that is lost upon conversion to PrP^{Sc} or when in contact with PrP^{Sc}. For instance, the loss of anti-apoptotic activity of PrP^{C} would lead directly to neuronal death. The "subversion-of-function" hypothesis (3, Scheme 24) proposes that the interaction with PrP^{Sc} converts PrP^{C} from a transducer of neuroprotective signals into a transducer of neurotoxic signals.

Genetic and biochemical evidence shows that the conversion of PrP^{C} to PrP^{SC} develops through the formation of a PrP^{C}/PrP^{SC} complex. Two models explain the conformational conversion of PrP^{C} to PrP^{SC}, 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 PrP^{SC} and endogenous PrP^{C}, which is induced to transform itself into further PrP^{SC}. Spontaneous conversion from PrP^{C} into PrP^{SC} may be prevented by a high-energy barrier.[1] The second model (2, Scheme 25), the so called "seeded nucleation" hypothesis, proposes that PrP^{C} and PrP^{SC} are in reversible thermodynamic equilibrium, where only a minimum amount of PrP^{SC} exists. The monomeric are mounted into a highly ordered seed and further monomeric PrP^{SC} can be recruited and eventually aggregate to amyloid. Due to fragmentation the number of nuclei would increase to recruit further PrP^{SC}, resulting in apparent replication of the agent.[121]



Scheme 25: Replication of Prions[1]

Data suggest that PrP^{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^{C} into PrP^{SC} seems to be the most attractive therapeutic target.[122] Understanding how PrP^{C} unfolds and refolds into PrP^{SC} is also of great importance to study other degenerative illnesses. Knowledge of PrP^{SC} formation may also help developing effective therapies for the more common neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease.[118]

4.2 Structure of the Prion Protein

The normal cellular prion protein (PrP^{C}) is a glycoprotein containing 209 AA (23-231) with α-helices as the predominant feature of the secondary structure.[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 α-helix and the Asn197 glycan located on a loop.[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^{Sc} from the brains of syrian hamsters contains the same set of glycans as PrP^{C}, but higher proportion of tri-and tetra-antennary sugars.[124]

Prion protein is usually, but not perpetually,[125] attached to the neuronal cell membrane via a GPI anchor at Ser231.[125] 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,[125] 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.

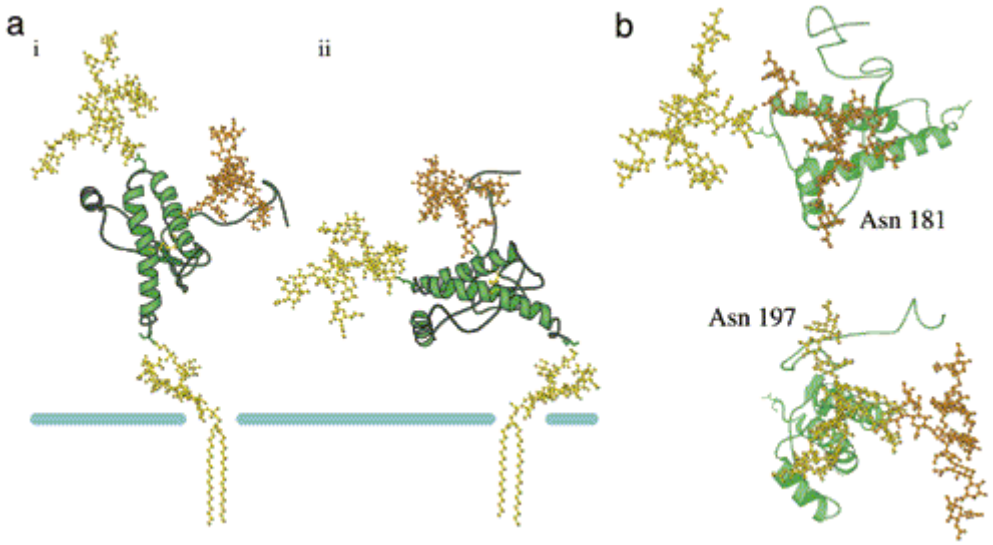
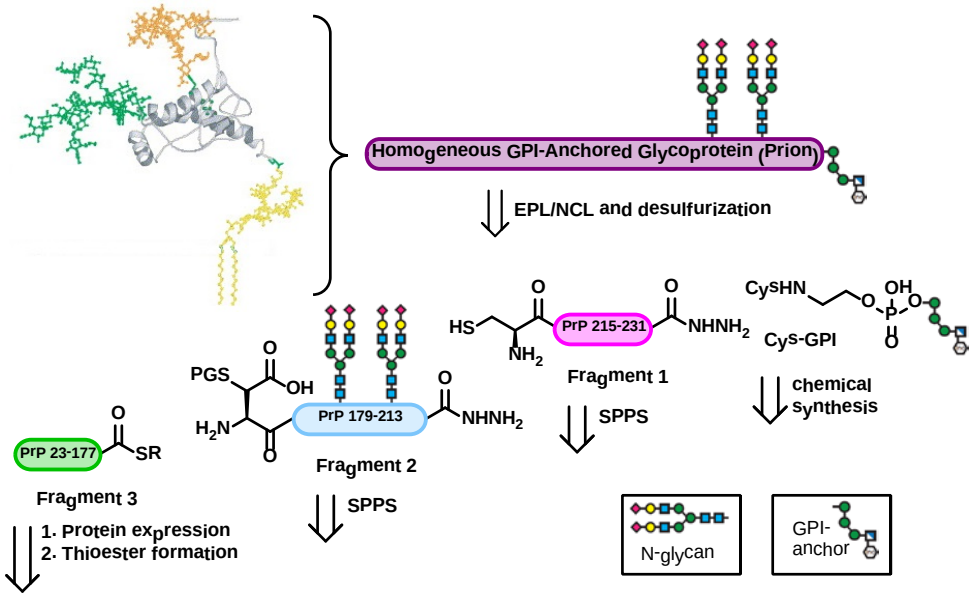


Figure 20: Proposed structure of PrP^{C}; [111, 126-127] a) The GPI anchor is shown in two orientations[127] b) two orthogonal views showing the areas of the protein surface covered by the gylcans at Asn181 (orange) and Asn197 (yellow)[127]

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 thioesterformation 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.



Scheme 26: General retrosynthetic strategy to obtain a homogeneous GPI-anchored *N*-glycosylated prion protein

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.[49] Therefore it is crucial to find the optimal conditions for thioester formation and choose the appropriate strategy; either the Dawson linker,[49] peptide hydrazides[51] or peptide sulfonamides[52] as thioester precursor or the use of fully protected peptides for thioester formation[50]. 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.[47, 128] 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.

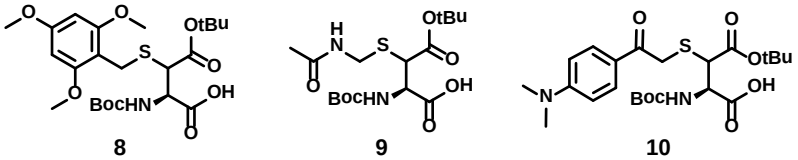
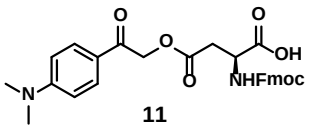


Figure 21: β-mercapto aspartic acid building blocks for ligation-desulfurization strategy

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.[67, 69] 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

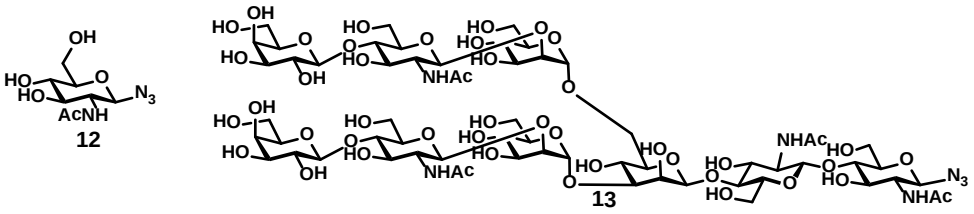
orthogonal protecting group, which is removable in presence of the commonly used allyl protecting group on aspartic acid, is needed. This second orthogonal handle, will allow the installation of one glycosylation at a time and install two different glycans at each glycosylation site. Unfortunately, no 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.



11

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.[69]



12 **13**

Figure 23: Glycan azides for glycopeptide synthesis using Lansbury aspartylation

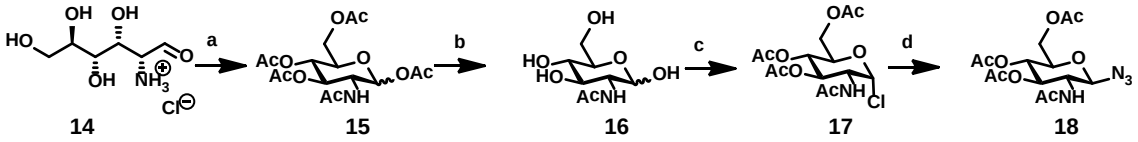
RESULTS AND DISCUSSION

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.[67, 69] 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

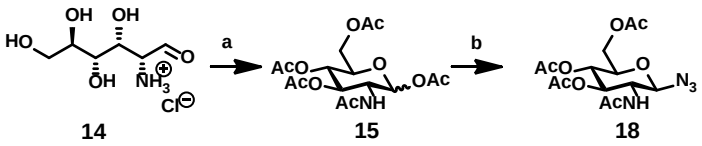
To synthezise *N*-glycopeptides, *N*-acetylglucosamine-azide (β-D-GlcNAc-azide), the simplest glycan-azide, is typically used to establish reactivity, selectivity and reaction conditions. Moreover, it gives *N*-glycopeptides to investigate the role of smaller glycans and can also be elongated using enzymatic methods to obtain larger *N*-glycans.[3] Until recently, β-D-GlcNAc-azide was not commercially available for a reasonable price, and therefore, had to be synthesized.



Scheme 27: Synthesis of 18: a) Ac_2O, pyridine, quant., b) NaOMe, MeOH, 84%, c) AcCl, HCl, 45%, d) NaN_3, DMF, 93%

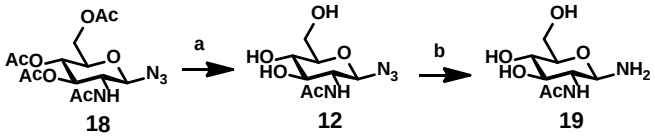
Starting from the hydrochloride of D-glucosamine, *N*-acetylglucosamine **16** was synthesized in a two stepped strategy.[129] Generating **16** directly from D-glucosamine with sodium methoxide and acetic anhydride only gave the product in low yields. Thus, peracetylated *N*-acetylglucosamine **15** 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, Ac_2O, quant., b) TMS-N_3, Sn(IV)Cl_4, EtOAc, 0°C, 68%

The second approach[130] (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, $H_2\uparrow$, 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.[131] 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.[95] Using this method, complex bianntenary *N*-glycans and asparagine building blocks containing complex bianntenary *N*-glycans can be obtained.[95, 132]

Initial reports described the isolation of a sialylglycopeptide from fresh eggs by separating insoluble proteins and lipids by precipitation with phenol.[95] Here, however, a method well-known from glycomic analysis in large scale using egg yolk powder was used.[133] 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.

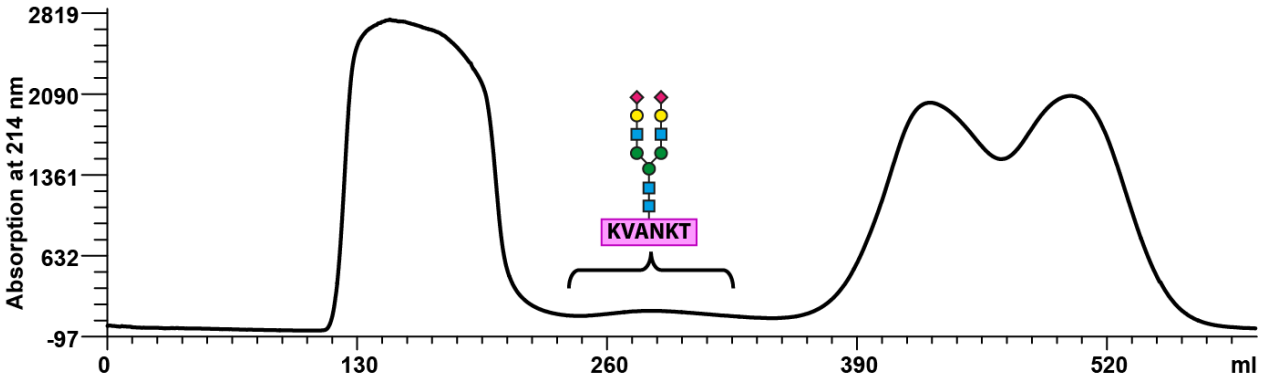
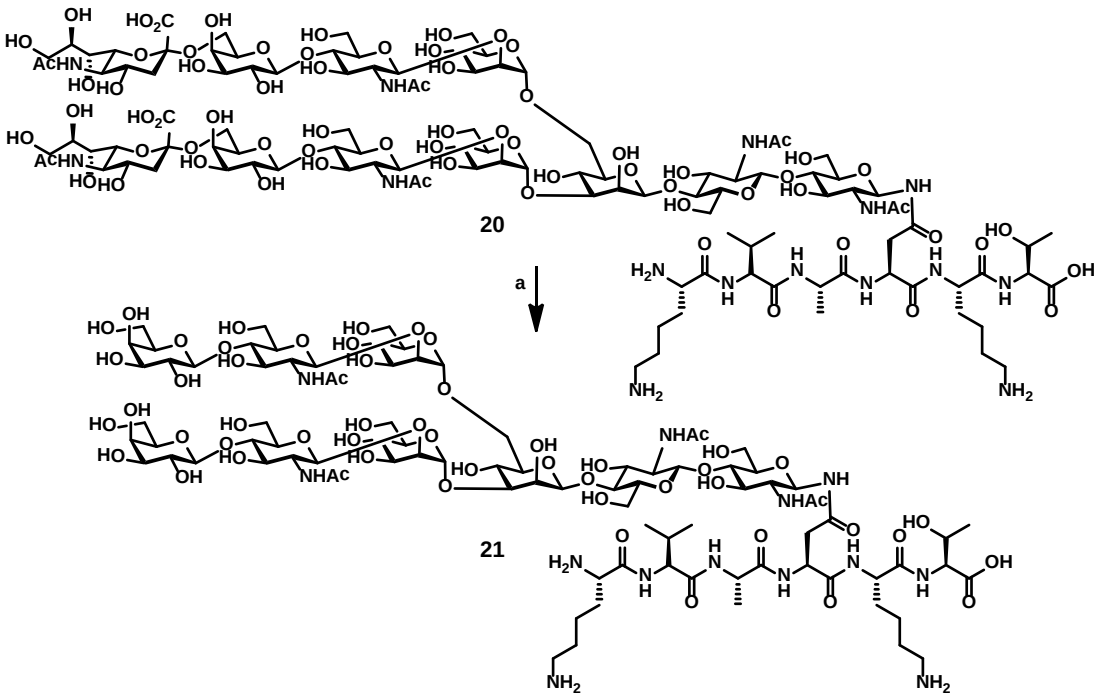


Figure 24: Chromatographic profile of the elution of sialylglycopeptide **20** on a Sephadex G50 column.

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,[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).



Scheme 30: Synthesis of 21: a) 10 mM TFA (pH=3), 80°C, 20 h, 38%

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%.

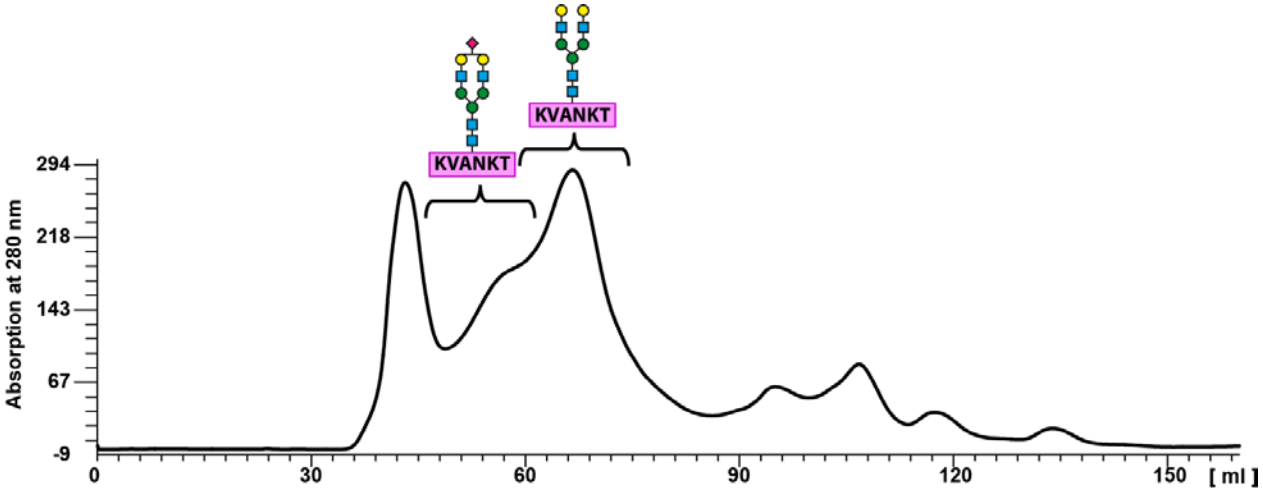
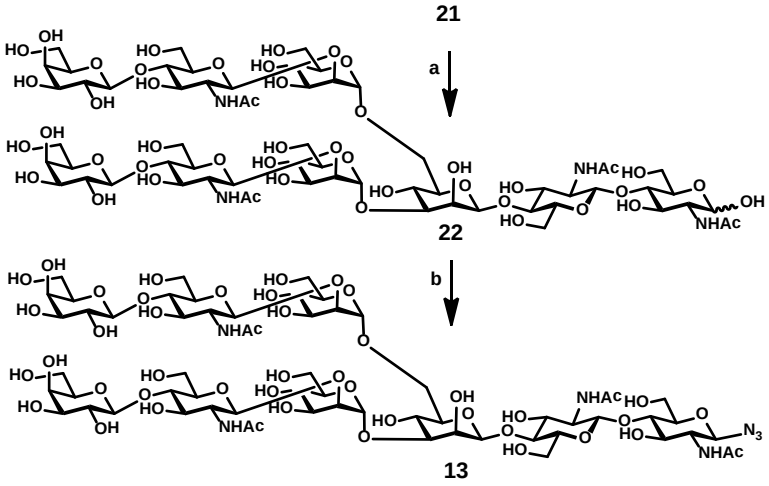


Figure 25: Chromatographic profile of the elution of glycopeptide **22** performed on a superdex 30 column

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.[134] Nona-azide **13** was

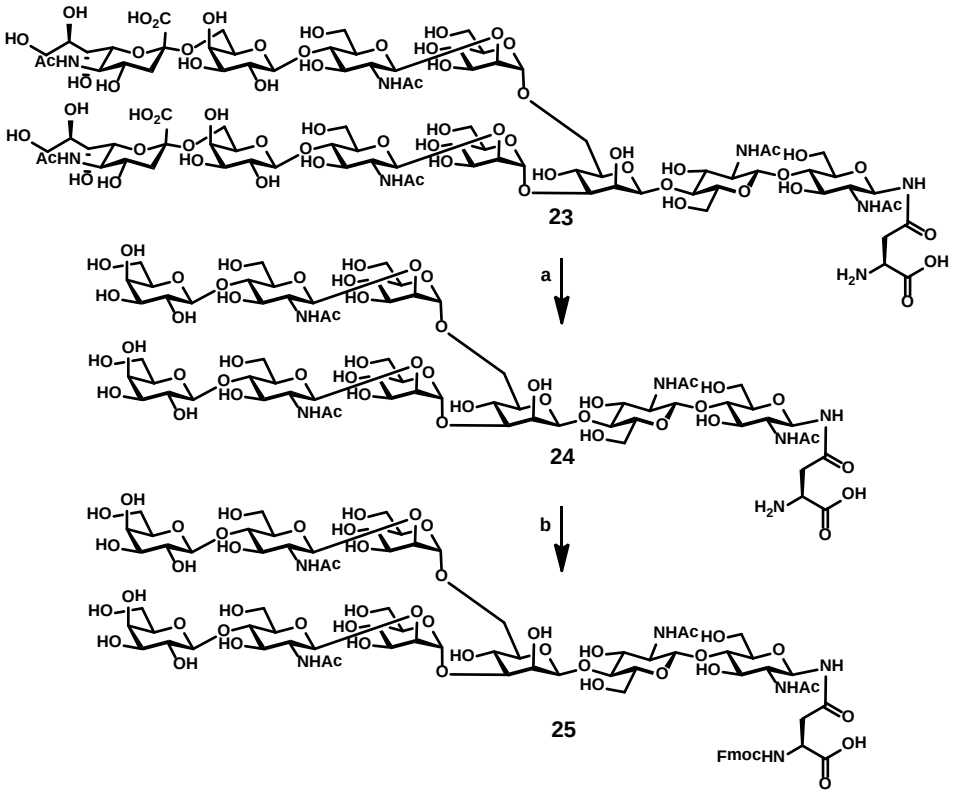
purified on a Sephadex G25 eluting with 5% ethanol in water to give the desired product in 82% yield. ^{1}H-NMR analysis of the product showed nearly identical chemical shifts compared to reported data[69] and verified the success of the purification and reactions.



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^{α}-function of asparagine, **24** was reacted with 9-Fluorenylmethyl *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.



Scheme 32: Synthesis of 25: a) 10 mM TFA (pH=3), 80°C, 20 h, 94%, b) Fmoc-OSu, TEA, 16h, 52%

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)Cl_4 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.[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.[69, 134]

Additionally, Fmoc-Asn-nona **25** was synthesized from glycosylated asparagine **23**. After acidic hydrolysis of the sialic acid residues the N^{α}-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^{α} 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.[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.

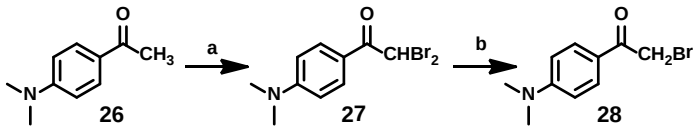
Considering the available protecting groups, a photolabile protection on aspartic acid would serve impeccably the necessities, though no such protecting group stable during SPPS is known.[57] 4,5-dimethoxynitrobenzyl (Dmnb) has been described as photocleavable protecting group on aspartic acid. However, 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.[137] In contrast, photoremovable protecting groups to mask the α-carbonyl function of amino acids have been described[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.[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.*[141] in 1982. This method uses amino acid copper(II) complexes and the corresponding halides of the protecting group for alkylation.

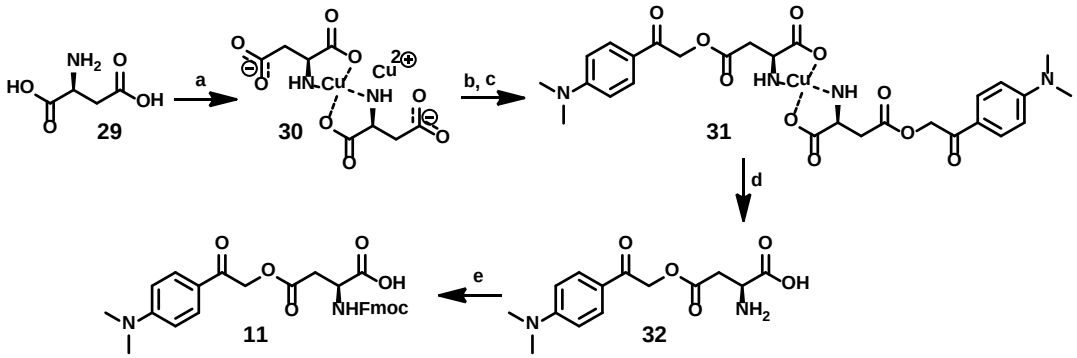
The required Map-bromide **28** can be synthesized in a two stepped high yielding fashion by bromination and debromination of 4-(dimethylamino)-acetophenone.[142]

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_2SO_4, Br_2, rt, 14 h, 90%, b) diethylphosphite, TEA, THF, 0°C to rt, 14 h, 43%

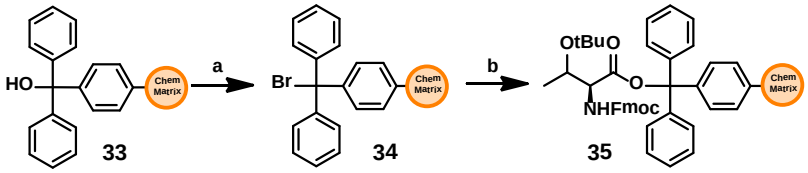
Having the suitable halide **28** in hand, L-aspartic acid was converted into a copper(II) complex **30** using copper(II)acetate in water, with a yield of 90% as a blue solid. After the formation of a L-aspartic acid copper (II) complex **30**, *N*,*N*,*N´*,*N´*-Tetramethylguanidinium was added to the 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).



Scheme 34: Synthesis of 11: a) $Cu(CO_2CH_3)_2$, water, 70°C, 48h, 90%, b) *N,N,N'N'*-Tetramethylguanidinium, rt, 1 h, DMF/H_2O 10:1, c) **28**, d) EDTA, $NaHCO_3$, water, 60% over 3 steps, e) FmocOSu, Na_2CO_3, THF/water 1:1, rt, 14 h, 70%

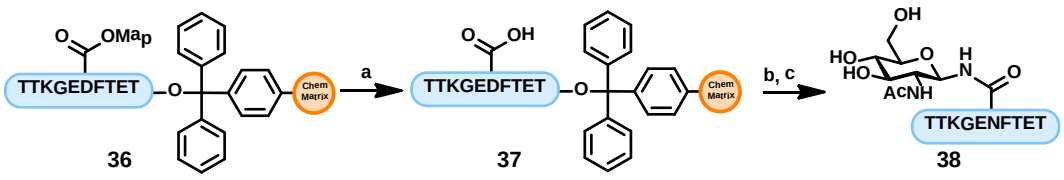
2.2 Glycopeptide synthesis using Fmoc-Asp(OMap)-OH

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 **34** 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).



Scheme 35: Bromination of trityl linker 33 and coupling of first amino acid: a) 10% AcBr in DCM, 4h, b) Fmoc-Thr(OtBu)-OH, DIPEA, 14h

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.



Scheme 36: Photolysis of Map on aspartic acid and coupling of GlcNAc: a) hv>300 nm, 18 min, b) PyBOP, DIPEA, 19, 14 h, c) TFA/TIPS/water (90:5:5)

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.

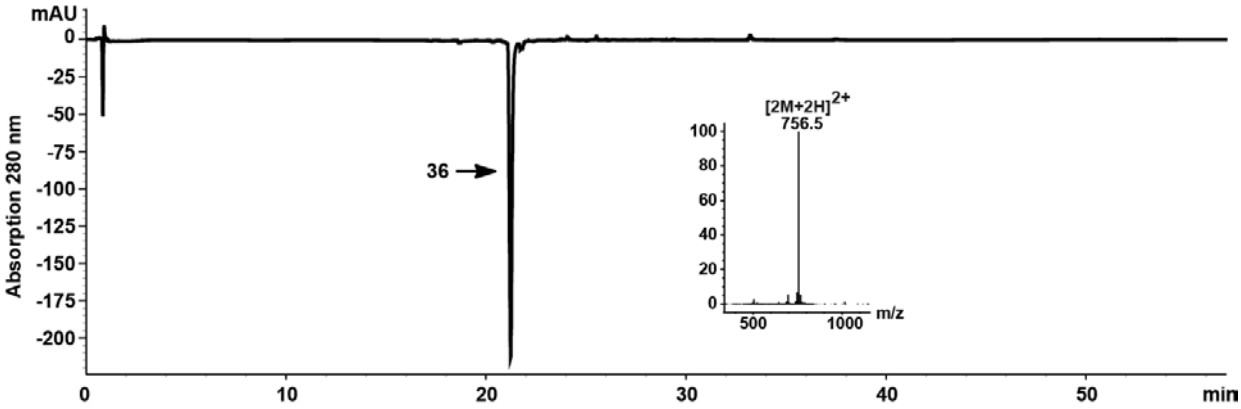


Figure 26: LC-MS analysis of 36 [LC-MS on Agilent 1200: C_{18} X-bridge [150x5mm; 5µm], 5% ACN to 95% ACN in H_2O (0.1% FA) in 30 min]

The photolysis in pure ethanol as proposed by Ueda *et al.*[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 (hv>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.

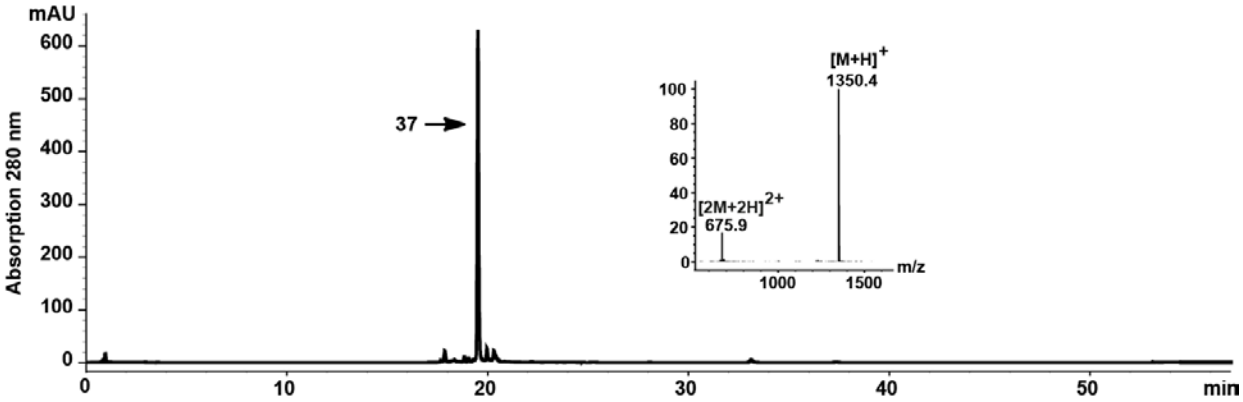


Figure 27: LC-MS analysis of 37 [LC-MS on Agilent 1200: C_{18} X-bridge [150x mm; 5µm], 5% ACN to 95% ACN in H_2O (0.1% FA) in 30 min]

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).

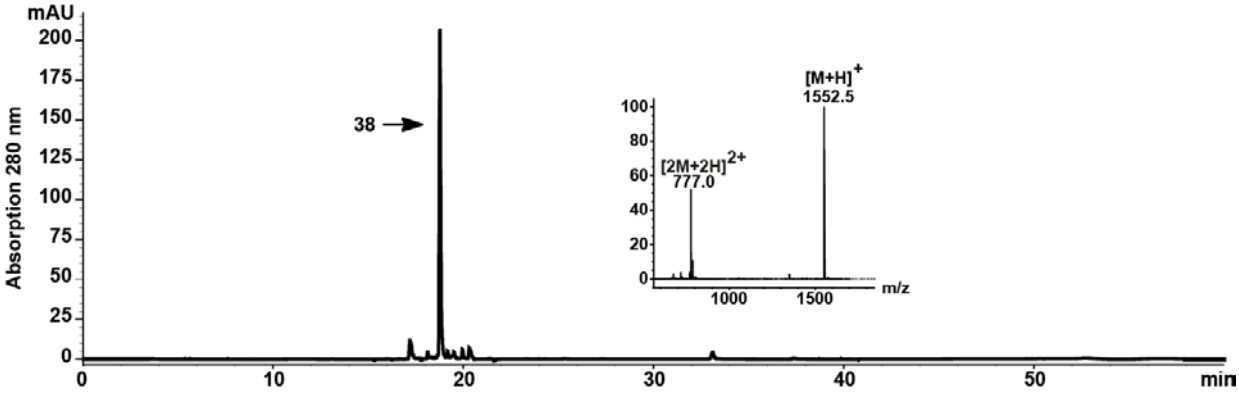


Figure 28: LC-MS analysis of 38 [LC-MS on Agilent 1200: C_{18} X-bridge [150x5mm; 5µm], 5% ACN to 95% ACN in H_2O (0.1% FA) in 30 min]

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

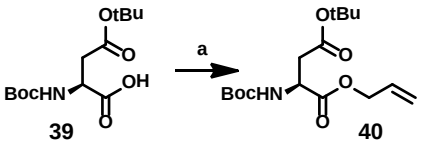
protected using a method introduced by Feijen and coworkers.[141] After decomplexation of **31** using EDTA, H-Asp(OMap)-OH **32** was observed in 60% yield over three steps. The N^{α}function of Asp was protected with Fmoc to furnish the desired Fmoc-Asp(OMap)-OH **11**.
After the successful synthesis of **11**, the building block was used for the generation of glycopeptide **38**. **11** was incorporated in the sequence of peptide **36**. During the coupling and peptide elongation using Fmoc-SPPS no aspartimide formation and no deletion sequences were observed, proving building block **11** to be optimal for SPPS and to be far superior to the known Fmoc-Asp(ODmnb)-OH.[57] Additionally, the removal of the Map-group was selective using a high-pressure Hg-lamp (hν>300 nm) in flow and a retention time of 18 minutes. The free acid function was coupled with amino glycan **19** to give a model glycopeptide **38** showing that Fmoc-Asp(OMap)-OH **11** 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.[39, 43] 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.[47, 128] 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(O*t*Bu)-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(O*t*Bu)-OH **39** by reaction with allyl bromide in DMF under basic conditions. The desired product **40** was observed in quantitative yield.

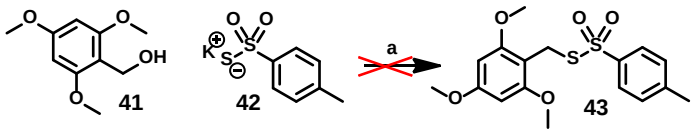


Scheme 37: Synthesis of 40: a) Allylbromide, DIPEA, DMF, quant.

Having the desired fully protected aspartic acid building block **40** in hand, three β-mercapto amino acids were synthesized.

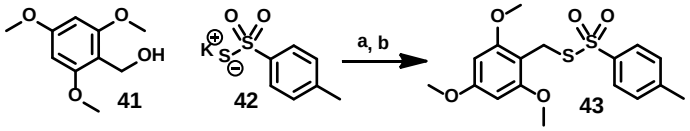
3.2 Synthesis of Boc-Asp(O*t*Bu;STmob)-OH 8

The protocol for the synthesis of required trimethoxybenzyl (Tmob) protected β-mercapto aspartic acid (Boc-Asp(O*t*Bu;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.



Scheme 38: Synthesis of 43: a) TFA, MeOH, 0 °C, 20 min

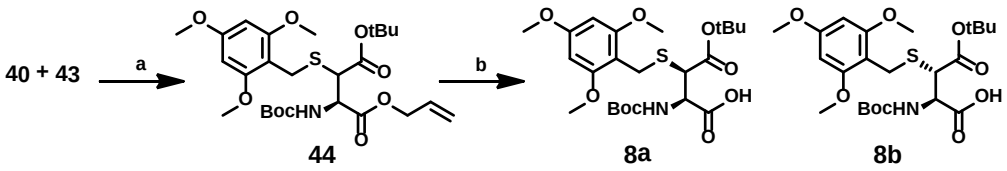
A stereocontrolled protocol for protected β-mercapto aspartic acids using a similar sulfenylating reagent was also published by Shibata *et al.* (Scheme 39).[128] and the strategy was used to synthesize **43**.



Scheme 39: Synthesis of 43: a) TEA, rt, 15 min, b) TFAA, 0°C to rt, 16 h, 72% over 2 steps

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.



Scheme 40: Synthesis of 8a and 8b: a) LiHMDS, -78°C, 2 h, 76%, b) $Pd(PPh_3)_4$, rt, 1 h, 80%

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 $Pd(PPh_3)_4$ 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 ^{1}H-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.[47, 128] As reported, during ligation both isomers show the same reactivity and therefore can be used in ligation reactions.[47]

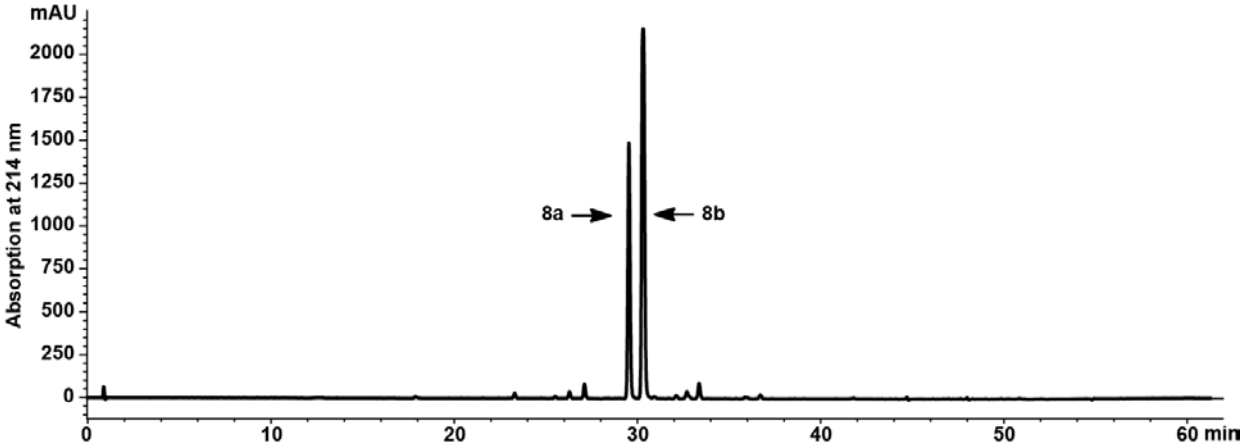
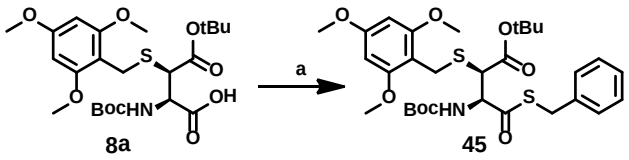


Figure 29: LC analysis of 8 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 0% ACN to 100% ACN in H_2O (0.1% TFA) in 40 min]

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(O*t*Bu,STmob) to PrP fragment 2 (179-213), since amino acid 179 is a cysteine. Thus a Boc-Asp(O*t*Bu,STmob) thioester is required.



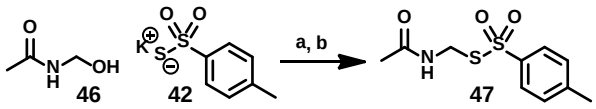
Scheme 41: Synthesis of 45: a) PyBOP, DIPEA, 0°C, 1h, quant.

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(O*t*Bu;SAcm)-OH

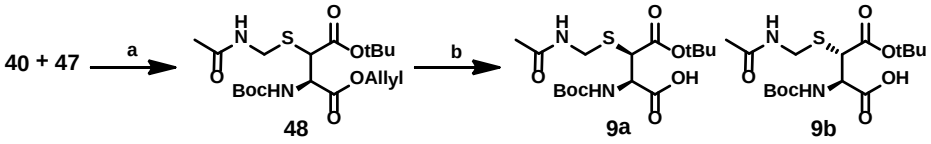
In addition to the previously introduced ligation strategy, ligation in C to N direction is also possible when the thiol function on the N-terminal amino acids bears an orthogonal protection group. A well described orthogonal protection group for thiols in Fmoc-SPPS is acetamidomethyl (Acm).

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



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 Shibataand coworkers.[128] The sulfenylating reagent **47** was isolated in 52% yield.



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.

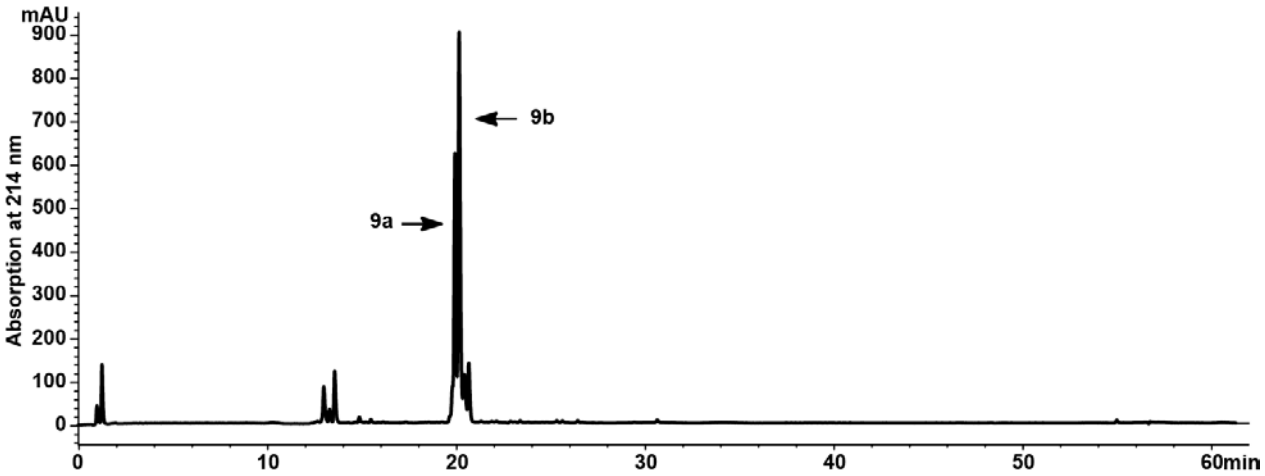
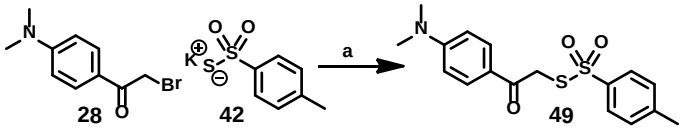


Figure 30: LC analysis of 9 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 0% ACN to 100% ACN in H_2O (0.1% TFA) in 40 min]

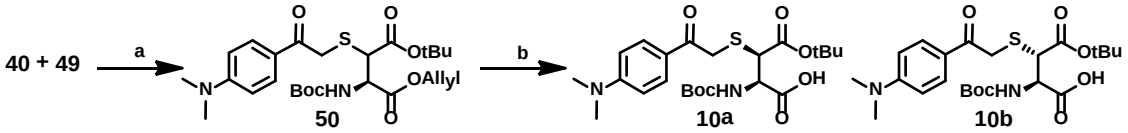
3.4 Synthesis of Boc-Asp(O*t*Bu;SMap)-OH 10

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



Scheme 44: Synthesis of 49: a) DMF, 16 h, 38%

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]



Scheme 45: Synthesis of 10a and 10b: a) LiHMDS, -78°C, 2 h, 62%, b) $Pd(PPh_3)_4$, rt, 1 h, 72%

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.

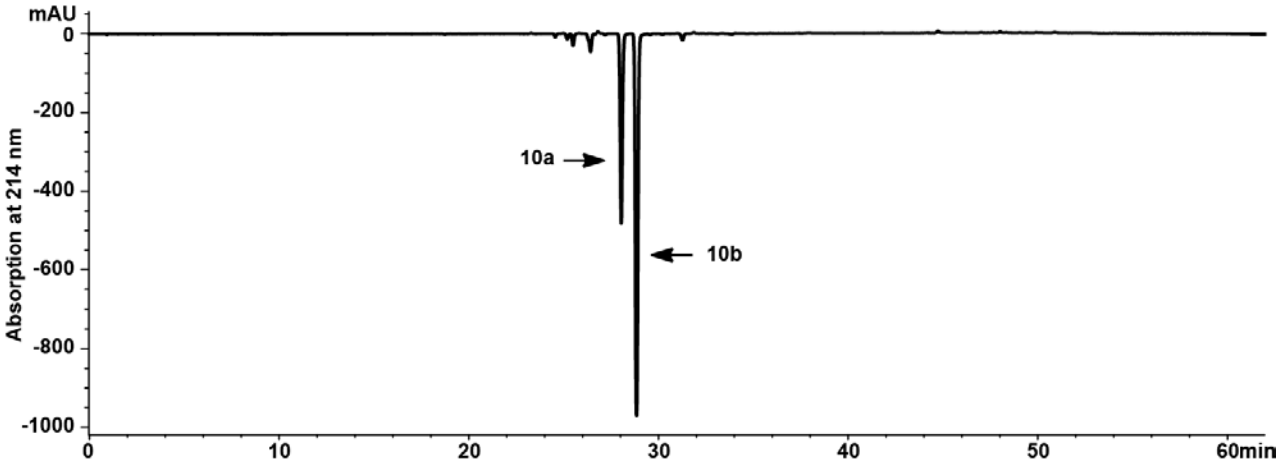


Figure 31: LC analysis of 10 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 0% ACN to 100% ACN in H_2O (0.1% TFA) in 40 min]

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(O*t*Bu,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[47, 128] 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.

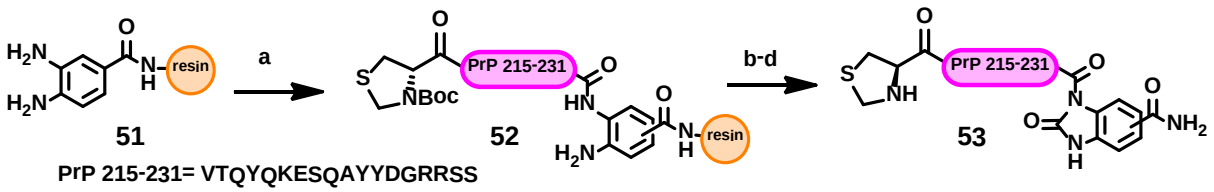
A third strategy for a β-mercapto aspartic acid with the photolabile Map as orthogonal protecting group, masking the thiol, was evaluated. Sulfenylating reagent **49** was generated by reacting bromide **28** and potassium 4-methylbenzenesulfonothioate **42** in DMF in 38%.[143] By reacting **40** with **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.[3] 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.*[49] 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: 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).

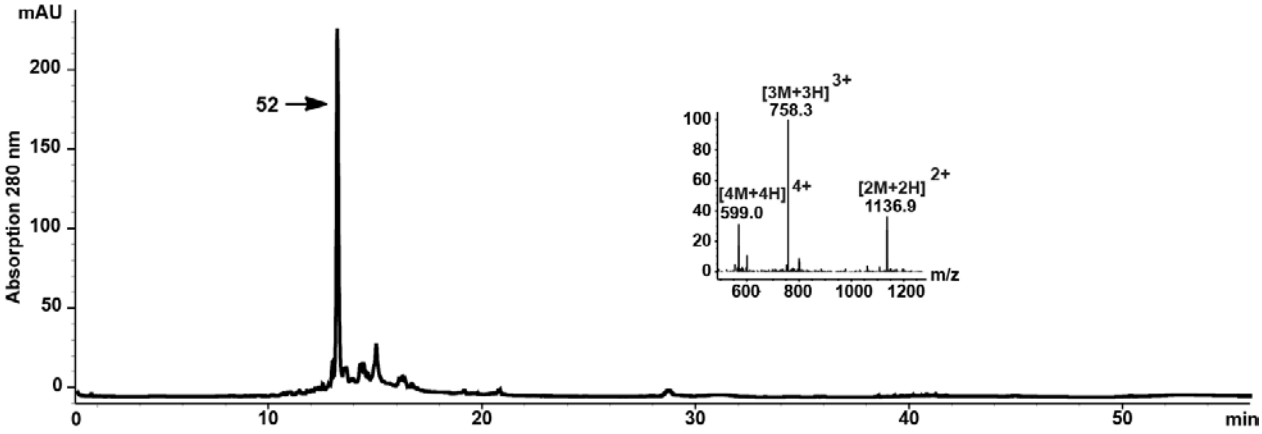


Figure 32: LC-MS analysis of 52 [LC-MS on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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).

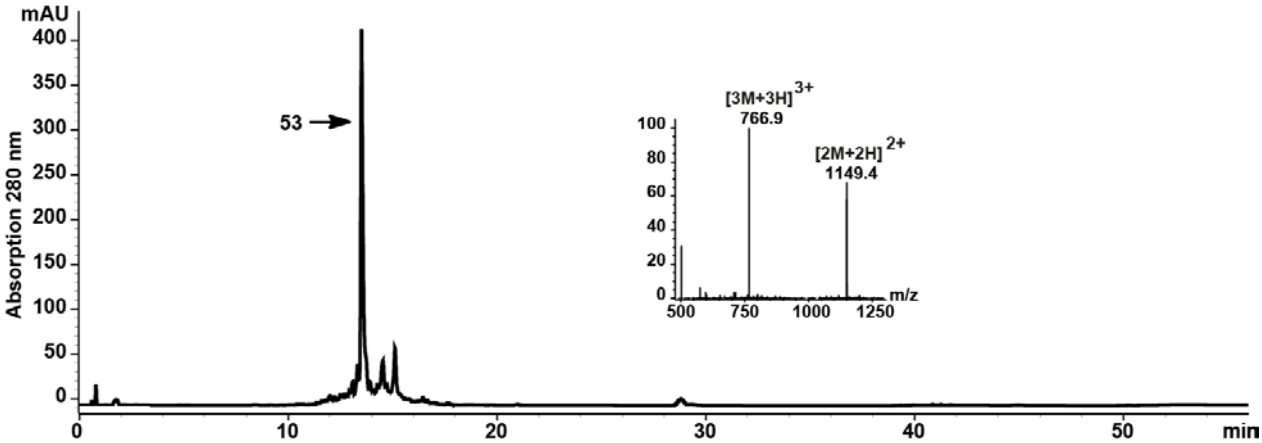


Figure 33: LC-MS analysis of 53 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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_2HPO_4, 200 mM 4-Mercaptophenylacetic acid (MPAA) and 20 mM TCEP·HCl

(TCEP=tris(carboxyethyl)phosphine) at pH 7].[49] 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.

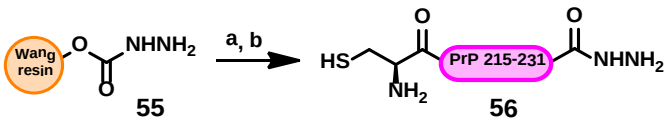
4.2 Peptide hydrazides as thioester precursor

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).[51]

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™ 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.



PrP 215-231= 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).

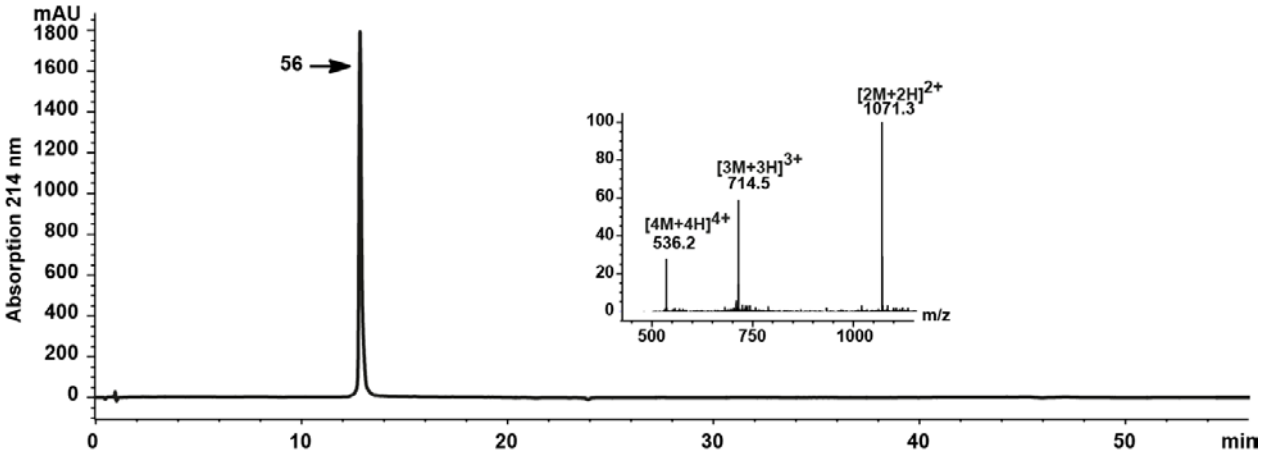
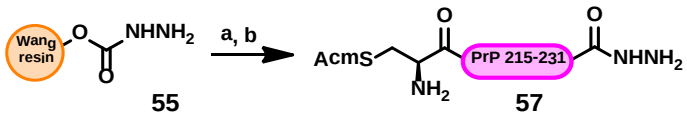


Figure 34: LC-MS analysis of 56 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

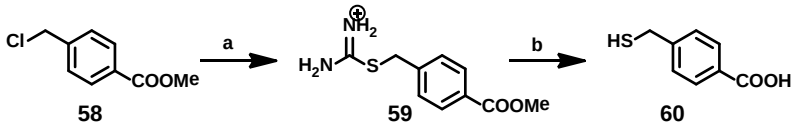
4.5 Fragment 1 for ligation in N to C direction

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-thioproline was not performed, since side reactions during oxidation of the hydrazide occur.[66] 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)

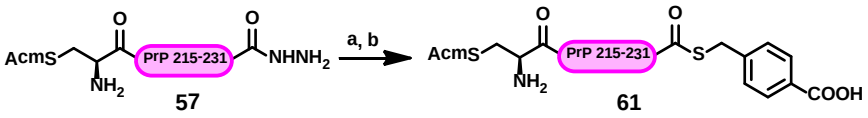
The desired product **57** was synthesized in a high crude purity. The crude peptide hydrazide was transformed into a thioester using $NaNO_2$ at pH 3 as oxidative condition and 4-(mercaptomethyl)benzoic acid (MMBA) as thiol.[144] The benzylic thiol MMBA was preferred over MPAA, since the thioester formed shows a similar reactivity but is more stable.[144] Despite being commercially available, because of its high price, MMBA was synthesized in a two stepped synthesis from methyl 4-(chloromethyl)benzoate (Scheme 50).[144]



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 $NaNO_2$ 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: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 0.02 M $NaNO_2$, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min, 25%

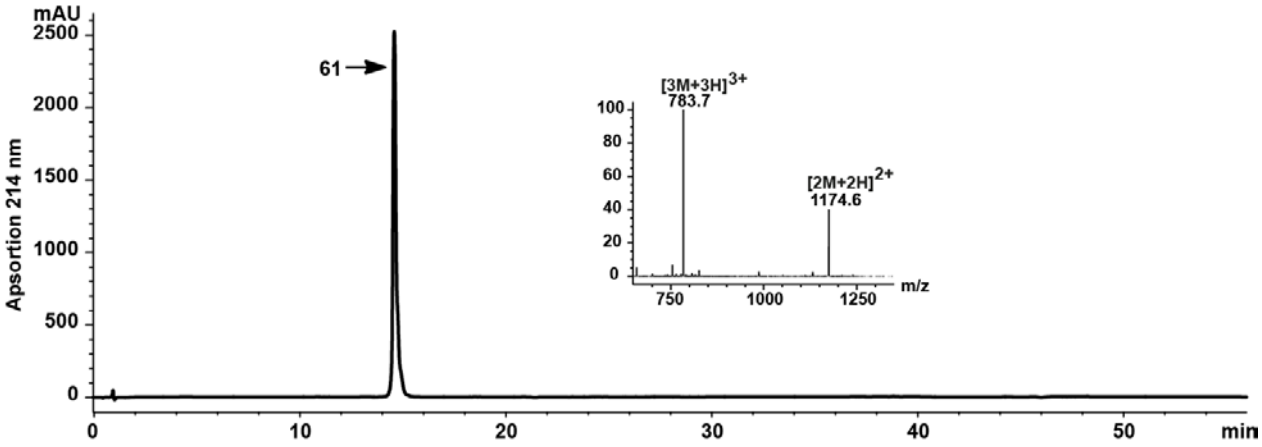
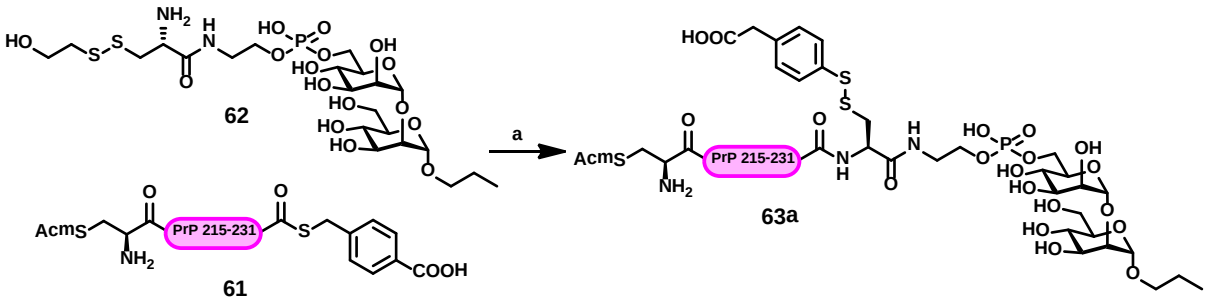


Figure 35: LC-MS analysis of 61 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.

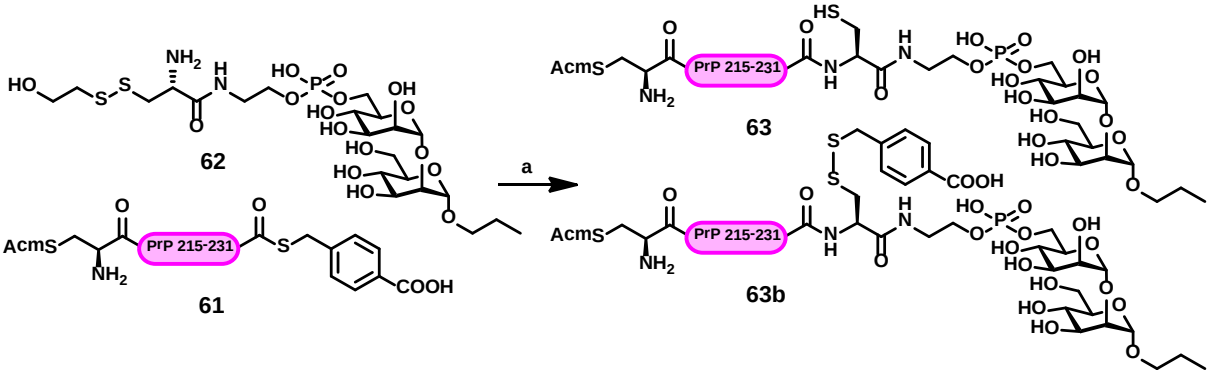
4.6 Ligation of 61 to cysteine modified Di-Mannose

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 Na_2HPO_4, 0.05 M MPAA, 5 mM TCEP at pH 7 was used (Scheme 52).[144] 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.



Scheme 52: Ligation of thioester 61 with Cys-DiMan 62: a) 6 M GdmCl, 0.1 M Na_2HPO_4, 0.05 M MPAA, 5 mM TCEP, pH 7

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. Occurance 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.



Scheme 53: Ligation of thioester 61 with Cys-DiMan 62: a) 6 M GdmCl, 0.1 M Na_2HPO_4, 5 eq. MMBA and 30 eq. TCEP, pH 7

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 Na_2HPO_4, 5 eq MMBA and 30 eq TCEP (Scheme 53). The progress of the reaction was monitored using HPLC.

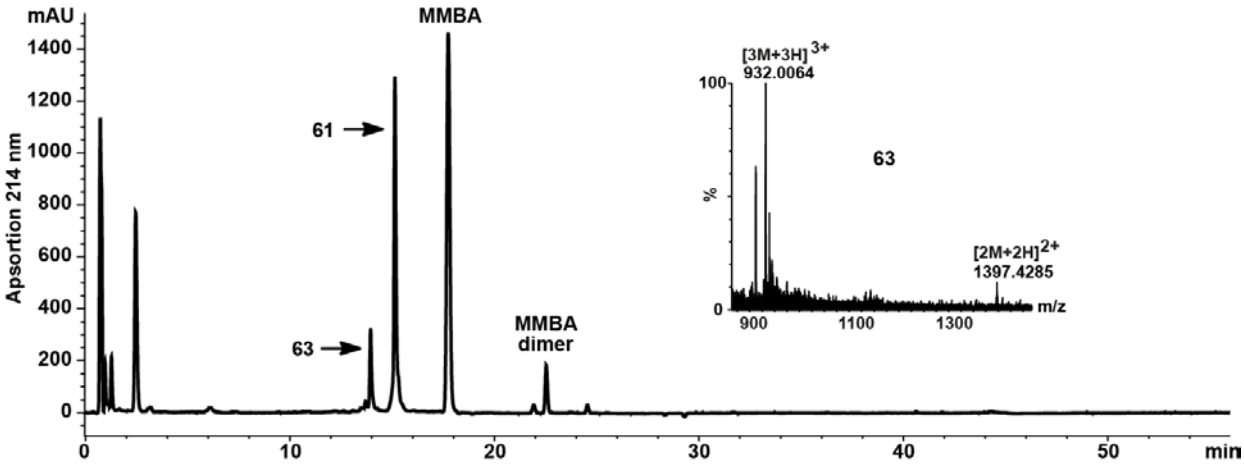


Figure 36: LC-MS analysis of ligation PrP I thioester 61 with Cys-DiMan 62 after 1 hour [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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).

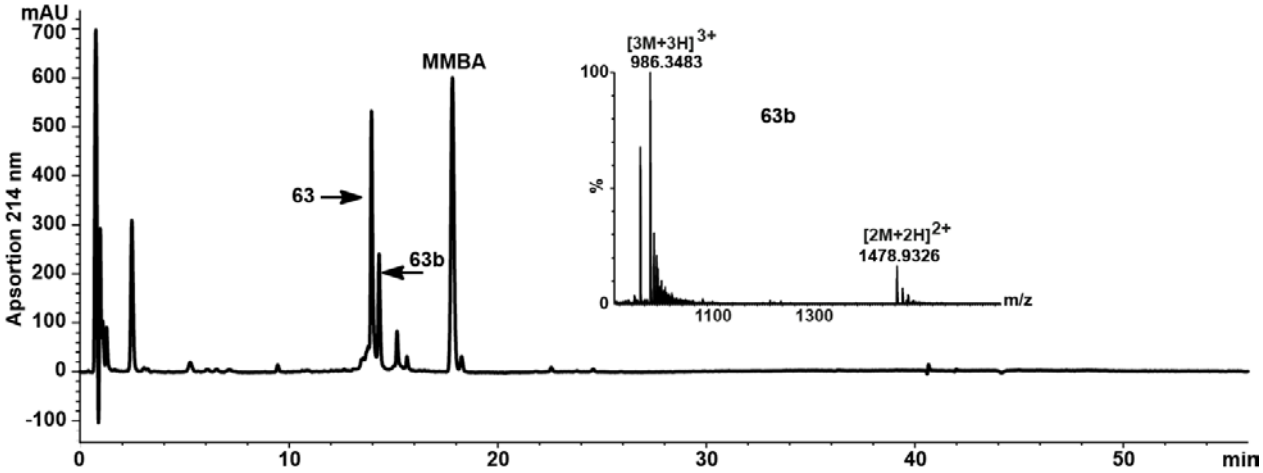


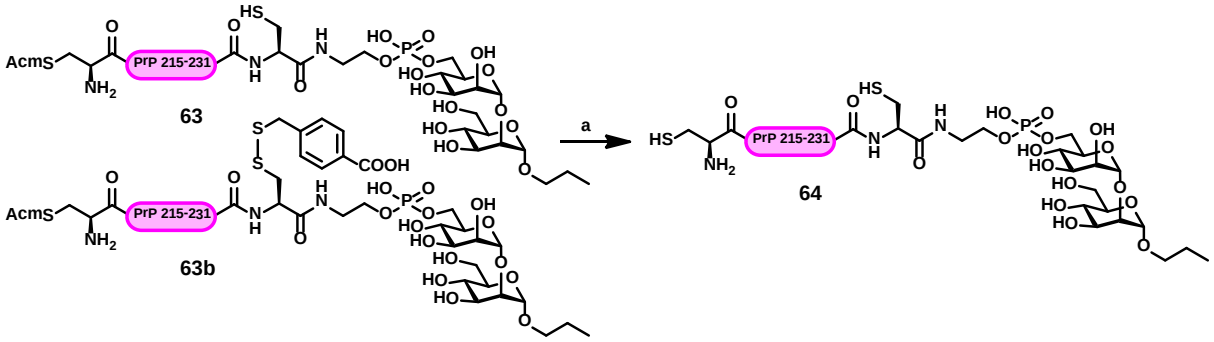
Figure 37: LC-MS analysis of ligation PrP I thioester 61 with Cys-DiMan 62 after 24 hours [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 I_2 in 80-95% acetic acid are required.[66] Recently, Brik and coworkers[66] published an efficient palladium-assisted deprotection to remove the Acm moiety on cysteine in water. Different

Pd(2) complexes were established. However, $PdCl_2$ 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.

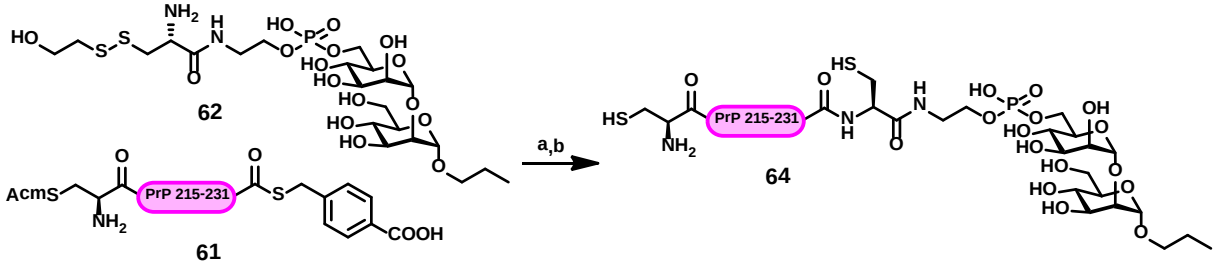
Thus **63** and **63b** were dissolved in water and 10 eq. $PdCl_2$ were added (Scheme 54). The reaction was incubated at 37°C and the removal was monitored with HPLC. A small amount was taken from the reaction mixture, quenched with DTT and analyzed using HPLC.



Scheme 54: Synthesis of 64: a) $PdCl_2$, water, 37°C, 16 h, 5%

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 $PdCl_2$ was shown, a one-pot ligation and Acm removal reaction was evaluated (Scheme **55**).



Scheme 55: Synthesis of 64: a) 6 M GdmCl, 0.1 M Na_2HPO_4, 5 eq. MMBA and 30 eq. TCEP, pH 7, b) $PdCl_2$, water, 37°C, 16 h, 6%

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. $PdCl_2$ 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 $PdCl_2$ 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).

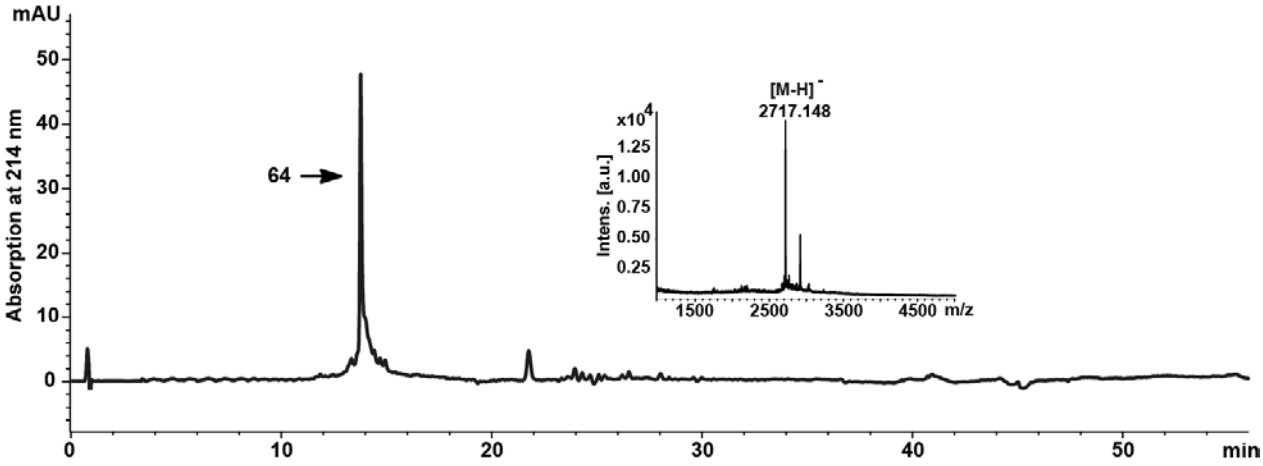
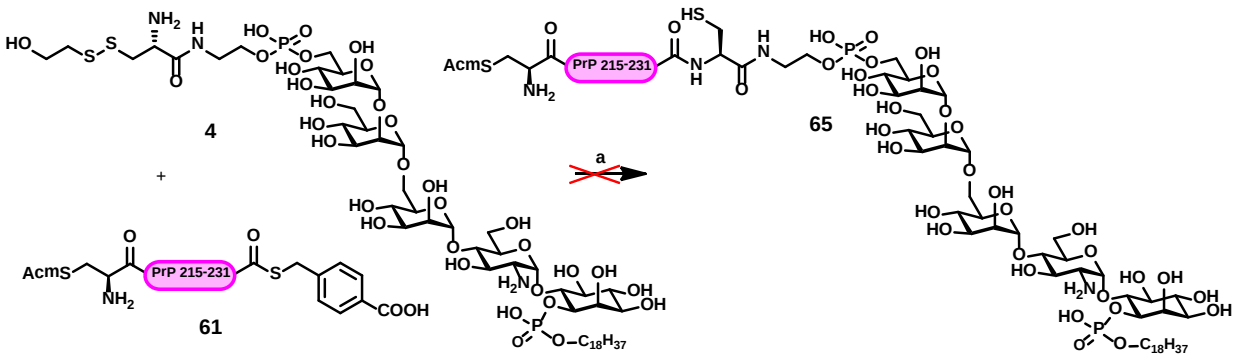


Figure 38: LC and MALDI-TOF analysis of 64 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.



Scheme 56: Ligation of thioester 61 with Cys-GPI 4: a) 6 M GdmCl, 0.1 M Na_2HPO_4, 5 eq MMBA and 30 eq TCEP, pH 7, rt for one week and 37°C for one week

To facilitate the formation of GPI-anchored PrPI **65** 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 (PrPI [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.[49] 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 $PdCl_2$[66] 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*t*Bu,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*t*Bu,SAcm)-OH **9** or Boc-Asp(O*t*Bu,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).

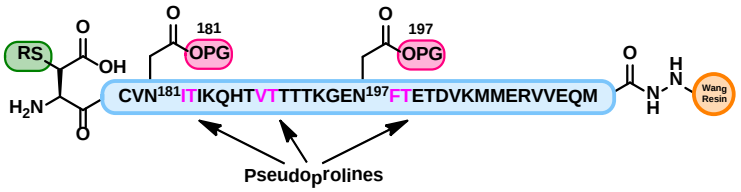
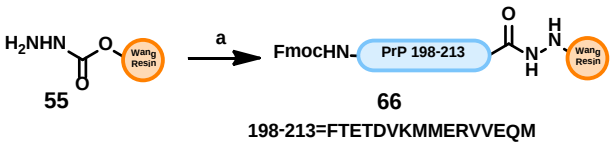


Figure 39: Installed pseudoprolines to circumvent aggregation and aspartimide formation

Starting from Wang hydrazide resin **55**, fragment 2 was synthesized using SPPS on the automated microwave assited 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.



Scheme 57: Synthesis of 66: 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

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.

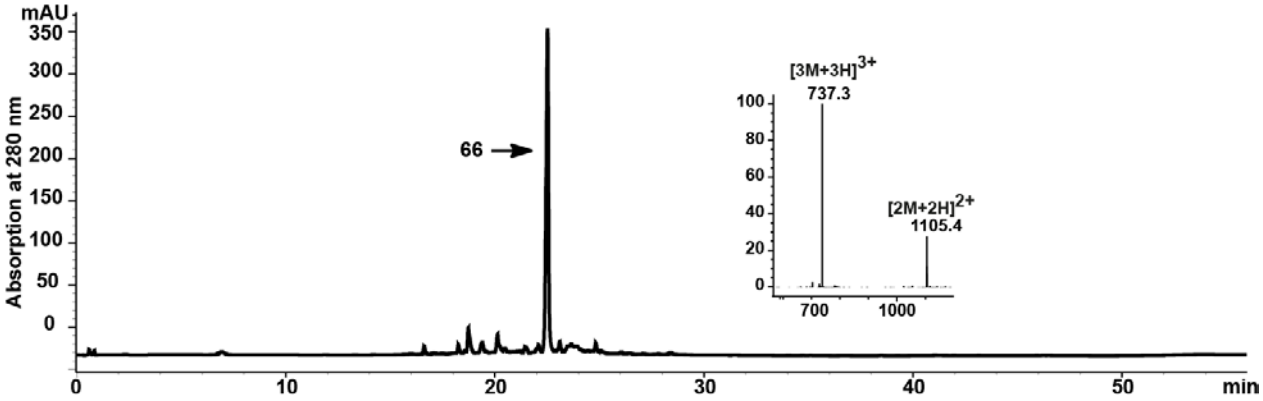


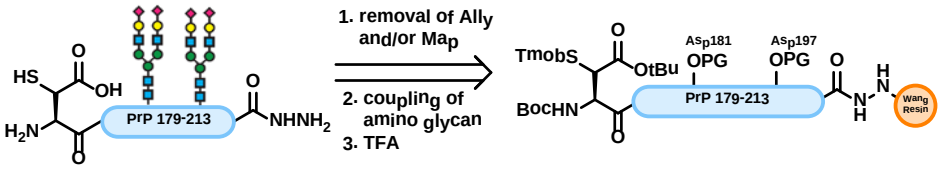
Figure 40: LC-MS analysis 66 [LC-MS on Agilent 1200: Hydrosphere C18 [50x3mm; 3µm], 5%A CN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.[71] 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(O*t*Bu,STmob)-OH **9**. Additionally, Asp181 and/or Asp197 were equipped with an orthogonal protecting group to enable Lansbury coupling after complete SPPS.

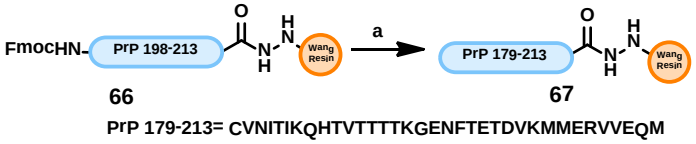


Scheme 58: Retrosynthetic analysis of strategy 1: fragment 2 for N to C ligation.

Starting from **66**, fragment 2 was synthesized using SPPS on the automated peptide synthesizer Liberty Bule™ using the opimized conditions. Four different peptides were generated to give non-, mono- and diglycosylated structures.

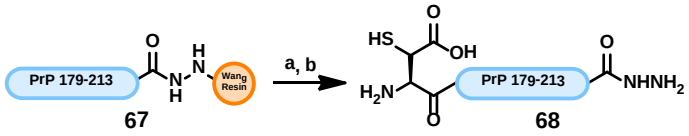
5.3 Synthesis of fragment 2 for N to C Ligation without glycosylation

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



Scheme 59: Synthesis of 67: a) Fmoc-SPPS: 1. 2x 10 eq. Fmoc-AA-OH, 10 eq. DIC/Oxyma (0.1M DIPEA), 90°C 2. 2x 20% piperidine, 90°C

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).



Scheme 60: Synthesis of 68: a) **8**, PyBOP, DIPEA, DMF, rt, 16h, b) TFA/water/TIPS (190/5/5, v/v/v)

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).

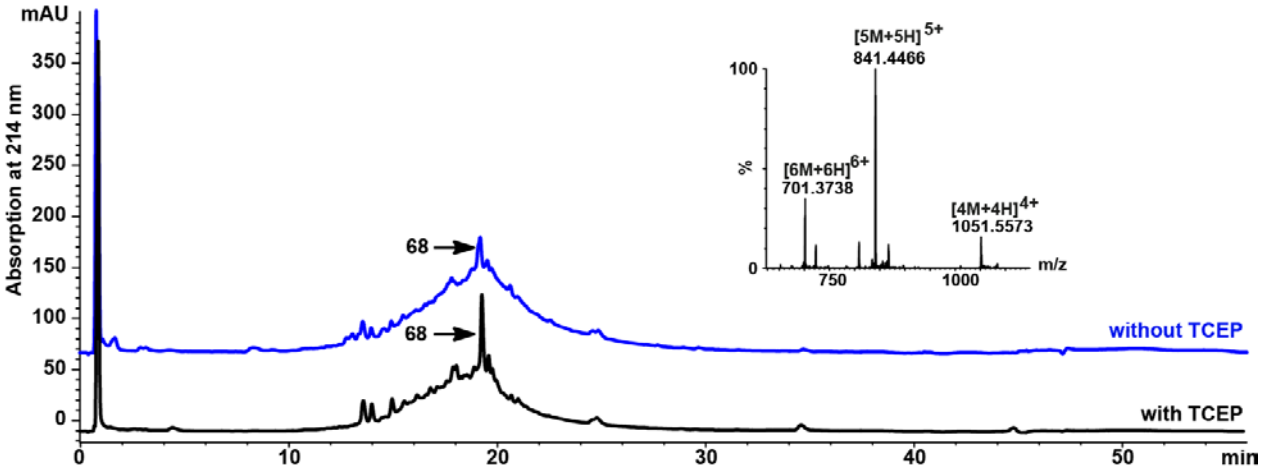
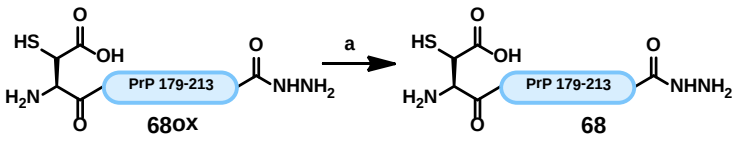


Figure 41: LC-MS analysis of 68 with and without additional TCEP [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70%ACN in H_2O (0.1% TFA) in 30 min]

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)].[76] 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.[77] Taking into account that **68ox** contains three methionine residues, a variety of oxidized species can be present in the crude peptide.



Scheme 61: Reduction of 68ox: a) NH_4I, DMS, TFA, 0°C, 20 min

To reduce the sulfoxide moieties, **68ox** was treated with NH_4I and dimethyl sulfide (DMS) in TFA (Scheme 61).[79] 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).

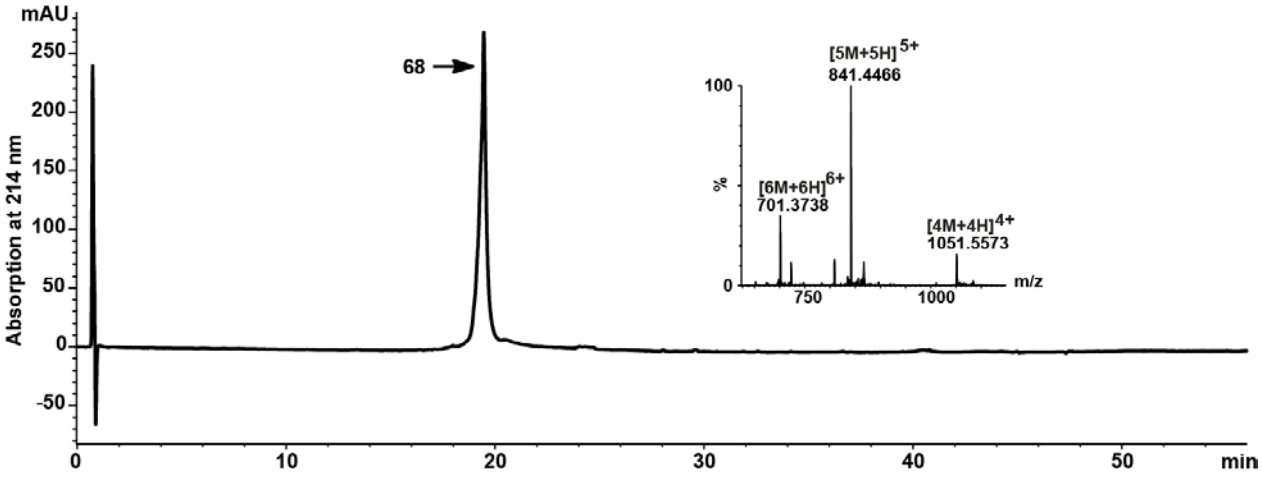
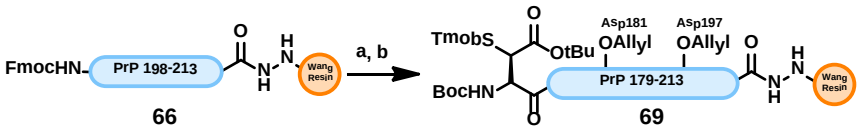


Figure 42: LC-MS analysis of purified 68 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

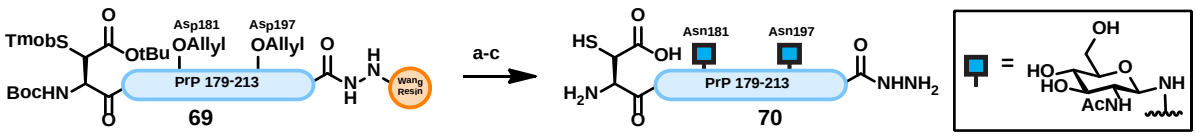
5.4 Synthesis of fragment 2 for N to C Ligation with glycosylation at Asn197 and/or Asn181

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 syntheziser (Scheme 62).



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

A testcleavage 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 intra- and intermolecular disulfides and oxidized methionine residues. Thereafter, the allyl ester was cleaved to give a free aspartic acid side chain. Using $Pd(PPh_3)_4$ 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.



Scheme 63: Synthesis of 70: a) $Pd(PPh_3)_4$, 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 glycoeptide **72** having a GlcNAc unit at Asn197.

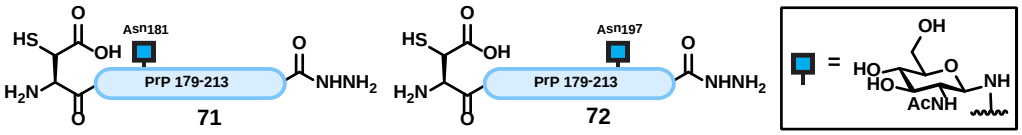


Figure 43: Glycopeptide 71 and 72

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.

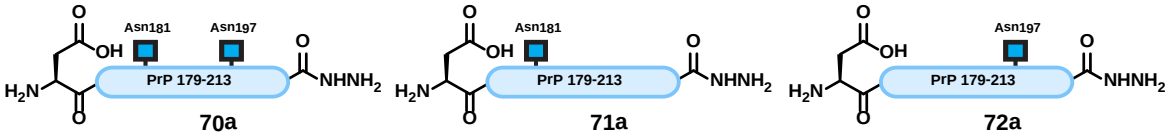


Figure 44: Desulfurized glycopeptides 70a, 71a and 72a

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.

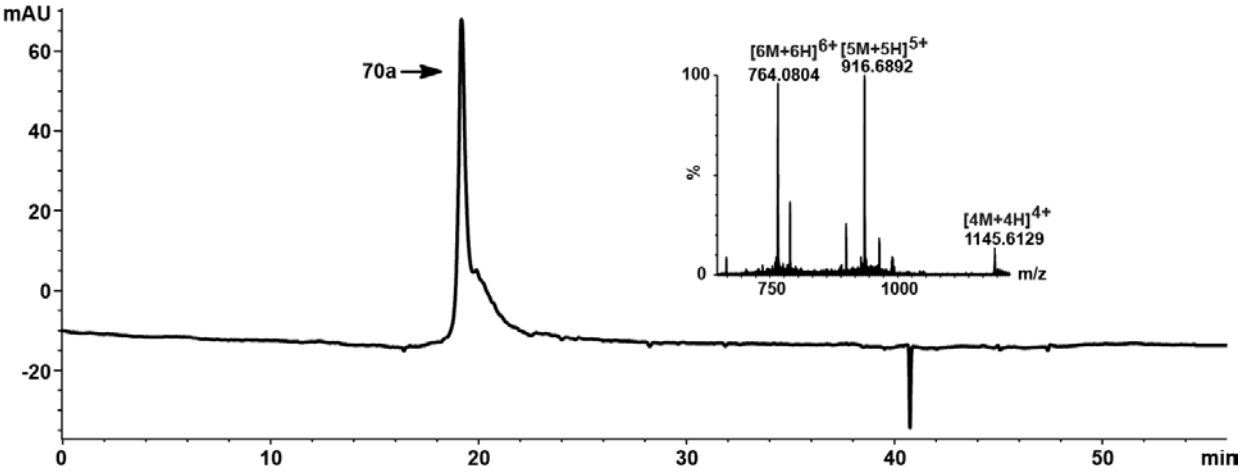
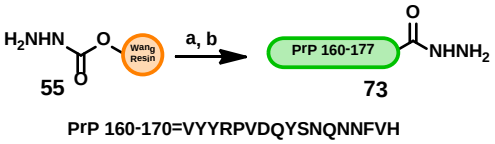


Figure 45: LC-MS analysis of purified 70a [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.



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).

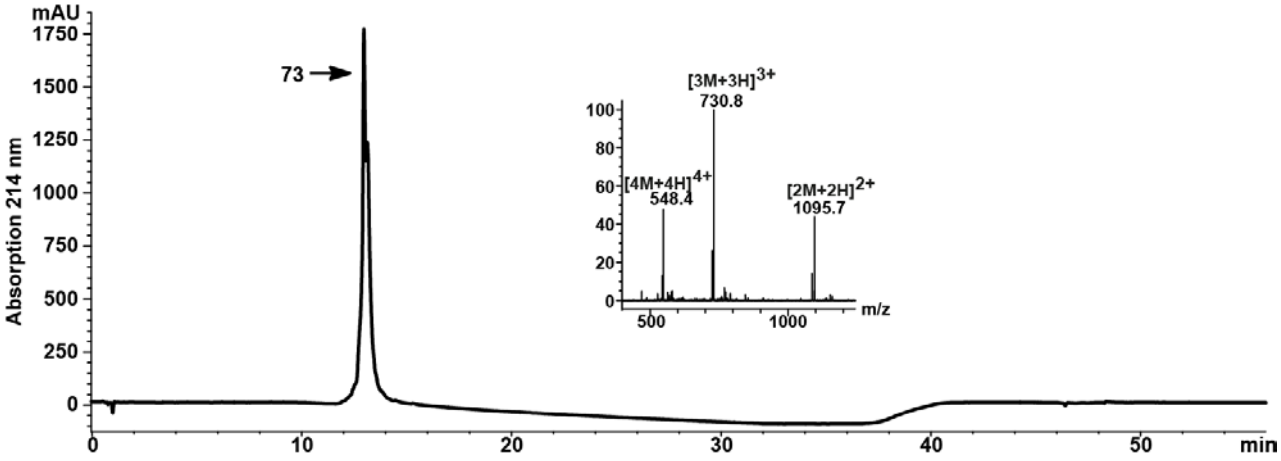
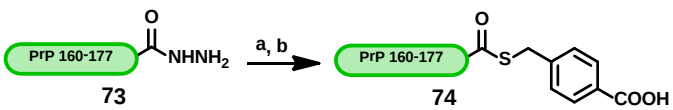


Figure 46: LC-MS analysis of 73 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

Peptide hydrazide **73** was transformed into a thioester using $NaNO_2$ at pH 3 as oxidative condition and MMBA at pH 7 (Scheme 65).[144] Surprisingly, the reaction showed only a 25% conversion.



Scheme 65: Synthesis of 74: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 0.02 M $NaNO_2$, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min, 10% (40% brsm)

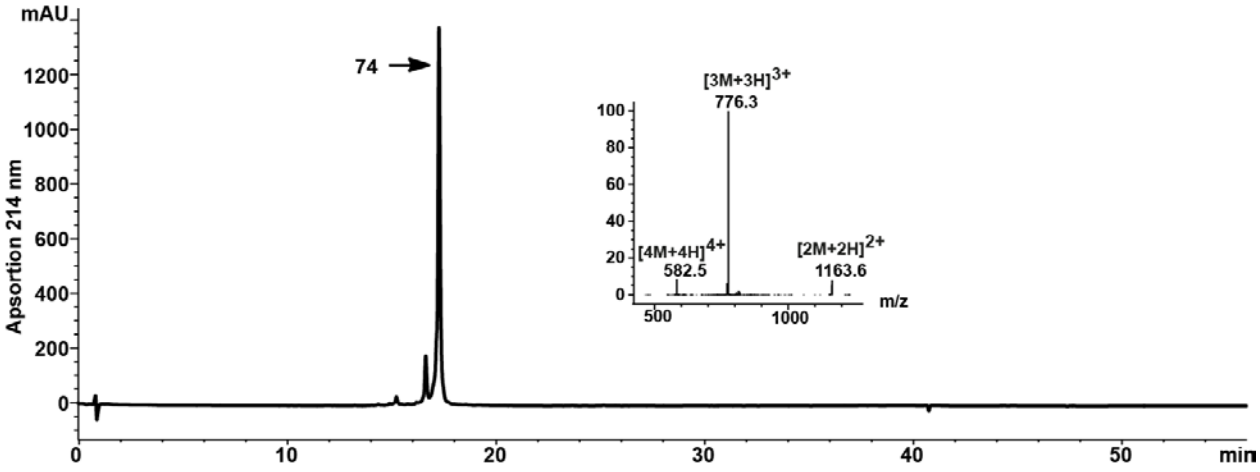
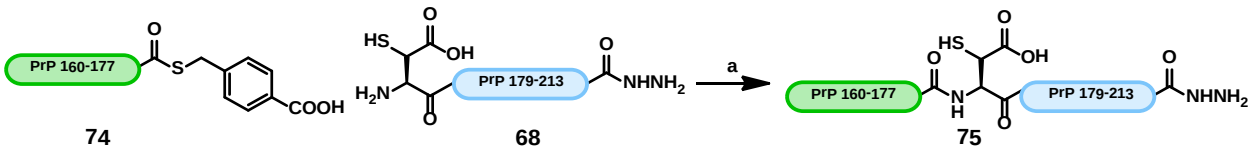


Figure 47: LC-MS analysis of 74 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 Na_2HPO_4, 5 eq. TCEP, 37°C, 2 days

The ligation was conducted using a buffer with 6 M GdmCl, 0.2 M Na_2HPO_4 and 5 eq. TCEP at pH 7 as proposed by Payne and coworkers.[47] 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.

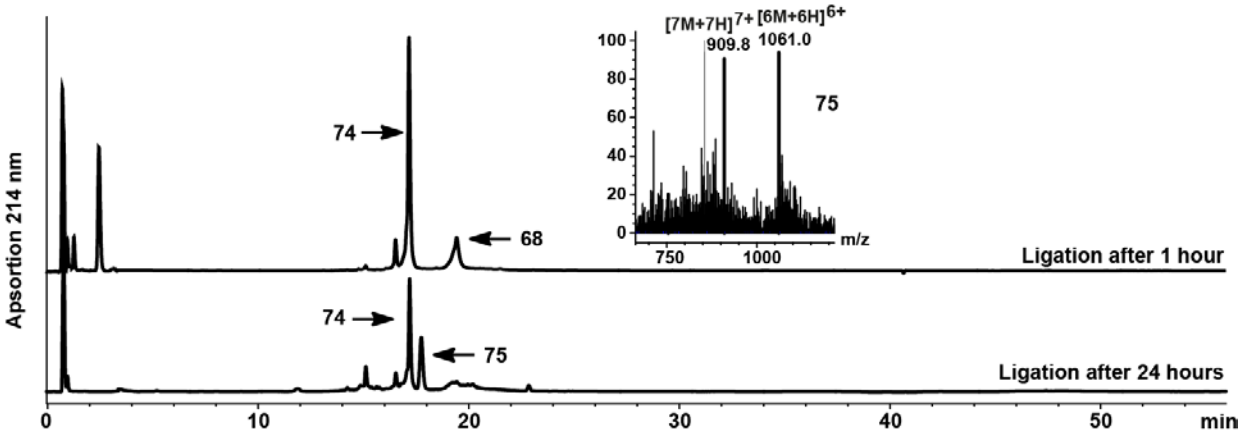


Figure 48: LC-MS analysis of the ligation between 68 and 74 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

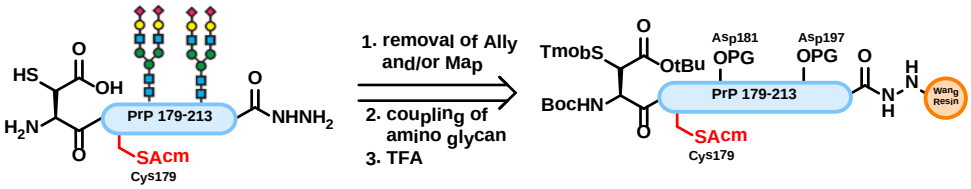
Within 24 hours peptide **68** was completely consumed and LC-MS analysis showed traces of the desired product (Figure 48). The reaction was incubated for additional 24 hours and purified using gel filtration (Superdex™ peptide 10/300) eluting with 20% ACN in water. Unfortunately, only an inseparable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



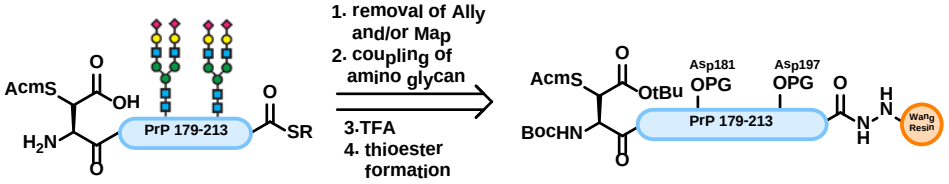
Scheme 67: New Retrosynthetic analysis of strategy 1: fragment 2 for N to C ligation.

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(O*t*Bu;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.

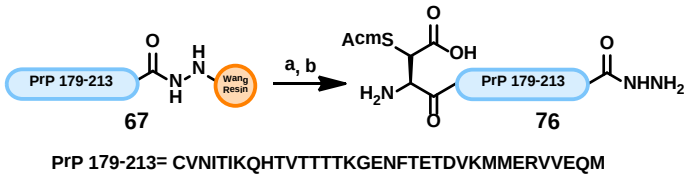


Scheme 68: Retrosynthetic analysis of strategy 2: fragment 2 for C to N ligation.

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(O*t*Bu;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).



PrP 179-213= CVNITIKQHTVTTTTKGENFTETDVKMMERVVEQM

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.

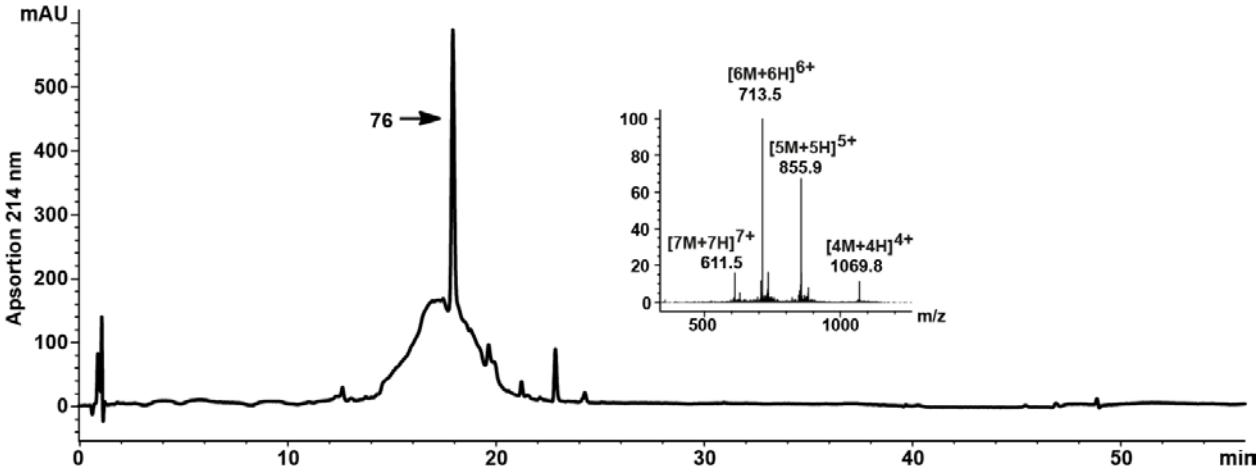
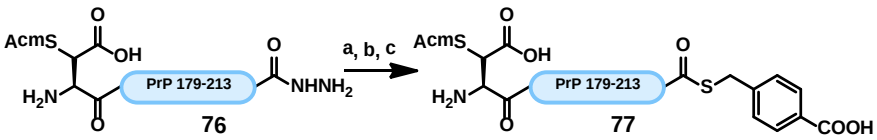


Figure 49: LC-MS analysis of 76 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.



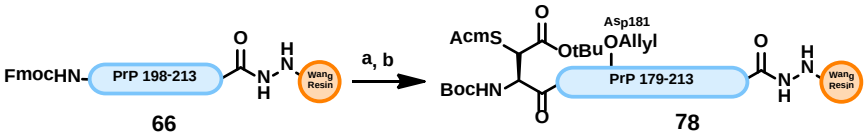
Scheme 70: Synthesis of 77: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 0.02 M $NaNO_2$, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min, 10% (40% brsm) c) NH_4I, DMS, TFA, 0°C, 20 min

Thioesterformation was conducted using $NaNO_2$ 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 NH_4I 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(O*t*Bu;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.



Scheme 71: Synthesis of 78: a) Fmoc-SPPS: 1. 2x 10 eq. Fmoc-AA-OH, 10 eq. DIC/Oxyma (0.1M DIPEA), 90°C 2. 2x 20% piperidine, 90°C b) **9**, PyBOP, DIPEA, DMF, rt, 16h

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

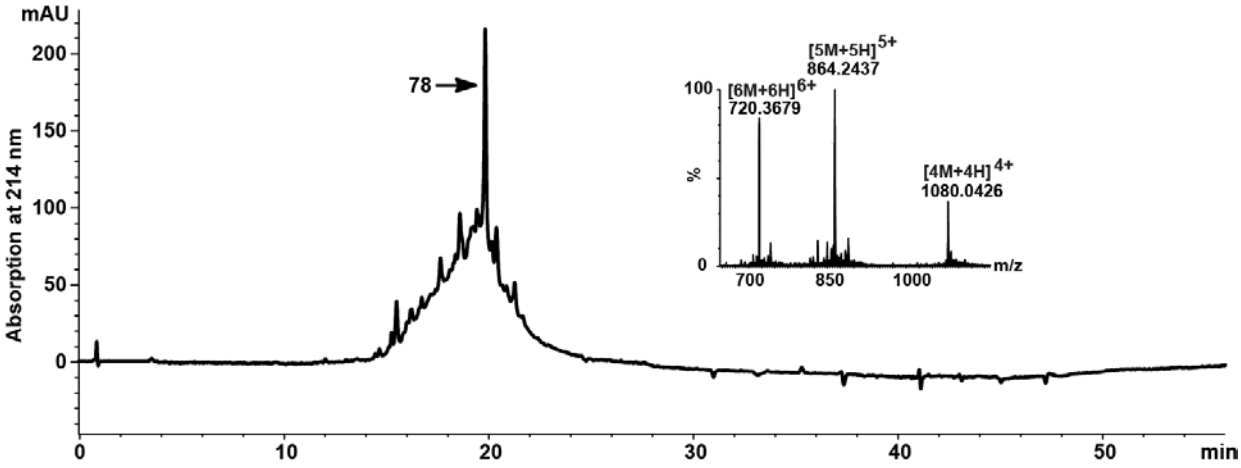
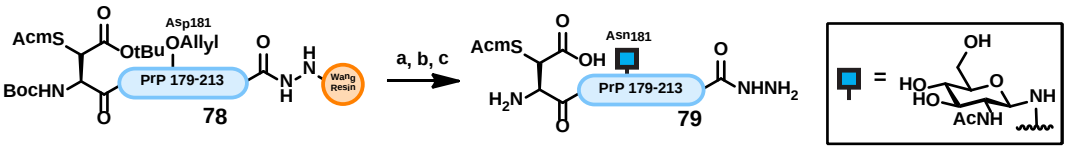


Figure 50: LC-MS analysis of 78 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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).



Scheme 72: Synthesis of 79: a) $Pd(PPh_3)_4$, 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 desired product was found together with oxidized peptide species as was observed previously. To reduce the sulfoxide moieties, **79** was treated with NH_4I and DMS in TFA.[79] 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**).

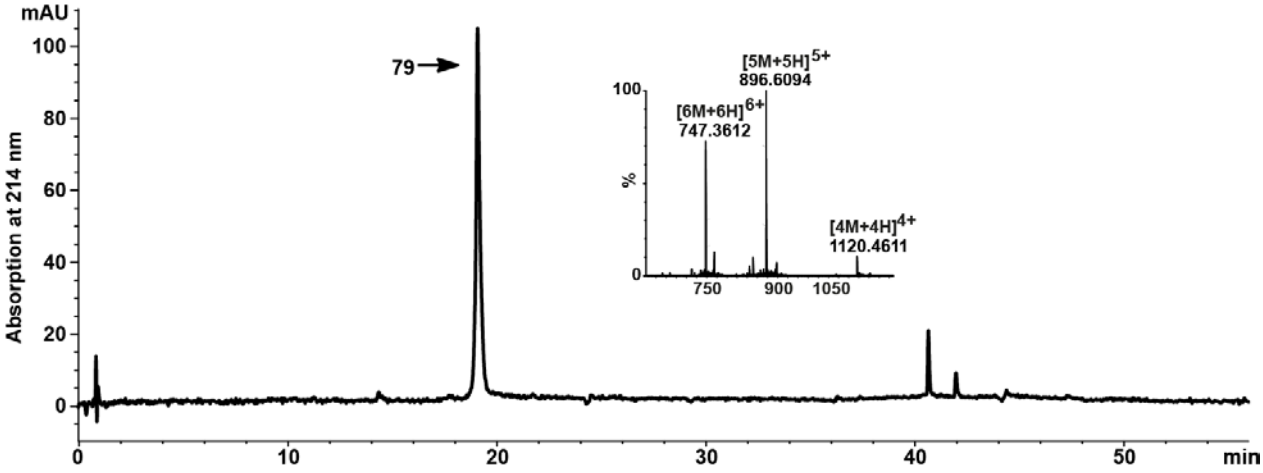
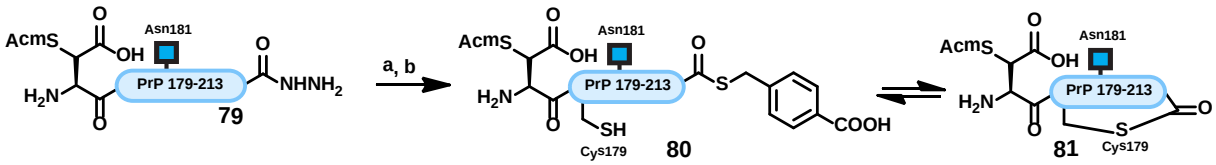


Figure 51: LC-MS analysis of 79 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

Peptide hydrazide **79** was transformed into a thioester **80** using $NaNO_2$ at pH 3 as oxidative condition and MMBA at pH 7 (Scheme 73).[144]



Scheme 73: Synthesis of 80: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 0.02 M $NaNO_2$, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min

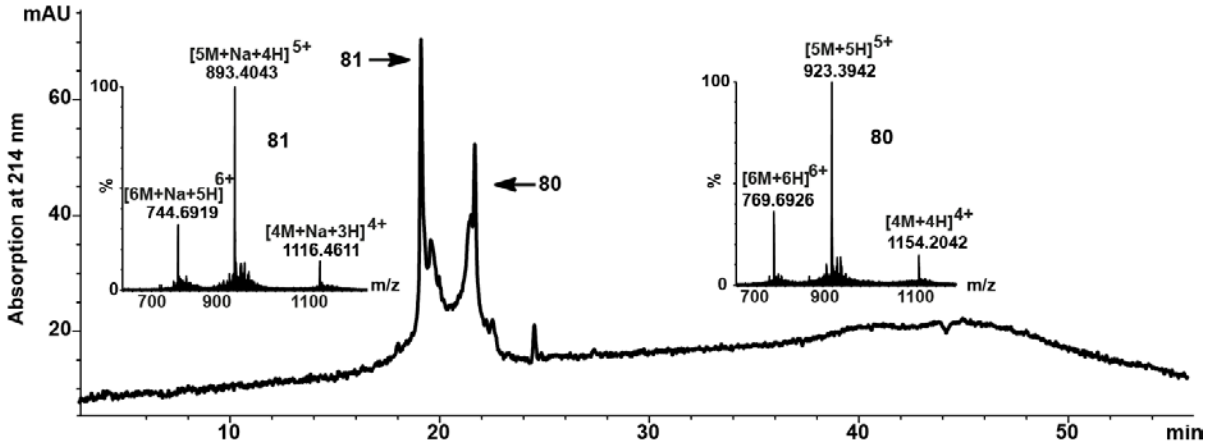
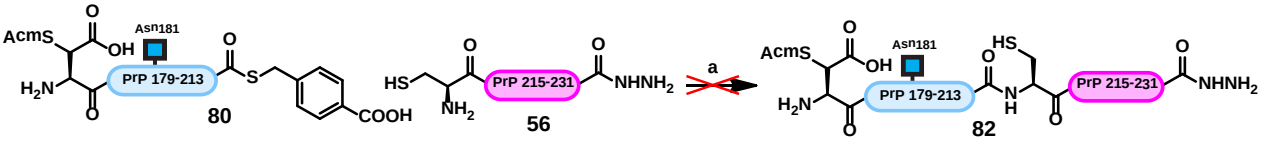


Figure 52: LC-MS analysis of 80 and 81 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.

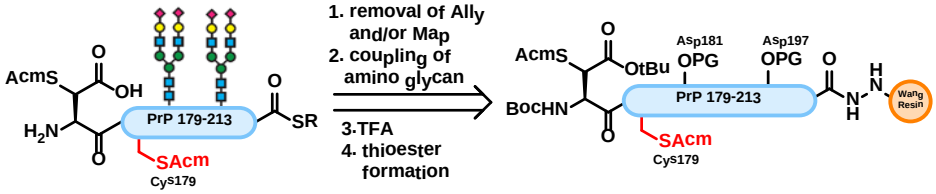


Scheme 74: Synthesis of 82: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 30 eq. TCEP, 5 eq MMBA 72 h, 5 eq MPAA 24h

The ligation was conducted using a buffer the optimized buffer with 6 M GdmCl, 0.2 M Na_2HPO_4, 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).

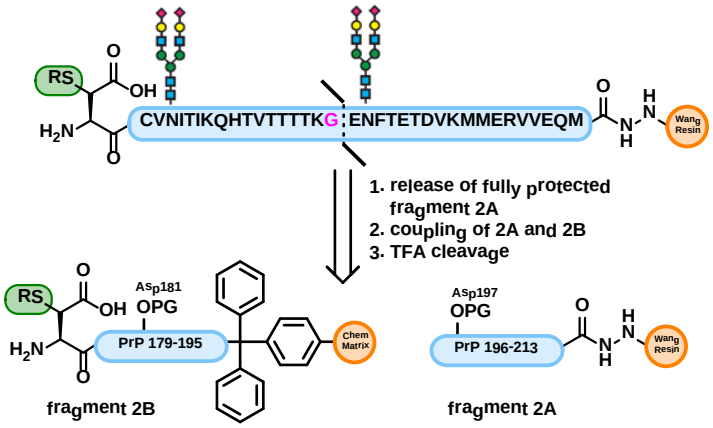


Scheme 75: New Retrosynthetic analysis of strategy 1: fragment 2 for C to N ligation

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.



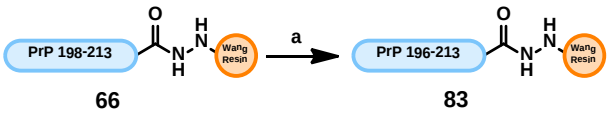
Scheme 76: Retrosynthetic analysis of strategy 3: fragment 2 via SPFC

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 thioesterformation and ligation to fragment 1, fragment 2B was synthesized as peptide hydrazide.[39, 51] 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 testcleavage 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 77: Synthesis of 83: a) Fmoc-SPPS: 1. 2x 10 eq. Fmoc-AA-OH PyBOP/DIPEA 2. 2x 20% piperidine

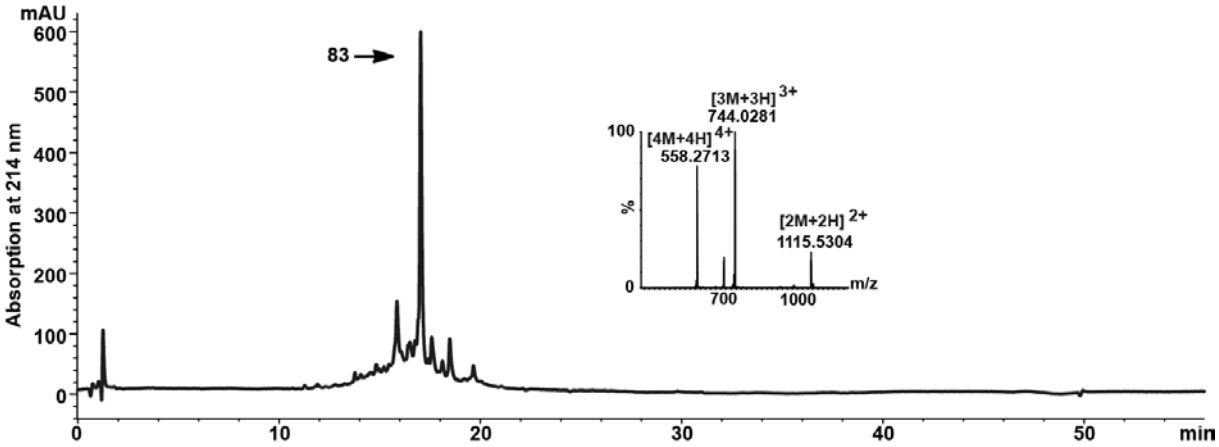
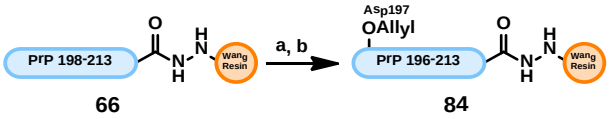


Figure 53: LC-MS analysis of 83: LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 testcleavage 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).

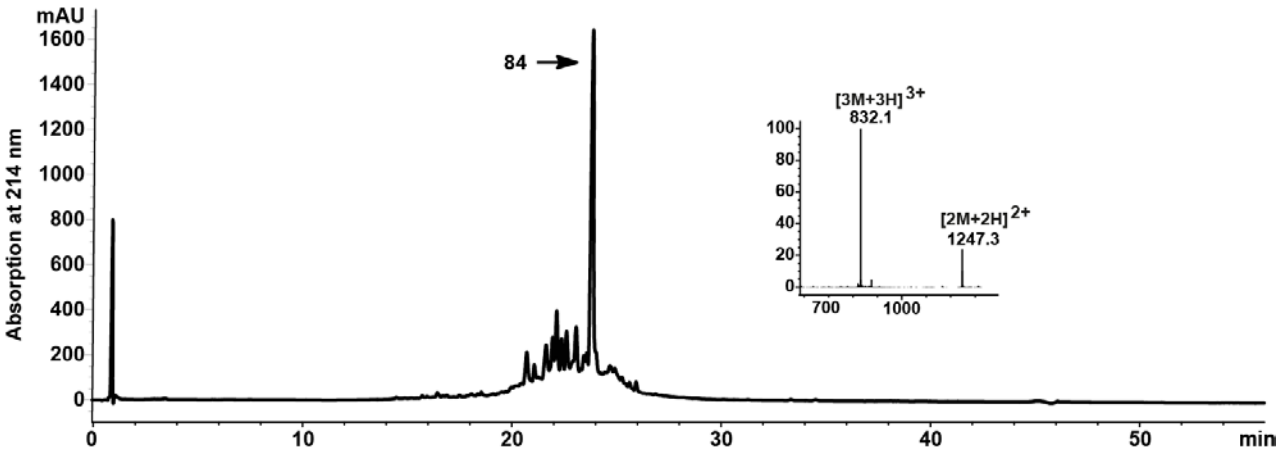
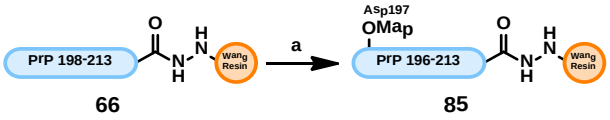


Figure 54: LC-MS analysis of 84: LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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: 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

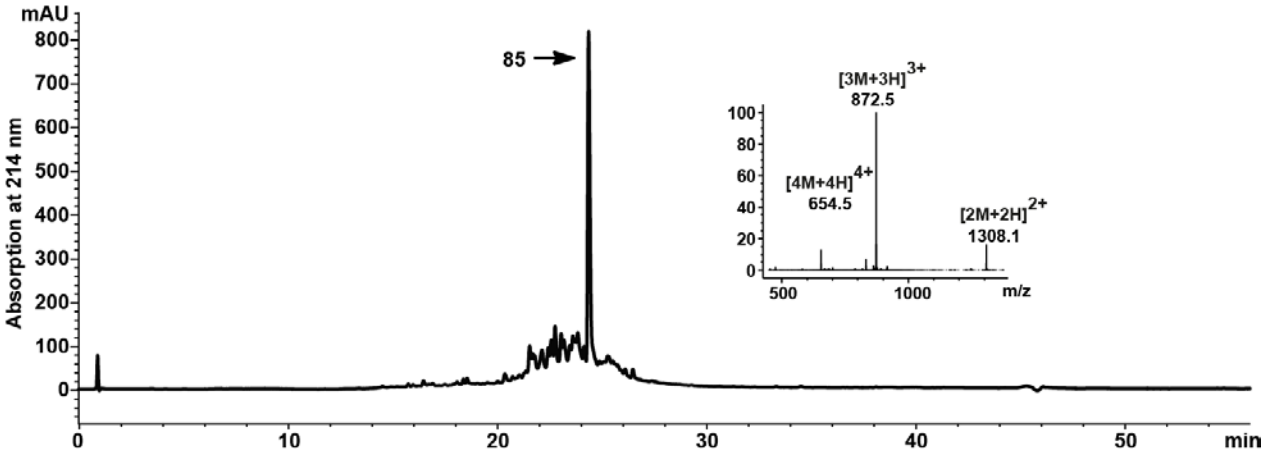
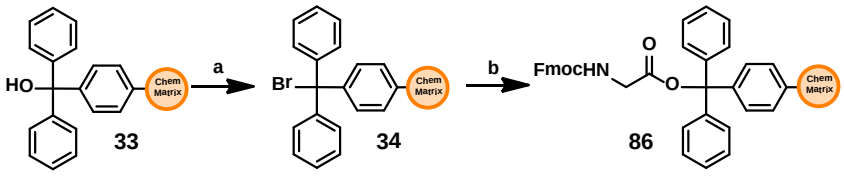


Figure 55: LC-MS analysis of 85: LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

5.15 Synthesis of fragment 2B

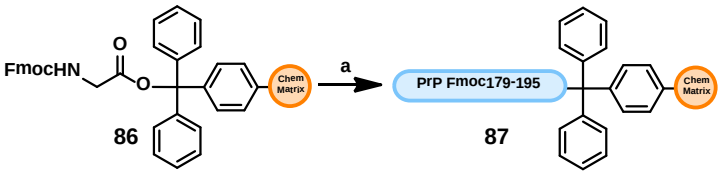
To start, commercially available trityl-ChemMatrix® (loading: 0.6-1.2 mmol/g) was brominated using 10% AcBr in anhydrous DCM under argon atmosphere for four hours. Thereafter, the first amino acid (Fmoc-Gly-OH) was coupled to the activated resin using DIPEA as base. After coupling, the resin loading 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: a) 10% AcBr in DCM, 4h, b) Fmoc-Gly-OH, DIPEA, 14h

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 testcleavage 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

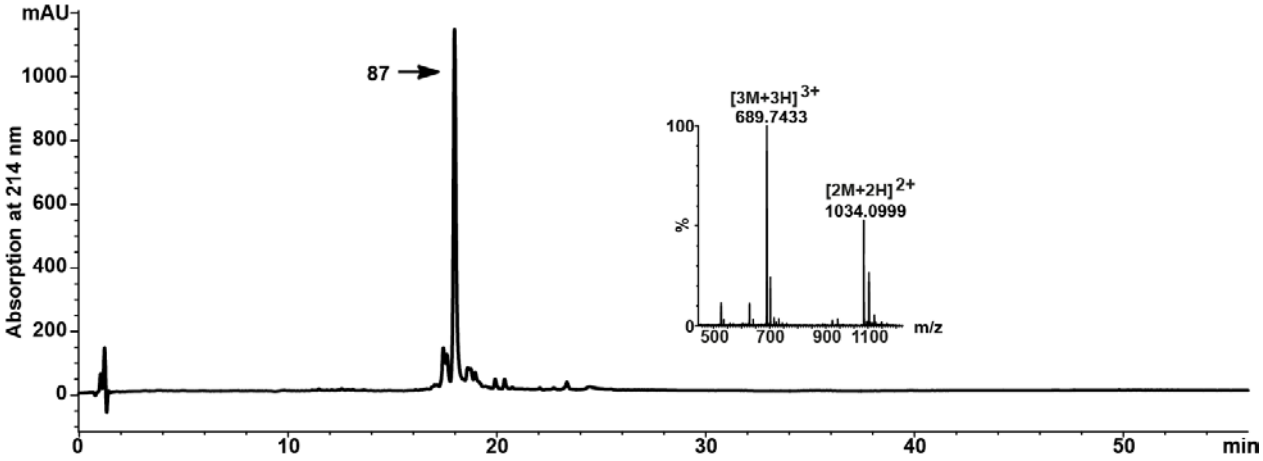
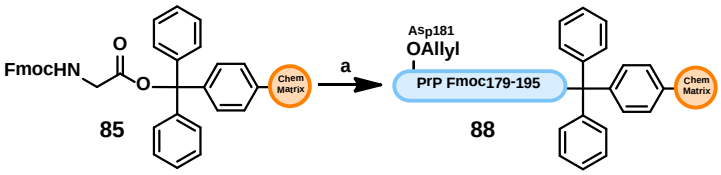


Figure 56: LC-MS analysis of 87: LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 testcleavage 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).

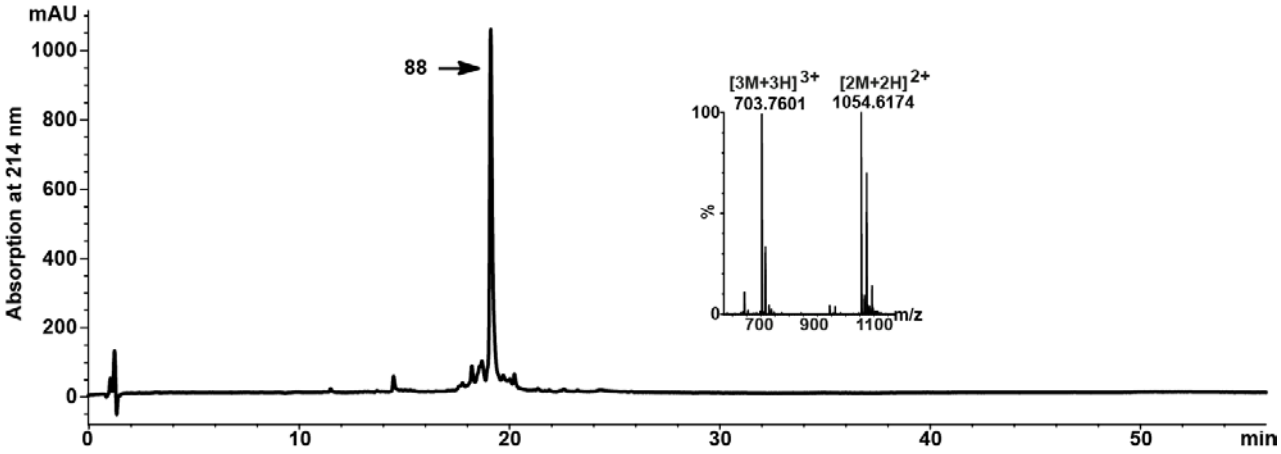
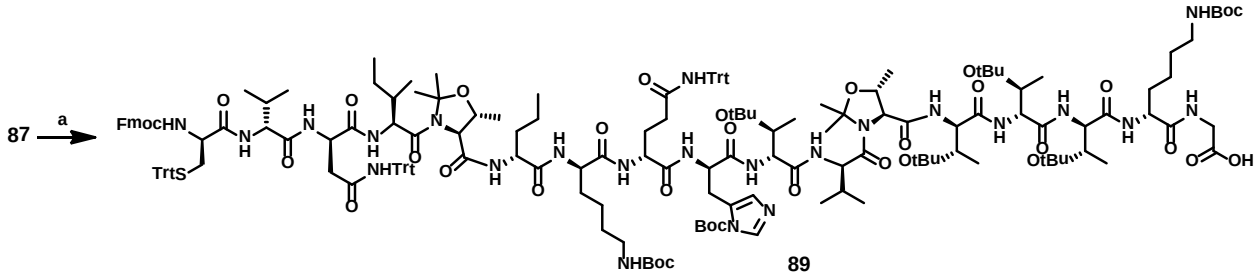


Figure 57: LC-MS analysis of 88: LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.

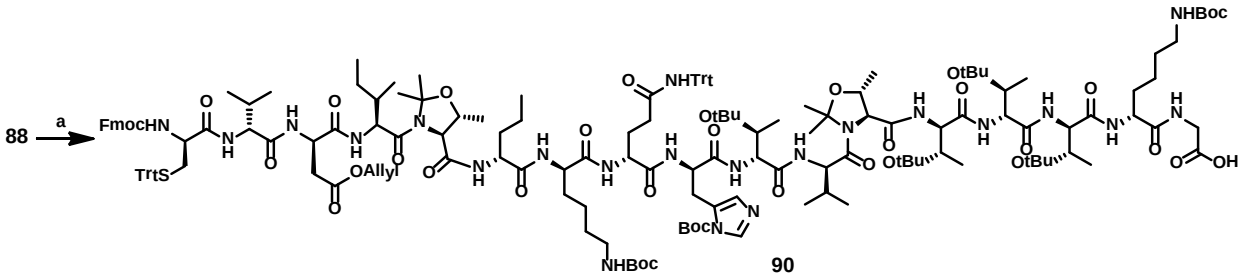
The resin was treated three times with 10 ml 1% TFA in DCM for 3 min and immediately quenched with DIPEA in DCM. The resin was washed with DCM and methanol and concentrated under reduced pressure. The resulting solid was dissolved in DCM and extracted with sat. NaCl-solution and water. The organic layer was dried over $NaSO_4$, concentrated under reduced pressure and crude peptide **89** was obtained in 39% yield.



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.



Scheme 84: Synthesis of 90: a) 1% TFA in DCM, 3x3min

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.

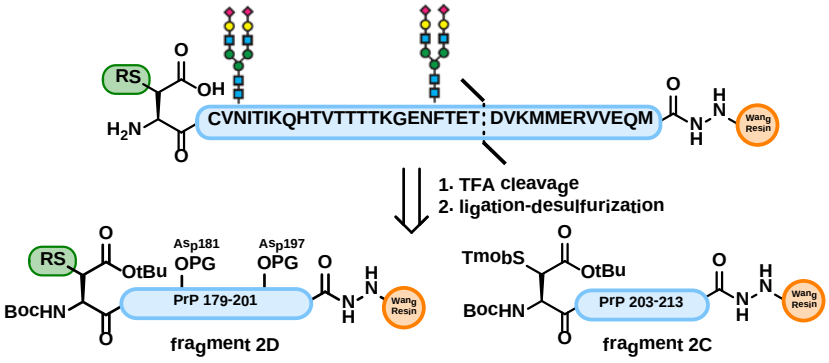
Additionally, two PrP fragments 2B (179-195) were generated, including one peptide having an allyl protecting group on Asp181. Both peptides, **87** and **88**, were observed in a high crude purity with only minor deletion sequences. Unfortunately, when the peptide was cleaved from the resin using mild 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(O*t*Bu,STmob)-OH **8** was already synthesized for installation at Asp178 and could also be used at Asp202 to facilitate a second ligation-desulfurization step.



Scheme 85: Retrosynthetic analysis of strategy 4: synthesis of fragment 2 using ligation-desulfurization

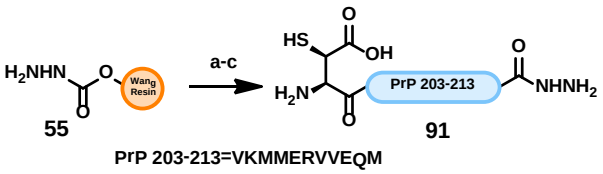
Thus, to synthesize fragment 2 using a ligation-desulfurization, the sequence was separated in two fragments: fragment 2C PrP (203-213) with Boc-Asp(OtBu,STmob)-OH **8** installed as *N*-terminal amino acid and fragments 2D PrP (178-204). Fragment 2D contains the two glycosylation sites and Asp178 is installed as orthogonal protected β-mercapto 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).

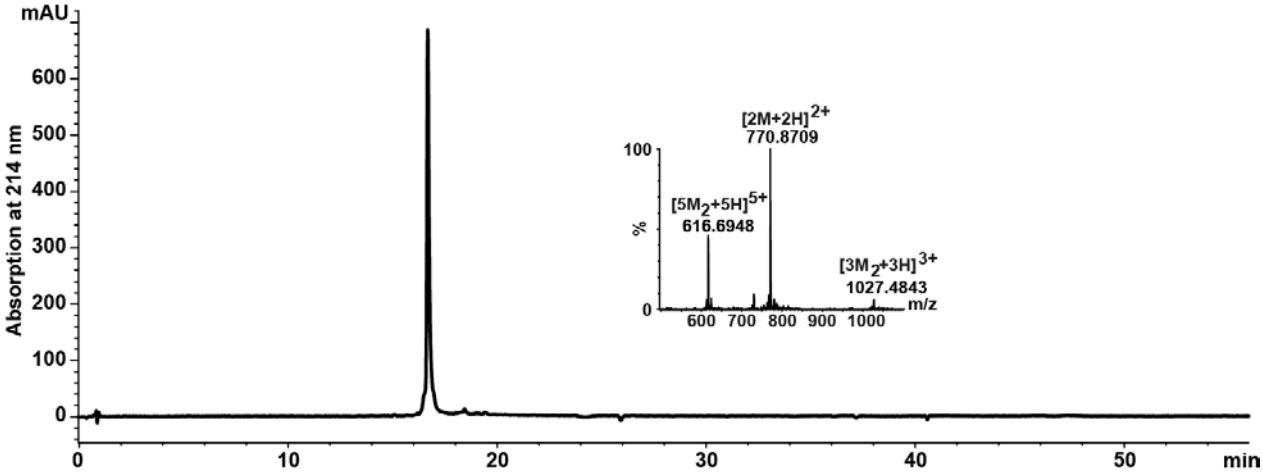
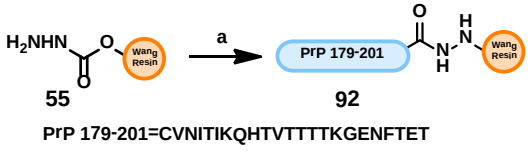


Figure 58: LC-MS analysis of purified 91 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 testcleavage 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).

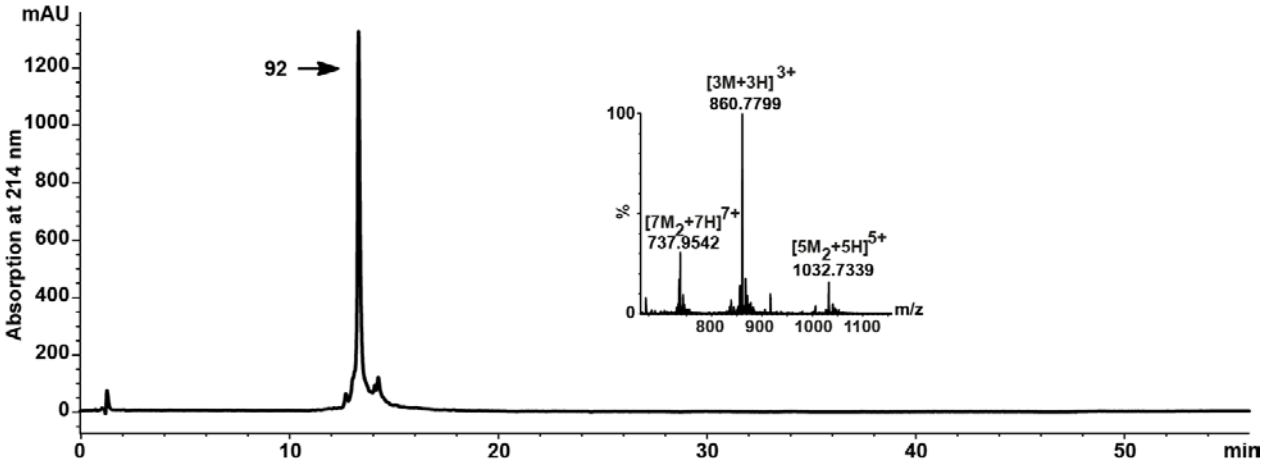
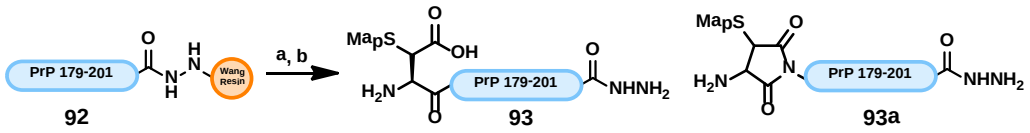


Figure 59: LC-MS analysis of 92 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

Boc-Asp(O*t*Bu,SMap)-OH **11** was coupled manually using PyBOP as coupling reagent and DIPEA as base (Scheme 88). After complete assembly, a testcleavage 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.

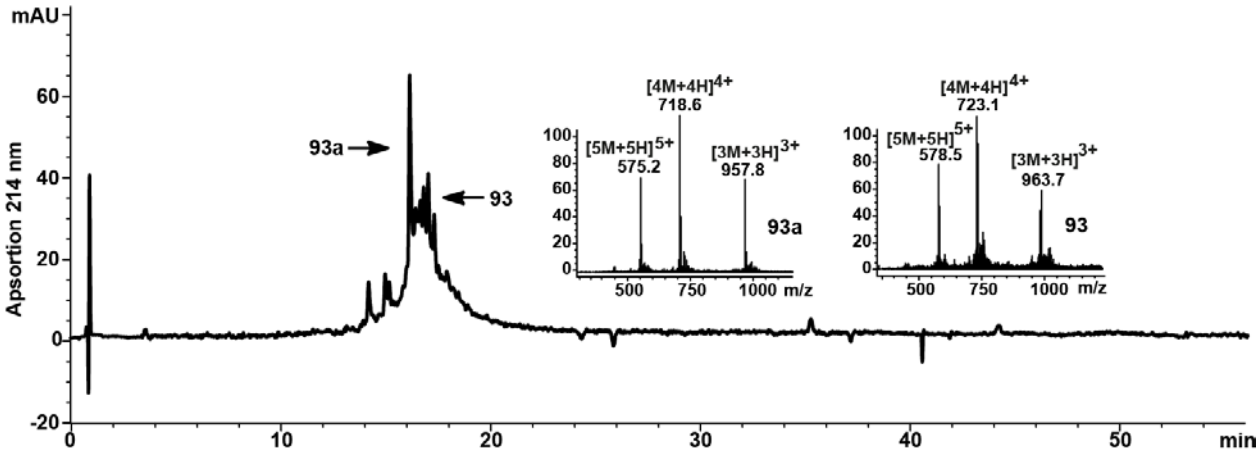
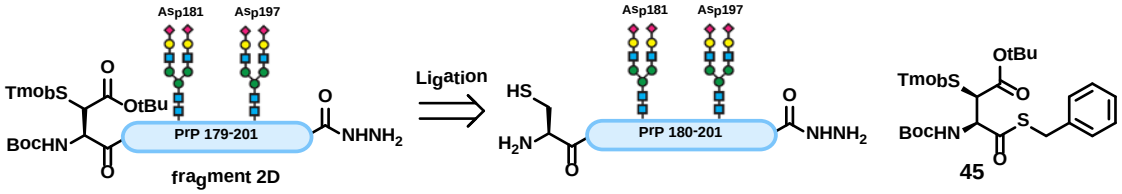


Figure 60: LC-MS analysis of 93 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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(O*t*Bu,STmob) thioester **45**.



Scheme 89: Retrosynthetic analysis of fragment 2D using ligation to 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).

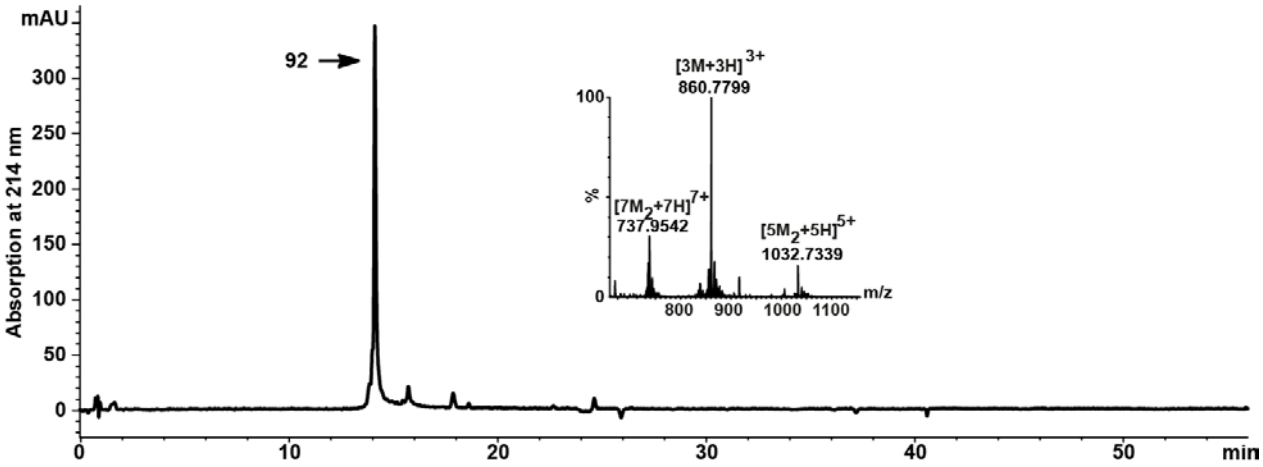
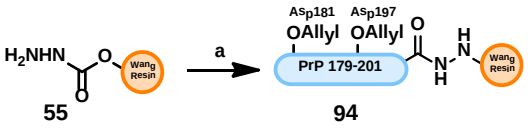


Figure 61: LC-MS analysis of 92 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 syntheziser 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).



Scheme 90: Synthesis of 94: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 50-90°C 2. 2x 20% piperidine 90°C

A testcleavage 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 N^{α}-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**.

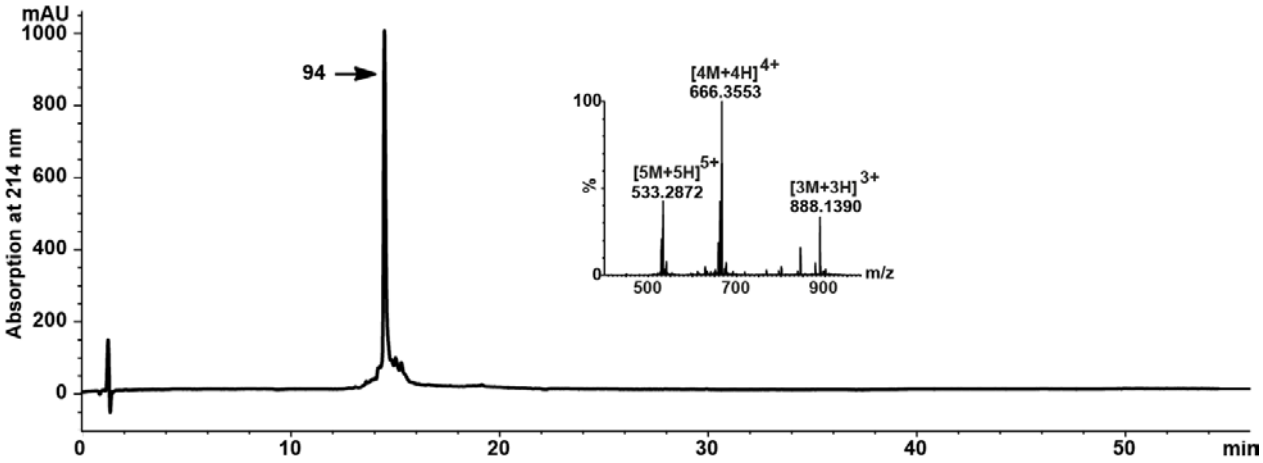
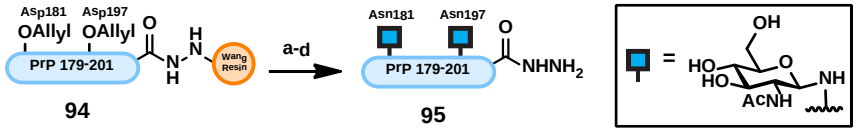


Figure 62: LC-MS analysis of 94 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

Thereafter, the allyl ester was cleaved to give a free aspartic acid side chain, using $Pd(PPh_3)_4$ and phenylsilane as scavenger in DCM. A testcleavage 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) Boc_2O, pyridine, DMF, 20 min b) $Pd(PPh_3)_4$, 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).

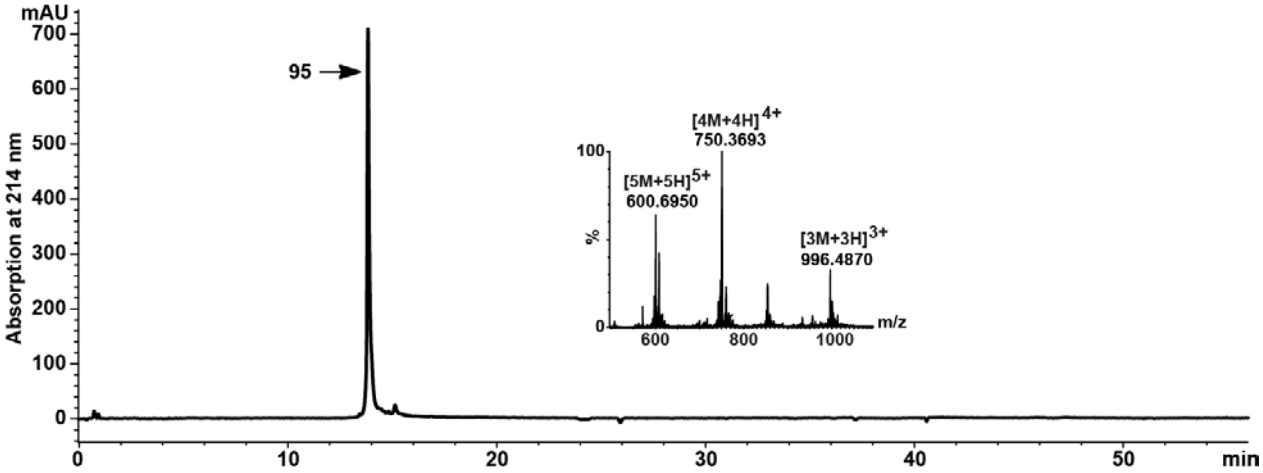


Figure 63: LC-MS analysis of 95 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 50-90°C 2. 2x 20% piperidine 90°C b) Boc_2O, pyridine, DMF, 20 min c) $Pd(PPh_3)_4$, phenylsilane, DCM, 2 h, quant. d) **19**, PyBOP, DIPEA, DMF/DMSO, 16 h, quant e) TFA/water/TIPS (190/5/5, v/v/v)

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

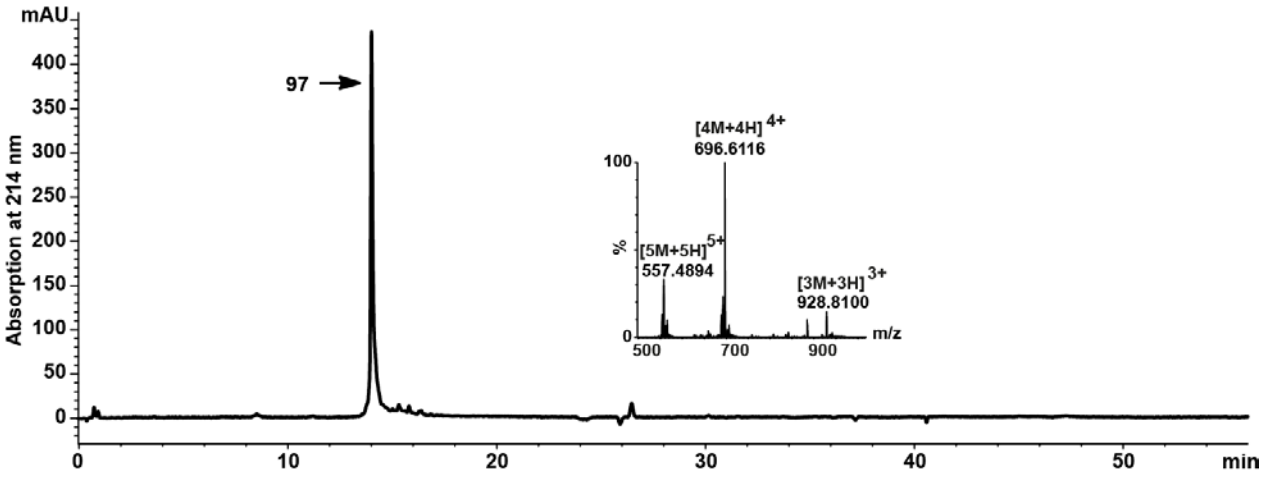
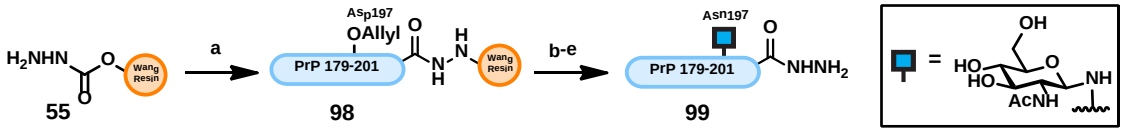


Figure 64: LC-MS analysis of 97 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

After complete assembly, the free N^{α}-function was capped using Boc-anhydride. Thereafter, the allyl ester was cleaved using $Pd(PPh_3)_4$ 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**.



Scheme 93: Synthesis of 99: a) Fmoc-SPPS: 1. 2x 5 eq. Fmoc-AA-OH, 5 eq. DIC/Oxyma (0.1M DIPEA), 50-90°C 2. 2x 20% piperidine 90°C b) Boc_2O, pyridine, DMF, 20 min c) $Pd(PPh_3)_4$, phenylsilane, DCM, 2 h, quant. d) **19**, PyBOP, DIPEA, DMF/DMSO, 16 h, quant e) TFA/water/TIPS (190/5/5, v/v/v)

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 $Pd(PPh_3)_4$ and amino glycan **19** was coupled using PYBOP and DIPEA in DMF/DMSO.

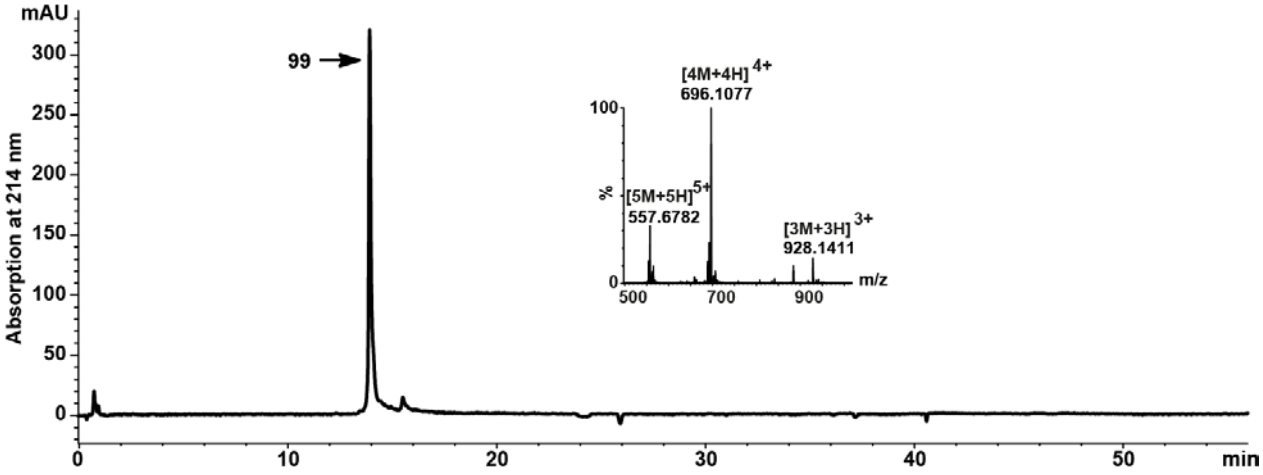
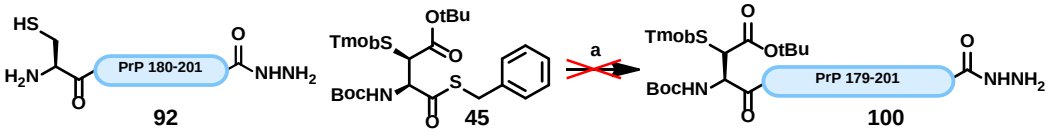


Figure 65: LC-MS analysis of 99 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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.

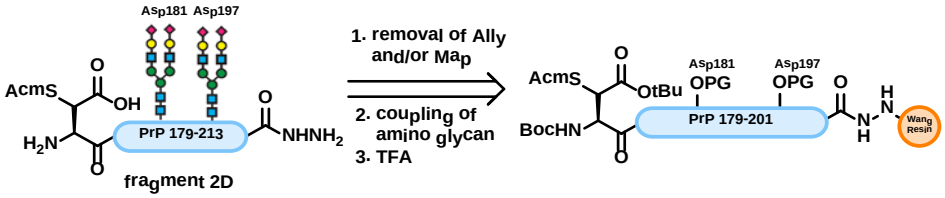


Scheme 94: Synthesis of 100: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 30 eq. TCEP, 5 eq MMBA, 10 eq **45** in THF

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

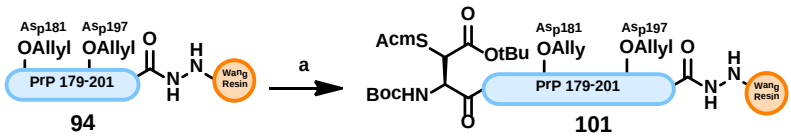
To have access to PrP (178-201) bearing a protected thiol function on the *N*-terminal β-mercapto Asp178, a strategy using orthogonal protected aspartic acid building block **9** was evaluated (Scheme 95). After successful ligation the orthogonal Acm group can be removed and the peptide can be ligation 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(O*t*Bu,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**.

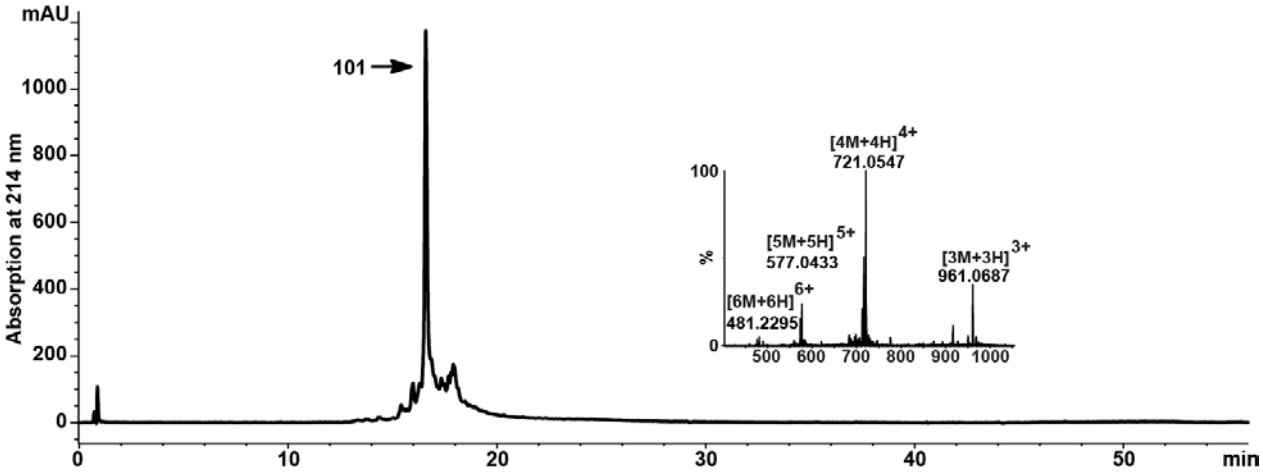
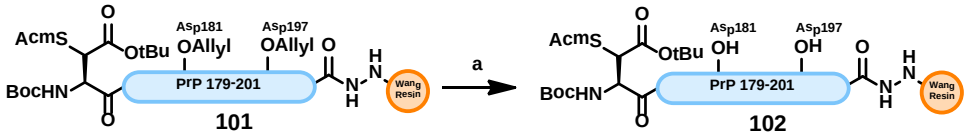


Figure 66: LC-MS analysis 101 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

The allyl ester was cleaved to give a free aspartic acid side chain using $Pd(PPh_3)_4$ and phenylsilane as scavenger in DCM (Scheme 97).



Scheme 97: Synthesis of 102: a) $Pd(PPh_3)_4$, phenylsilane, DCM, 2 h, quant

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

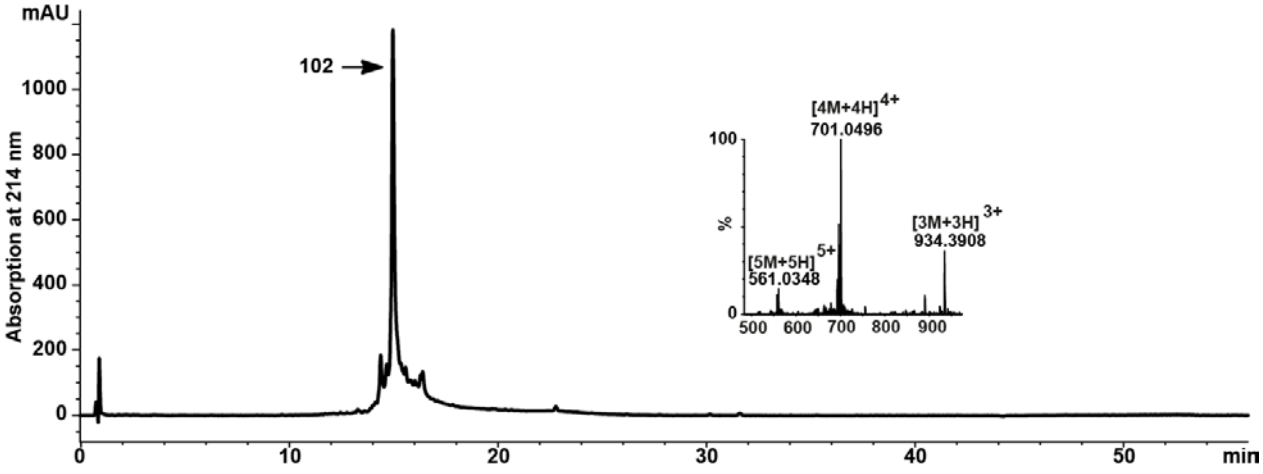
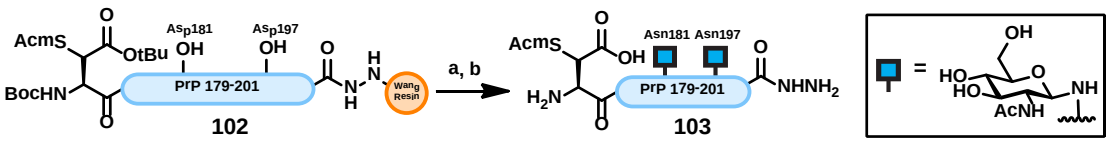


Figure 67: LC-MS analysis of 102 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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).



Scheme 98: Synthesis of 103: a) **19**, PyBOP, DIPEA, DMF/DMSO, 16 h, quant b) TFA/water/TIPS (190/5/5, v/v/v)

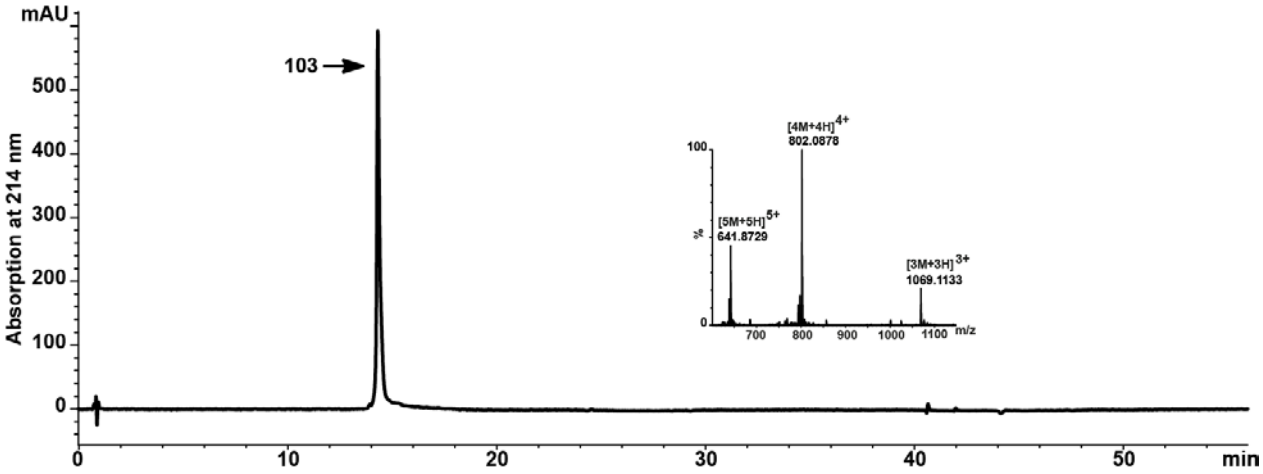
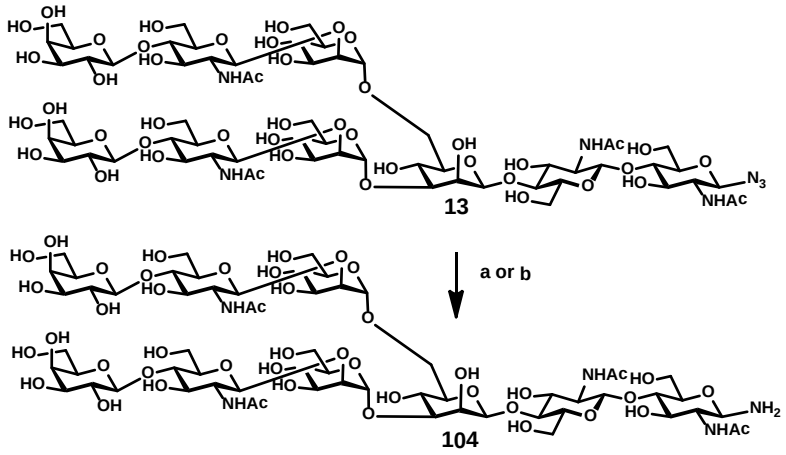


Figure 68: LC-MS analysis of 103 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

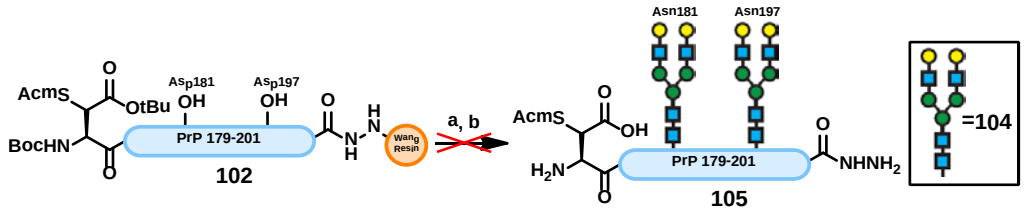
Thereafter, the synthesis of fragment 2D [178-201] bearing two large *N*-glycans was evaluated. The required amino nonasaccharide **104** was generated starting from **13** using reductive conditions. In a first synthesis, azide **13** was reduced using the same condition established for **19**.

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).



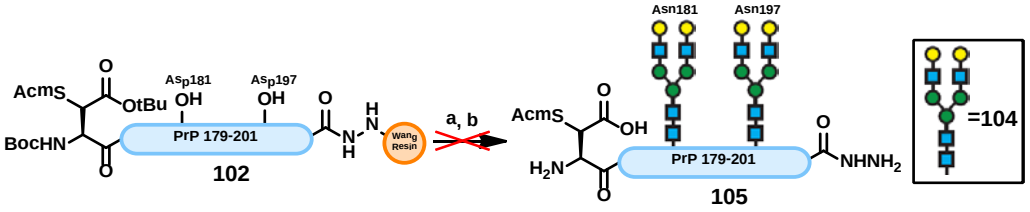
Scheme 99: Synthesis of 104: a) Pd/C, H_2↑, MeOH 2 h b) ethanedithiol, DIPEA, MeOH

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 testcleavage 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.[69] 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 testcleavage showed that no product was observed. Thereafter, HATU and HOBt were used as coupling reagents and DIPEA as base (Scheme 101).



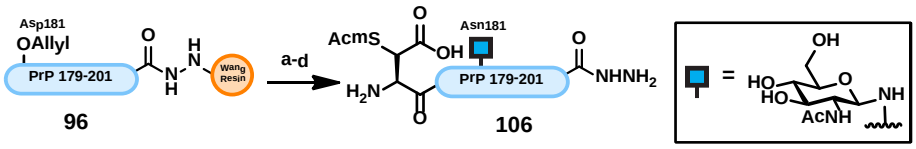
Scheme 101: Synthesis of 105: a) **104**, HATU, HOBt, DIPEA, DMF/DMSO, 16 h b) TFA/water/TIPS (190/5/5, v/v/v)

A testcleavage 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 testcleavage 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. DIPEA, DMF, 16 h b) $Pd(PPh_3)_4$, 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).

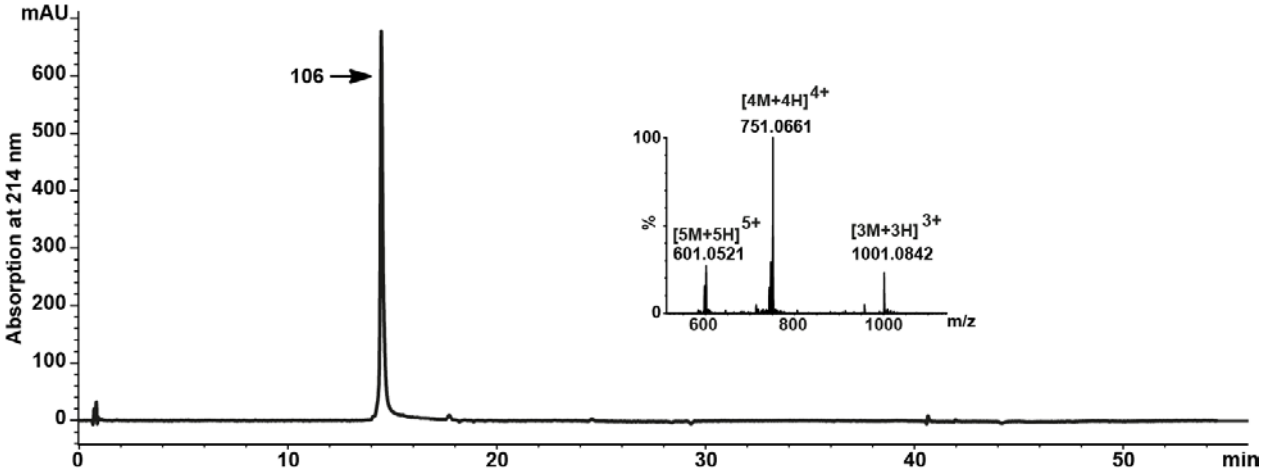
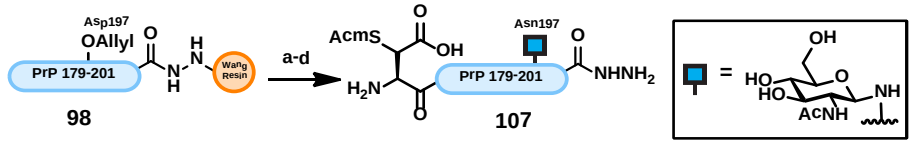


Figure 69: LC-MS analysis 106 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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) $Pd(PPh_3)_4$, 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).

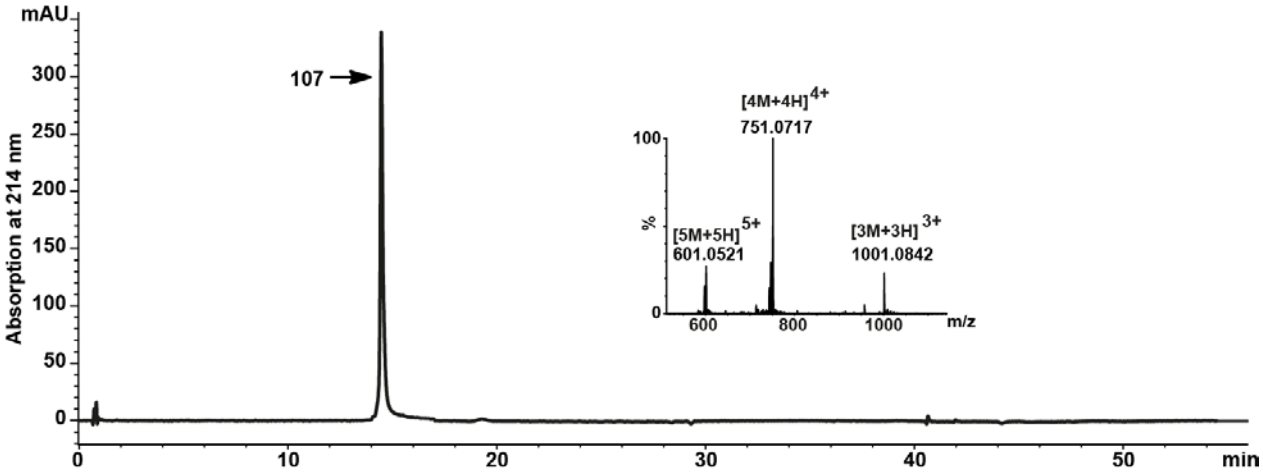
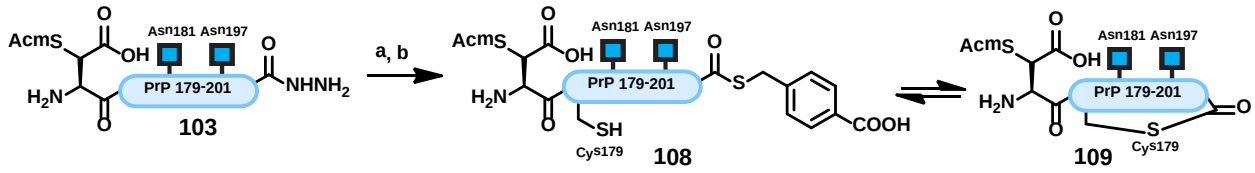


Figure 70: LC-MS analysis 107[LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 $NaNO_2$ at pH 3 as oxidative condition and MMBA at pH 7 (Scheme 104).[144]



Scheme 104: Synthesis of 108: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 0.02 M $NaNO_2$, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min

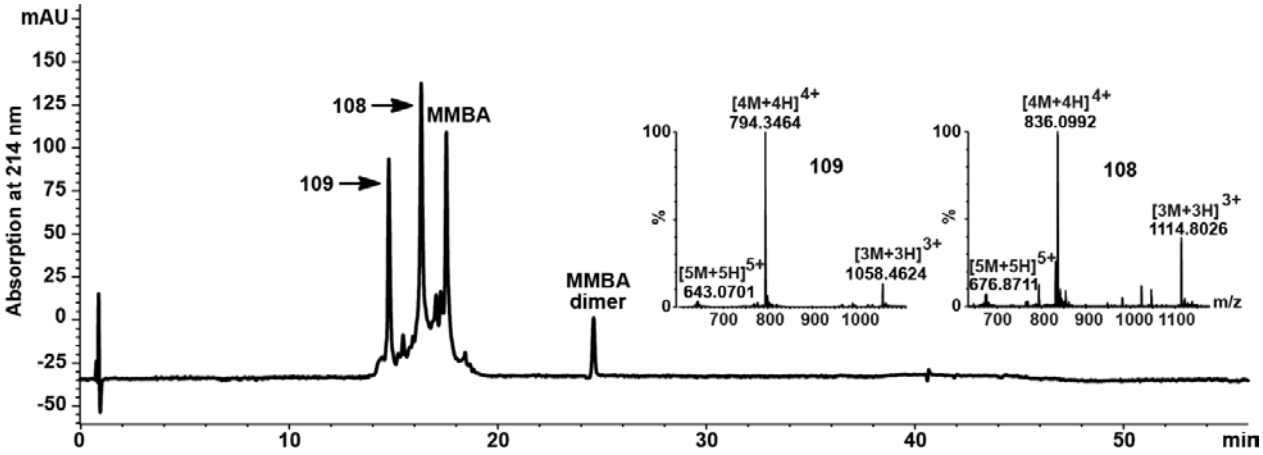
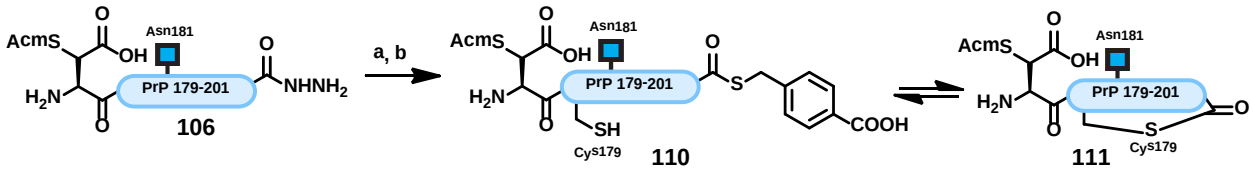


Figure 71: LC-MS analysis of 108 and 109 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 $NaNO_2$ at pH 3 and MMBA at pH 7 (Scheme 105)



Scheme 105: Synthesis of 110: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 0.02 M $NaNO_2$, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min

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

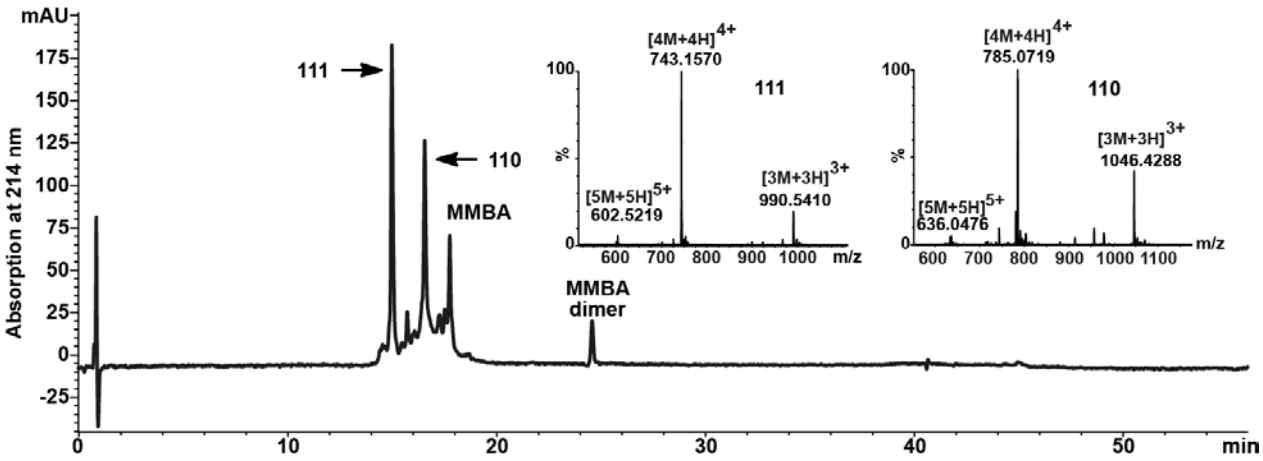
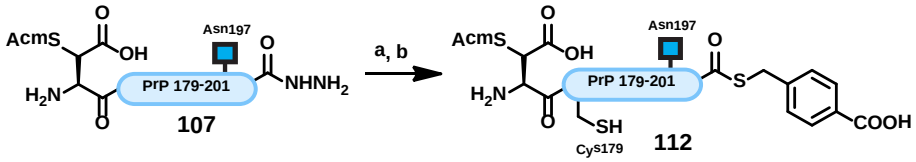


Figure 72: LC-MS analysis of 110 and 111 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

Additionally, glycopeptide hydrazide **107** was used to generate thioester **112**. Peptide hydrazide **107** was treated with $NaNO_2$ containing diazotation buffer at pH 3 and MMBA at pH 7 (Scheme 106).



Scheme 106: Synthesis of 112: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 0.02 M $NaNO_2$, pH 3, -10°C, 20 min, b) MMBA, pH 7, rt, 30 min

The reaction was purified using gel filtration and thioester **112** was obtained in 64% yield (Figure 73).

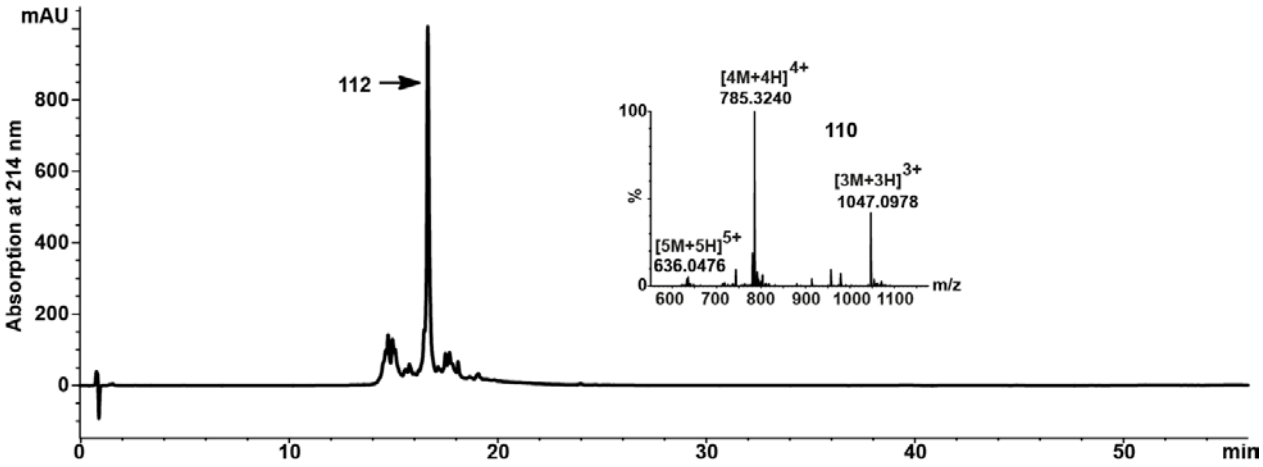


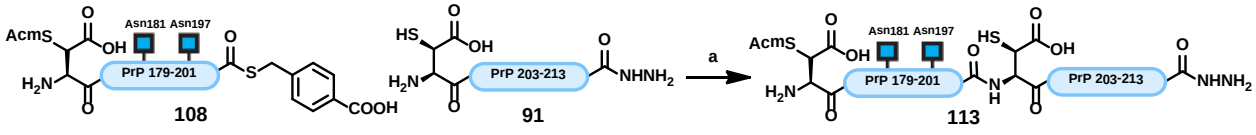
Figure 73: LC-MS analysis of 112 [LC on Agilent 1100: Hydrosphere C18 [50x3mm; 3µm], 5% ACN to 70% ACN in H_2O (0.1% TFA) in 30 min]

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 Na_2HPO_4 and 5 eq. TCEP at pH 7 as proposed by Payne and coworkers[47] 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 thiolacatone **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 Na_2HPO_4, 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.



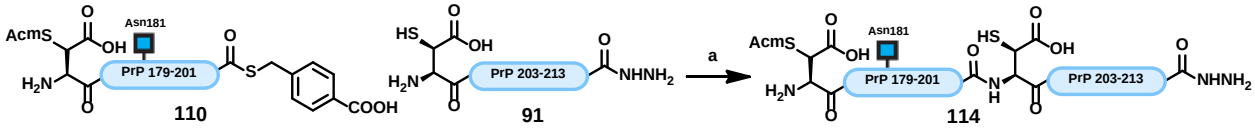
Scheme 107: Synthesis of 113: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 1 eq. MMBA (or MPAA), 10 eq. TCEP, pH 7, 37°C

Taking these observations into account, the ligation buffer was further optimized and 6 M GdmCl, 0.2 M Na_2HPO_4, 1 eq. MMBA and 10 eq. TCEP at pH 7 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 Na_2HPO_4, 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.

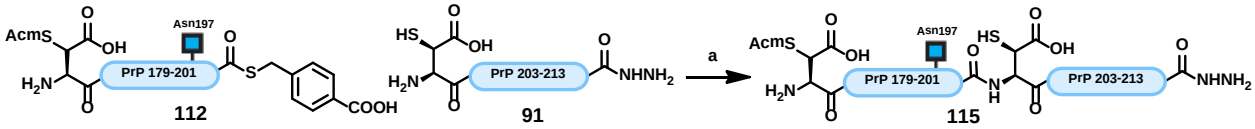
The ligation conditions were also tested for thioester/thiolactone **110/111** and β-mercapto aspartic acid containing peptide **91**. 6 M GdmCl, 0.2 M Na_2HPO_4, 1 eq. MPAA and 10 eq. TCEP at pH 7 were used at 37°C.



Scheme 108: Synthesis of 113: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 1 eq. MPAA, 10 eq. TCEP, pH 7, 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 Na_2HPO_4, 1 eq. MPAA and 10 eq. TCEP at pH 7 and 37°C.



Scheme 109: Synthesis of 115: a) 6 M GdmCl, 0.2 M Na_2HPO_4, 1 eq. MPAA, 10 eq. TCEP, pH 7, 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

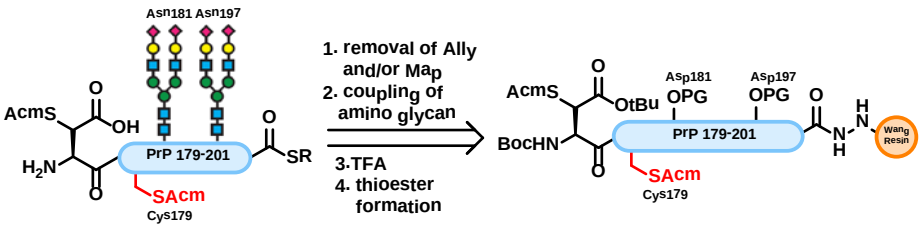
The synthesis of fragment 2 was evaluated using a ligation-desulfurization approach. The β-mercapto aspartic acid containing PrP (202-213) was synthesized successfully using the optimized conditions for fragment 2. After purification desired peptide **91** was obtained in 25% yield and used for ligation 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,

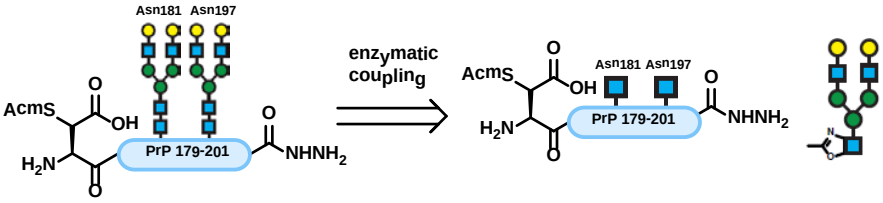
the desired ligation product was observable. Unfortunately, the ligation showed only a very low conversion, and after prolonged reaction the thioester/thiolactone mixture hydrolyzed and the thiol function of β-mercapto Asp178 formed disulfides. Although LC-MS analysis showed the formation of the desired 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 **91** formed MMBA.



Scheme 110: New Retrosynthetic analysis of strategy 1: fragment 2D for a ligation-desulfurization strategy

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 **19** using Lansbury coupling resulted in quantitative conversion. However, the installation nona-saccharide **104** did not give the desired product, independent of the coupling conditions.



Scheme 111: New Retrosynthetic analysis for the coupling of large glycans

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_2O (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 λ 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 Na_2HPO_4, 0.02 M $NaNO_2$, pH 3

Ligation Buffer(A): 6 M GdmCl, 0.1 M Na_2HPO_4, 0.05 M MPAA, 5 mM TCEP, pH 7

Ligation Buffer(B): 6 M GdmCl, 0.1 M Na_2HPO_4, 0.05 M MMBA, 5 mM TCEP, pH 7

Ligation Buffer(C): 6 M GdmCl, 0.1 M Na_2HPO_4, 5 equivalents MPAA, 5 equivalents per thiol TCEP, pH 7

Ligation Buffer(D): 6 M GdmCl, 0.1 M Na_2HPO_4, 5 equivalents MMBA, 5 equivalents per thiol TCEP, pH 7

Ligation Buffer(E): 6 M GdmCl, 0.1 M Na_2HPO_4, 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:

$$substitution\left[{}^{mmol}/_{g}\right] = \frac{[Abs\ 290\ nm]}{mg\ resin\ x\ 1{,}75}$$

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-methylmprpholine 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;SAcm)-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 testcleavage 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 testcleavage 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_4I 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-*t*-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/NH_4I
(81:2.5:5:5:3:2:1.5/v:v:v:w:v:v:w)

Test cleavages

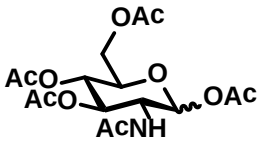
Depending on the sequence, 200 µl for short sequences, *i.e.* up to 20 amino acids, and 300 µl for long sequences, *i.e.* more than 20 amino acids, of reagent B, K or H were added to some bits of dry resin in a 1.5 ml Eppendorf tube and shaken for 60 minutes. The peptide was crushed out in ice cold ether, 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.

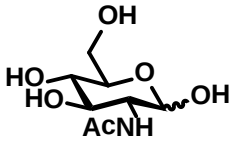
4. SYNTHESIS OF GLYCAN BUILDING BLOCKS FOR GLYCOPEPTIDE SYNTHESIS

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



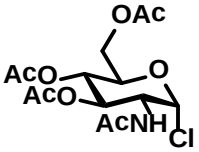
20 g 2-amino-2-deoxy-glucose hydrochloride (112 mmol) were suspended in 20 ml pyridine and 105 ml acetic anhydride were added. The mixture was stirred for 16 hours and concentrated under reduced pressure. The resulting oil was dissolved in DCM and washed with sat. $NaHCO_3$-solution and sat. NaCl-solution. The combined organic phases were dried over $NaSO_4$. The solvent was removed to give **15** in quantitative yield as yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$): 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: $Mass_{calc}$ for $C_{16}H_{23}NO_{10}$: 390.140 $[M+H]^+$; $Mass_{obs}$: 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. ^{1}H-NMR (400 MHz, Methanol-d_4): δ = 5.10 (d, *J* = 3.4 Hz, 1H), 3.92 – 3.57 (m, 6H), 1.99 (s, 3H) ppm.

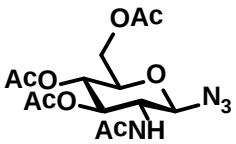
3,4,6-Tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside-1-chloride 17



10 g of **16** were dissolved in 50 ml acetyl chloride and 14 µl conc. HCl ware 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. $NaHCO_3$-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 $NaSO_4$, concentrated and purified using column chromatography to give the desired product **17** in 45% yield as white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.17 (d, *J* = 3.7 Hz, 1H), 5.88 (d, *J* = 8.7 Hz, 1H), 5.31 (dd, *J* = 10.5 Hz, 9.5 Hz, 1H), 5.22 - 5.17 (m, 1H), 4.52 (ddd, *J* = 10.7 Hz, 8.7 Hz, 3.7 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 Hz, 3H) ppm; ESI-MS: $Mass_{calc}$ for $C_{14}H_{20}ClNO_8$: 366.096 $[M+H]^+$; $Mass_{obs}$: 366.080 $[M+H]^+$

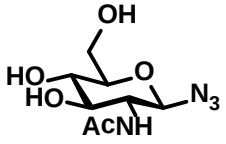
3,4,6-Tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside-1-azide 18



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 $NaSO_4$. 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 $SnCl_4$ 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}H-NMR (400 MHz, $CDCl_3$): δ = 6.39 (d, *J* = 9.0 Hz, 1H), 5.26 (dd, *J* = 10.5 Hz, 9.4 Hz, 1H), 5.07 - 5.02 (m, 1H), 4.81 (d, *J* = 9.3 Hz, 1H), 4.23 (dd, *J* = 12.4 Hz, 5.0 Hz, 1H), 4.15 - 4.11 (m, 1H), 3.89 (dt, *J* = 10.5 Hz, 9.1 Hz, 1H), 3.81 (ddd, *J* = 10.1 Hz, 4.9 Hz, 2.4 Hz, 1H), 2.05 (s, 3H), 2.00 (d, *J* = 3.5 Hz, 3H), 1.98 (s, 3H), 1.94 (s, 3H) ppm; ESI-MS: $Mass_{calc}$ for $C_{14}H_{20}N_4O_8$: 373.136 $[M+H]^+$; $Mass_{obs}$: 373.170 $[M+H]^+$

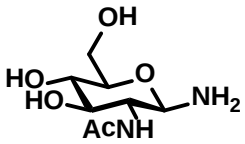
2-Acetamido-2-deoxy-β-D-glucopyranoside-1-azide 12



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): δ = 4.50 (d, *J* = 9.3 Hz, 1.1 Hz, 1H), 3.90 (d, *J* = 12.1 Hz, 1.8 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

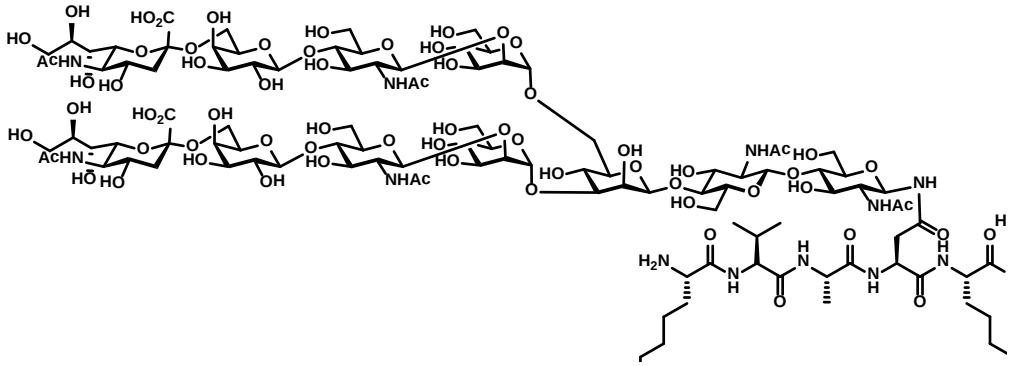


12 was dissolved in anhydrous methanol under argon atmosphere to give a 0.1 molar concentration and argon was bubbled through the solution. 0.1 eq. palladium on carbon were added and hydrogen was bubbled through the suspension. Afterwards the suspension was incubated under hydrogen atmosphere for 2 hours. 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[N^4-{5-*N*-Acetyl-D-neuraminyl-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-D-glucopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-*O*-[5-*N*-acetyl-α-D-neuraminyl-(2→6)-*O*-β-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-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



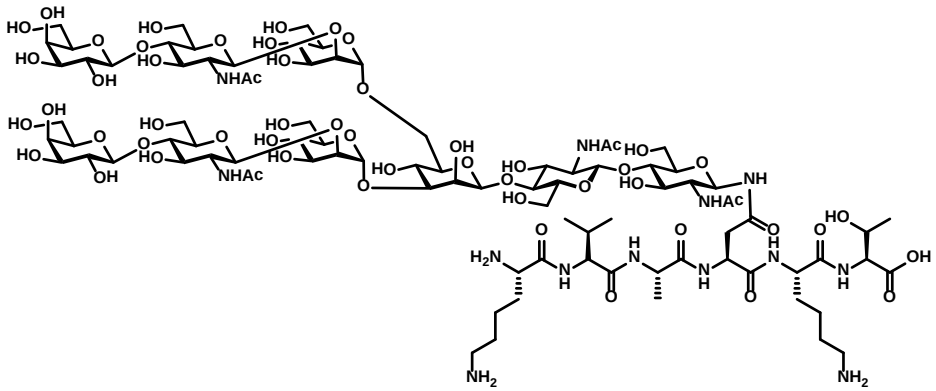
300 g egg yolk powder were suspended in 900 ml water and stirred for 16 hours. 450 ml MeOH was added. The suspension was separated into 40-ml fractions and centrifuged (4000 rpm for 10 minutes). 10 ml $CHCl_3$ were added to each fraction and the mixture was

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 $[M-H]^-$, 2865,177 $[M+H]^+$; $Mass_{obs}$: 2864.0779 $[M-H]^-$, 1431.5789$[2M-2H]^{2-}$, 2865.0669 $[M+H]^+$

Nona-gylcopeptide 21

L-lysine-L-valine-L-alanine[N^4-{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-β-D-glucopyranosyl)}]-L-asparagine-L-lysine-L-threonine

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



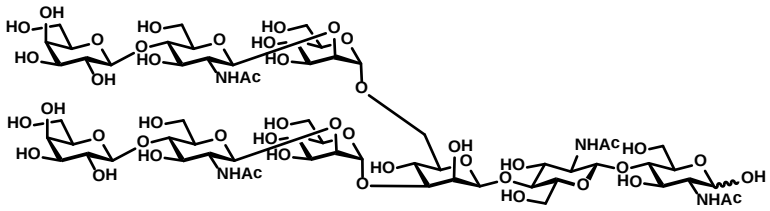
Crude **20** was dissolved in 10 mM TFA solution in water. The pH was adjusted to 3 using concentrated TFA. The solution was warmed to 80°C and monitored using MALDI-TOF. After 18 hours both sialic acids were removed and the mixture was lyophilized. Nonasaccharide containing hexapeptide **21** was purified 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. ^{1}H-NMR (400 MHz, D_2O): 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 Hz, 3H) ppm; ^{13}C-NMR (101 MHz, D_2O): δ = 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 $C_{90}H_{155}N_{13}O_{54}$: 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→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-β-D-glucopyranosyl

(Gal-GlcNAc-Man)$_2$-Man-GlcNAc-GlcNAc-OH

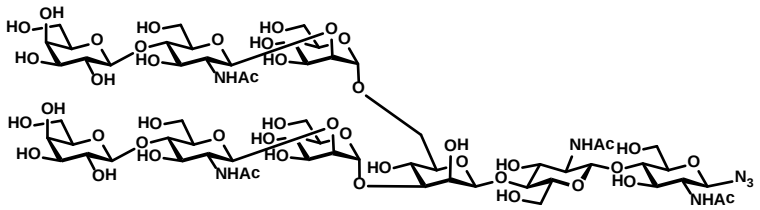


210 mg **21** were dissolved in 5 ml 100 mM ammonium acetate buffer. 100 units PNGase were added and the reaction was incubated at 37°C for 24 hours. The completeness of the reaction was confirmed using MALDI-TOF. The mixture was concentrated by freeze-drying and purified on a SiliaSep™ C18 cartridge eluting with water. The fractions were lyophilized and gave **22** in 44 % yield. ^{1}H-NMR (400 MHz, D_2O): δ = 5.04 (s, 1H), 4.98 (s, 1H), 4.78 (s, 1H), 4.44 (d, J = 8.6 Hz, 3H), 4.31 (t, J = 10.9 Hz, 3H), 4.20 (d, J = 13.7 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 Hz, 3H), 1.90 (s, 9H) ppm; ESI-MS: $Mass_{calc}$ for $C_{62}H_{104}N_4O_{46}$: 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

(Gal-GlcNAc-Man)$_2$-Man-GlcNAc-GlcNAc-N$_3$

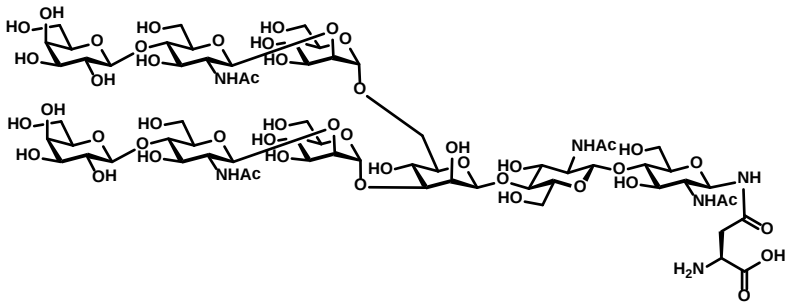


120 mg **22** were dissolved in 1.5 ml water and cooled to 2°C. 1,4 g sodium azide, 170 µl 2,6-lutidine and 124 mg DMC were added. The reaction was stirred at 2°C for one week. **13** was purified Sephadex G25 column eluting with 5% ethanol in water. The fractions were analyzed with MALDI-TOF and **13** containing fractions were lyophilized. **13** was observed in 82% yield. ^{1}H-NMR (400 MHz, D_2O): δ = 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_2O): δ = 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 $C_{62}H_{103}N_7O_{45}$: 1688.588 $[M+Na]^+$; $Mass_{obs}$: 1688.5905 $[M+Na]^+$

H-Asn-Nona 24

[N^4-{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-β-D-glucopyranosyl)}]-L-asparagine

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

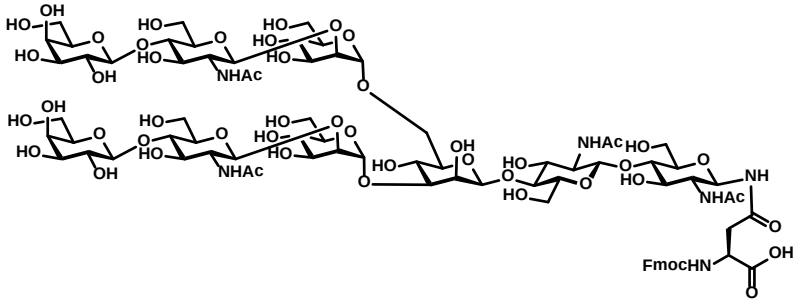


300 mg H-Asn-[(Neu5Ac-Gal-GlcNAc-Man)$_2$-Man-GlcNAc-GlcNAc]-OH **23** were dissolved in 10 mM TFA solution in water. The pH was adjusted to 3 using concentrated TFA. The solution was warmed to 80°C and monitored using MALDI-TOF. After 36 hours both sialic acids were removed and the mixture was lyophilized. **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 $C_{66}H_{110}N_6O_{48}$: 1753.627 $[M-H]^-$¸ 1752.6333 $[M-H]^-$

Fmoc-Asn-Nona 25

Fmoc-[N^4-{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-β-D-glucopyranosyl)}]-L-asparagine

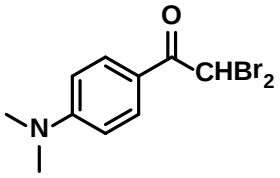
Fmoc-Asn-[(Gal-GlcNAc-Man)$_2$-Man-GlcNAc-GlcNAc]-OH



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_2O): δ = 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, 11H), 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_2O): δ = 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: $Mass_{calc}$ for $C_{81}H_{120}N_6O_{50}$: 1999,693 $[M+Na]^+$¸ 1999.6805 $[M+Na]^+$

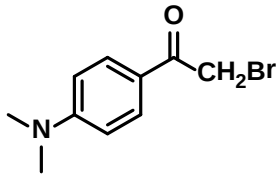
5. SYNTHESIS OF A PHOTOLABILE PROTECTED ASPARTIC ACID FOR LANSBURY ASPARTYLATION

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



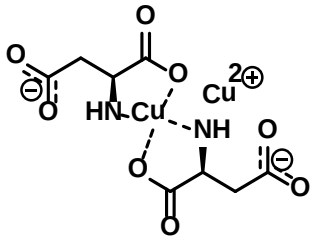
1-(4-(dimethylamino)phenyl)ethanone **26** (15 g, 92 mmol) was dissolved in 30 ml sulfuric acid and cooled to 0°C. Bromine (4.73 ml, 92 mmol) was added drop wise. The reaction mixture was stirred for 14 hours and poured on ice. The resulting green solid was separated via suction, dried and gave the desired product **27** in 90% yield (26.5 g, 82 mmol) as a green solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.98 (d, *J* = 9.1 Hz, 2H), 6.73 – 6.66 (m, 3H), 3.10 (s, 6H) ppm; ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 184.20, 154.17, 134.74, 132.20, 110.99, 40.66, 40.18 ppm; ESI-MS: $Mass_{calc}$ for $C_{10}H_{11}Br_2NO$: 319.929 $[M+H]^+$; $Mass_{obs}$: 320.030 $[M+H]^+$

2-bromo-1-(4-(dimethylamino)phenyl)ethanone 28



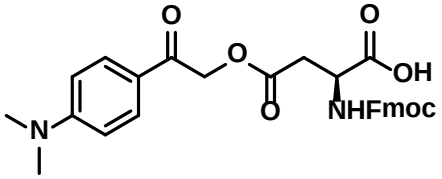
27 (24.45 g, 76 mmol) was dissolved in 200 ml THF and the mixture was cooled to 0°C. After adding diethyl phosphite (9.83 ml, 76 mmol) dropwise, TEA (15.60 ml, 76 mmol) in 50 ml THF was added. The mixture was stirred for 14 hours and poured on ice. The resulting solid was separated via suction. Purification 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. ^{1}H-NMR (400 MHz, $CDCl_3$): 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; ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 189.51, 153.92, 132.21, 131.43, 110.88, 40.18, 30.92 ppm; ESI-MS: $Mass_{calc}$ for $C_{10}H_{12}BrNO$: 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 NH_2-Asp(Map)-OH as a brown solid in 60.1% yield over three steps (5.77 g, 19.6 mmol). NH_2-Asp(Map)-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}H-NMR (400 MHz, DMSO): δ = 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}C-NMR (101 MHz, DMSO): δ = 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: $Mass_{calc}$ for $C_{29}H_{28}N_2O_7$ 539.179 $[M+Na]^+$; $Mass_{obs}$: 539.174 $[M+Na]^+$

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® 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: $Mass_{calc}$ for $C_{72}H_{96}N_{13}O_{24}$ 1511.583 $[M+H]^+$; $Mass_{obs}$: 756.6 $[2M+H]^{2+}$

Fmoc-Thr(OtBu)-Thr(OtBu)-Gly-Glu(OtBu)-Asp(OH)-Phe-Thr(psiMe,Mepro)-Glu(OtBu)-Thr(OtBu)-OH 37

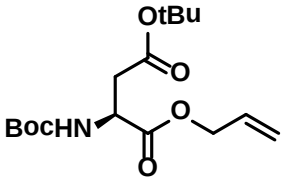
The **36** containing resin was swollen in a 1:1 mixture of ethanol and DCM. Photolysis of Map was performed in a continuous flow system using a high-pressure Hg-lamp (hν>300 nm) in flow (flow rate 1 ml/min, 18 ml loop, radiation time 18 min). A testcleavage was performed to confirm completion of the reaction. ESI-MS: $Mass_{calc}$ for $C_{62}H_{83}N_{11}O_{23}$ 1350.382 $[M+H]^+$; $Mass_{obs}$: 1350.4 $[M+H]^+$, 675.9 $[2M+H]^{2+}$

Fmoc-Thr(OtBu)-Thr(OtBu)-Gly-Glu(OtBu)-Asp(NHGlcNAc)-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: $Mass_{calc}$ for $C_{70}H_{98}N_{14}O_{26}$ 1552.613 $[M+H]^+$; $Mass_{obs}$: 1552.5 $[M+H]^+$, 777.0 $[2M+H]^{2+}$

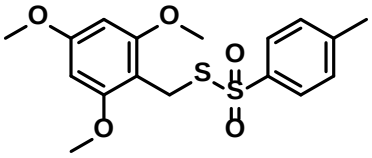
6. Synthesis of Aspartic Acid Building Blocks for Ligation-Desulfurization Strategies

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



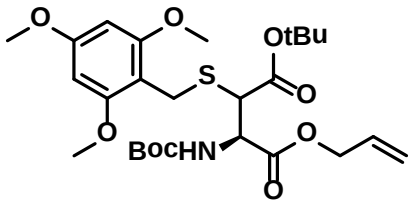
5 g (*S*)-4-(*tert*-Butoxy)-2-((*tert*-butoxycarbonyl)amino)-4-oxobutanoic acid were dissolved in 50 ml DMF and 4 ml DIPEA were added. To the mixture 2 ml allyl bromide were added and the reaction was stirred for 16 hours. The reaction was concentrated under reduced pressure, dissolved in DCM and washed 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: $Mass_{calc}$ for $C_{16}H_{27}NO_6$: 352,174 $[M+Na]^+$; $Mass_{obs}$: 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 $NaSO_4$, concentrated and purified using column chromatography to give the desired product **43** in 72% yield as yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$): 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; ^{13}C-NMR (101 MHz, $CDCl_3$): 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.

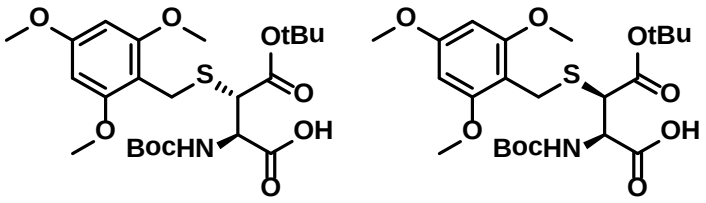
(2*R*)-1-Allyl-4-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-3-((2,4,6-trimethoxybenzyl)thio)succinate 44



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. NH_4Cl-solution. THF was removed under reduced pressure and the mixture was dissolved in ethyl acetate and washed with sat. NH_4Cl-solution. The combined organic were dried over $NaSO_4$, concentrated and purified using column chromatography to give the desired product **44** as mixture of enantiomers in 76% yield as yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$): 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; ^{13}C-NMR (101 MHz, $CDCl_3$): 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 $[M+Na]^+$

(2*R*)-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.

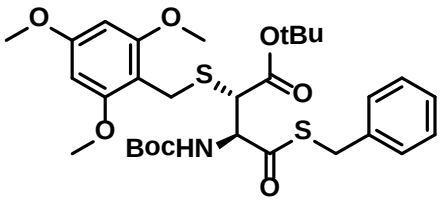
Peak 1 eluting at 21.1 minutes:

^{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 $C_{23}H_{35}NO_9S$: 524,193 $[M+Na]^+$; $Mass_{obs}$: 524.193 $[M+Na]^+$

Peak 2 eluting at 23.6 minutes:

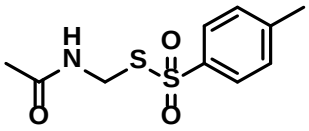
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.08 (s, 2H), 5.75 (d, *J* = 9.6 Hz, 1H), 4.72 (dd, *J* = 9.6 Hz, 4.4 Hz, 1H), 4.01 – 3.94 (m, 2H), 3.77 (s, 6H), 3.76 (s, 3H), 1.42 (s, 9H), 1.40 (s, 9H) ppm; ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 174.57, 171.46, 169.15, 160.69, 160.60, 158.94, 158.93, 158.87, 156.11, 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 $C_{23}H_{35}NO_9S$: 524,193 $[M+Na]^+$; $Mass_{obs}$: 524.193 $[M+Na]^+$

(3*S*)-*tert*-Butyl 4-(benzylthio)-3-((*tert*-butoxycarbonyl)amino)-4-oxo-2-((2,4,6-trimethoxybenzyl)thio)butanoate 45



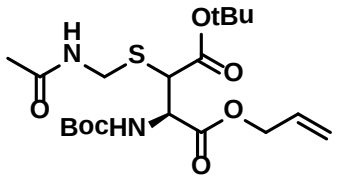
66 mg **8a** were dissolved in 2 ml anhydrous DMF and the mixture was cooled to 0°C. 137 mg PyBOP and 92 µl DIPEA were added and stirred for 15 minutes. To the mixture 31 µl benzyl mercaptan were added and the reaction was stirred for 1 hour at 0°C. The reaction was concentrated under reduced pressure and purified using column chromatography to give the desired product **45** in quantitative yield. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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 $C_{30}H_{40}NO_8S_2$: 630.217 $[M+H]^+$; $Mass_{obs}$: 630.200$[M+Na]^+$

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



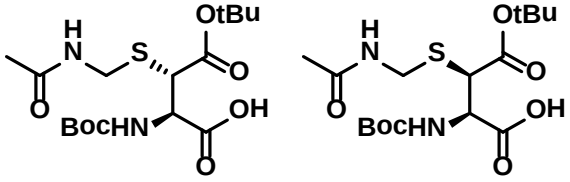
3 g *N*-(hydroxymethyl)acetamide were dissolved in 40 ml DCM and cooled to 0°C. To the mixture 6.7 ml DIPEA were added and 4.8 ml TFAA were added dropwise. The mixture was stirred for 15 minutes. 7.6 g Potassium 4-methylbenzenesulfonothioate were dissolved in 80 ml acetone and added to the reaction. 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 168.39, 144.69, 132.94, 129.11, 127.84, 109.15, 59.50, 21.86, 20.90 ppm.

(3*R*)-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. NH_4Cl-solution. THF was removed under reduced pressure and the mixture was dissolved in ethyl acetate and washed with sat. NH_4Cl-solution. The combined organic phases were dried over $NaSO_4$, 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. ^{1}H-NMR (400 MHz, Acetone-d_6): δ = 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; ^{13}C-NMR (101 MHz, Acetone-d_6): δ = 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 $C_{19}H_{32}N_2O_7S$: 455.183 $[M+Na]^+$; $Mass_{obs}$: 455.181 $[M+Na]^+$

(2*R*)-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 $Pd(PPh_3)_4$ 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.

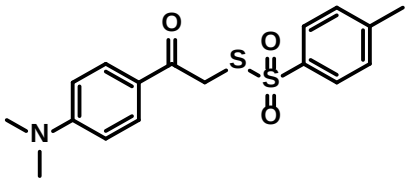
Peak 1 eluting at 30.8 minutes:

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 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 $C_{16}H_{28}N_2O_7S$: 391,154 $[M-H]^-$; $Mass_{obs}$: 391.0 $[M-H]^-$, 415,151 $[M+Na]^+$

Peak 2 eluting at 33.4 minutes:

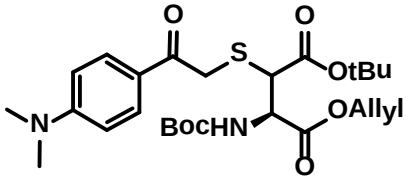
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.68 (d, *J* = 9.8 Hz, 1H), 4.74 (s, 1H), 4.62 – 4.54 (m, 1H), 4.51 – 4.40 (m, 1H), 4.02 (d, *J* = 4.6 Hz, 1H), 1.48 – 1.43 (d, 18H) ppm; ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 171.86, 170.16, 156.37, 83.81, 81.00, 54.82, 53.37, 49.90, 42.29, 28.23, 27.79, 27.72, 22.84 ppm; ESI-MS: $Mass_{calc}$ for $C_{16}H_{28}N_2O_7S$: 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, $CDCl_3$): δ = 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, $CDCl_3$): δ = 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.

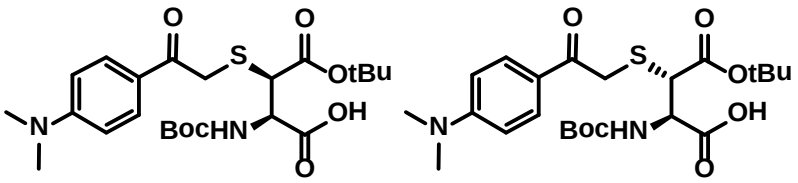
(3*R*)-4-Allyl-1-*tert*-butyl 2-((4-(dimethylamino)phenyl)thio)-3-((*tert*-butoxycarbonyl)amino) succinate 50



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. NH_4Cl-solution. THF was removed under reduced pressure and the mixture was dissolved in ethyl acetate and washed with sat. NH_4Cl-solution. The combined organic were dried over $NaSO_4$, concentrated and purified using column chromatography to give the desired product **50** as mixture of stereo isomers in 62% yield as yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$): 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; ^{13}C-NMR (101 MHz, $CDCl_3$): 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: $Mass_{calc}$ for $C_{26}H_{38}N_2O_7S$: 523,248 $[M+H]^+$; $Mass_{obs}$: 523.2 $[M+H]^+$, 546.0 $[M+Na]^+$

(3*R*)-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 $Pd(PPh_3)_4$ 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:

^{1}H-NMR (400 MHz, $CDCl_3$): 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; ^{13}C-NMR (101 MHz, $CDCl_3$): 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: $Mass_{calc}$ for $C_{23}H_{34}N_2O_7S$: 505,198 $[M+H]^+$; $Mass_{obs}$: 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 testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{98}H_{144}N_{28}O_{34}S$: 2271.001 $[M+H]^+$; $Mass_{obs}$: 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: $Mass_{calc}$ for $C_{99}H_{140}N_{28}O_{34}S$: 2296.981 $[M+H]^+$; $Mass_{obs}$: 573.2 $[4M+4H]^{4+}$, 766.9 $[3M+3H]^{3+}$, 1149.4 $[2M+2H]^{2+}$

PrP I [214-231] hydrazide 56

H-Cys-Val-Gln-Tyr-Gln-Lys-Glu-Ser-Gln-Ala-Tyr-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-$NHNH_2$

Starting from Wang ChemMatrix® hydrazide **55** the peptide was loaded according to procedure 3 (Loading of Wang hydrazide resin). The substitution grade was 0.63 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_2O (0.1% TFA) in 38 min or YMC hydrosphere RP C18 [250x10 mm; 5 µm] 5% ACN to 25% ACN in H_2O (0.1% TFA) in 25 min. The pure product **56** was obtained in 31% yield. ESI-MS: $Mass_{calc}$ for $C_{87}H_{132}N_{23}O_{26}S$: 2139.964 $[M+H]^+$; $Mass_{obs}$: 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-Tyr-Asp-Gly-Arg-Arg-Ser-Ser-$NHNH_2$

Starting from Wang ChemMatrix® hydrazide **55** the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.63 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: $Mass_{calc}$ for $C_{90}H_{137}N_{27}O_{31}S$: 2123.969 $[M+H]^+$; $Mass_{obs}$: 554.0 $[4M+4H]^{4+}$, 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 was separated via suction to give pure product **60** in 98% yield. ^{1}H-NMR (400 MHz, DMSO-d_6): 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; ^{13}C-NMR (101 MHz, DMSO-d_6): 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-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: $Mass_{calc}$ for $C_{87}H_{132}N_{23}O_{26}S$: 2139.964 $[M+H]^+$; $Mass_{obs}$: 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-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

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

a) To a mixture of **63** and **63a** in 0.2 ml water, 0.5 mg $PdCl_2$ 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 H_2O (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 $PdCl_2$ 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 H_2O (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-$NHNH_2$

Starting from Wang ChemMatrix® hydrazide **55** the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.58 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 testcleavage 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

NH_2-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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 testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{170}H_{287}N_{49}O_{57}S_4$: 4056,003 $[M+H]^+$; $Mass_{obs}$: 677.0393 $[6M+6H]^{6+}$, 812.0470 $[5M+5H]^{5+}$, 1015.0582 $[4M+4H]^{4+}$

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

NH_2-Asp(SH)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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 H_2O (0.1% TFA) in 35 min). After reduction and purification **68** was observed in 5% yield. ESI-MS: $Mass_{calc}$ for $C_{174}H_{292}N_{50}O_{60}S_5$: $[M+H]^+$; $Mass_{obs}$: 701.3738 $[6M+6H]^{6+}$, 841.4468 $[5M+5H]^{5+}$, 1051.5573 $[4M+4H]^{4+}$

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

NH_2-Asp(SH)-Cys-Val-Asn(GlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn(GlcNAc)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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 H_2O (0.1% TFA) in 35 min). After reduction and purification desulfurized product **70a** was observed. ESI-MS: $Mass_{calc}$ for $C_{190}H_{318}N_{52}O_{70}S_5$: 4608,153 $[M+H]^+$; $Mass_{obs}$(desulfurized product): 764.0804 $[6M+6H]^{6+}$, 916.6892 $[5M+5H]^{5+}$, 1145.6129 $[4M+4H]^{4+}$

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

NH_2-Asp(SH)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn(GlcNAc)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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 H_2O (0.1% TFA) in 35 min). After reduction and purification desulfurized product **71a** was observed. ESI-MS: $Mass_{calc}$ for $C_{185}H_{305}N_{51}O_{65}S_5$: 4405,073 $[M+H]^+$; $Mass_{obs}$(desulfurized product): 701.3738 $[6M+6H]^{6+}$, 841.4468 $[5M+5H]^{5+}$, 1051.5573 $[4M+4H]^{4+}$

PrP II [178Asp(SH)-213] 197GlcNAc 72

NH_2-Asp(SH)-Cys-Val-Asn(GlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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 H_2O (0.1% TFA) in 35 min). After reduction and purification desulfurized product **72a** was observed. ESI-MS: $Mass_{calc}$ for $C_{185}H_{305}N_{51}O_{65}S_5$: 4405,073 $[M+H]^+$; $Mass_{obs}$(desulfurized product): 701.3738 $[6M+6H]^{6+}$, 841.4568 $[5M+5H]^{5+}$, 1051.5573 $[4M+4H]^{4+}$

PrP III [161-177] hydrazide 73

NH_2-Val-Tyr-Tyr-Arg-Pro-Met-Asp-Glu-Tyr-Ser-Asn-Gln-Asn-Asn-Phe-Val-His-$NHNH_2$

Starting from Wang ChemMatrix® hydrazide **55** the peptide was loaded according to procedure 3 (Loading of Wang hydrazide Resin). The substitution grade was 0.63 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 H_2O (0.1% TFA) in 35 min). Product **73** was obtained in 25% yield. ESI-MS: $Mass_{calc}$ for $C_{97}H_{136}N_{28}O_{29}S$: 2189,983 $[M+H]^+$; $Mass_{obs}$: 548.5 $[4M+4H]^{4+}$, 776.3 $[3M+3H]^{3+}$, 1163.6 $[2M+2H]^{2+}$

PrP III [161-177] thioester 74

NH_2-Val-Tyr-Tyr-Arg-Pro-Met-Asp-Glu-Tyr-Ser-Asn-Gln-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

NH_2-Val-Tyr-Tyr-Arg-Pro-Met-Asp-Glu-Tyr-Ser-Asn-Gln-Asn-Asn-Phe-Val-His-Asp(SH)-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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

NH_2-Asp(SAcm)-Cys-Val-Asn(GlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

Starting from 0.012 mmol **67**, Boc-Asp(O*t*Bu;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 H_2O (0.1% TFA) in 35 min). After reduction and purification **76** was observed in 5% yield. ESI-MS: $Mass_{calc}$ for $C_{177}H_{297}N_{51}O_{61}S_5$: 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

NH_2-Asp(SAcm)-Cys-Val-Asp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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 testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{180}H_{300}N_{50}O_{62}S_5$: 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

NH_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-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

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 H_2O (0.1% TFA) in 35 min). After reduction and purification **79** was observed in 5% yield. ESI-MS: $Mass_{calc}$ for $C_{185}H_{310}N_{52}O_{66}S_5$: 4477,118 $[M+H]^+$; $Mass_{obs}$: 747.3612 $[6M+6H]^{6+}$, 896.6094 $[5M+5H]^{5+}$, 1120.4611 $[4M+4H]^{4+}$

PrP II [178Asp(SAcm)-213] 181GlcNAc thioester 80

NH_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-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: $Mass_{calc}$ for $C_{193}H_{314}N_{50}O_{68}S_5$: 4580,125 $[M+H]^+$; $Mass_{obs}$: 769.6926 $[6M+6H]^{6+}$, 923.3942 $[5M+5H]^{5+}$, 1154.2042 $[4M+4H]^{4+}$; ESI-MS: $Mass_{calc}$ for $C_{185}H_{306}N_{50}O_{66}S_5$: 4445,081 $[M+H]^+$; $Mass_{obs}$: 744.6919 $[6M+6H]^{6+}$, 893.4043 $[5M+5H]^{5+}$, 1116.4611 $[4M+4H]^{4+}$

PrPII [196-213] 83

NH_2-Glu-Asn-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

Starting from peptide **66**, the peptide was elongated procedure 7 (conditions for manual peptide elongation). A testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{92}H_{152}N_{26}O_{32}S_3$: 2230,031 $[M+H]^+$; $Mass_{obs}$: 558.2712 $[4M+4H]^{4+}$, 744.0281 $[3M+3H]^{3+}$, 1115.5304 $[2M+2H]^{2+}$

PrPII [196-213] Asp197Allyl 84

NH_2-Glu-Asp(OAllyl)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

Starting from peptide **66**, the peptide was elongated procedure 7 (conditions for manual peptide elongation). A testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{95}H_{155}N_{25}O_{33}S_3$: 2271,046 $[M+H]^+$; $Mass_{obs}$: 832.1 $[3M+3H]^{3+}$, 1247.3 $[2M+2H]^{2+}$

PrPII [196-213] Asp197Map 85

NH_2-Glu-Asp(OMap)-Phe-Thr-Glu-Thr-Asp-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

Starting from peptide **66**, the peptide was elongated procedure 7 (conditions for manual peptide elongation). A testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{102}H_{162}N_{26}O_{34}S_3$: 2392,099 $[M+H]^+$; $Mass_{obs}$: 654.5 $[4M+4H]^{4+}$, 872.5 $[3M+3H]^{3+}$, 1308.1 $[2M+2H]^{2+}$

PrPII [Fmoc179-195] 87

NHFmoc-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-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 testcleavage 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 $[2M+2H]^{2+}$

PrPII [Fmoc179-195] Asp181Allyl 88

NHFmoc-Cys-Val-Asp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-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 testcleavage 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 $[2M+2H]^{2+}$

PrPII [Fmoc179-195] 89

NHFmoc-Cys(STrt)-Val-Asn(NHTrt)-Ile-Thr(O*t*Bu)-Ile-Lys(NHBoc)-Gln(NHTrt)-His(NBoc)-Thr(O*t*Bu)-Val-Thr(O*t*Bu)-Thr(O*t*Bu)-Thr(O*t*Bu)-Thr(O*t*Bu)-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(O*t*Bu)-Ile-Lys(NHBoc)-Gln(NHTrt)-His(NBoc)-Thr(O*t*Bu)-Val-Thr(O*t*Bu)-Thr(O*t*Bu)-Thr(O*t*Bu)-Thr(O*t*Bu)-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: $Mass_{calc}$ for $C_{173}H_{250}N_{22}O_{35}S$: 3228,826 $[M+H]^+$; $Mass_{obs}$: 3121.5361 $[M(-Boc)+H]^+$

PrPII [179-201] 92

NH_2-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-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_2O (0.1% TFA) in 35 min). Product **92** was obtained in 8% yield. ESI-MS: $Mass_{calc}$ for $C_{109}H_{182}N_{32}O_{38}S$: 2579,301 $[M+H]^+$; $Mass_{obs}$: 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-Thr-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: $Mass_{calc}$ for $C_{123}H_{198}N_{34}O_{42}S_2$: 2888,392 $[M+H]^+$; $Mass_{obs}$: 578.5 $[5M+3H]^{5+}$; 723.1 $[4M+4H]^{4+}$; 963.7 $[3M+3H]^{3+}$; ESI-MS: $Mass_{calc}$ for $C_{123}H_{197}N_{33}O_{41}S_2$: 2870,382 $[M+H]^+$; $Mass_{obs}$: 575.2 $[5M+3H]^{5+}$; 718.6 $[4M+4H]^{4+}$; 957.8 $[3M+3H]^{3+}$

PrPII [179-201] l97Asp(OAllyl) and l81Asp(OAllyl) 94

NH_2-Cys-Val-AsPp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-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 testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{115}H_{188}N_{30}O_{40}S$: 2661,332 $[M+H]^+$; $Mass_{obs}$: 533.2872 $[5M+5H]^{5+}$, 666.3553 $[4M+4H]^{4+}$, 888.1390 $[3M+3H]^{3+}$

PrPII [179-201] 197Asp(OH) and l81Asp(OH) 94a

NH_2-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 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 testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{109}H_{180}N_{30}O_{40}S$: 2581,269 $[M+H]^+$; $Mass_{obs}$: 517.2756 $[5M+5H]^{5+}$, 646.3410 $[4M+4H]^{4+}$, 861.4529 $[3M+3H]^{3+}$

PrP 179-204 l97Asn(NHGlcNAc) and 181Asn(NHGlcNAc) 95

NH_2-Cys-Val-Asn(NHGlcNAc)-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 **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 H_2O (0.1% TFA) in 25 min). Product **95** was obtained in 9% yield. ESI-MS: $Mass_{calc}$ for $C_{125}H_{208}N_{34}O_{48}S$: 2985,460 $[M+H]^+$; 597.0926 $[5M+5H]^{5+}$, 747.6163 $[4M+4H]^{4+}$, 996.1514 $[3M+3H]^{3+}$

PrPII [179-201] l81Asp(OAllyl) 96

NH_2-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-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 testcleavage 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 $[2M+2H]^{2+}$

PrPII [179-201] l81Asp(OH) 96a

NH_2-Cys-Val-Asp(OH)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-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 testcleavage 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 $[3M+3H]^{3+}$

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

NH_2-Cys-Val-Asn(NHGlcNAc)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-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_2O (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 $[3M+3H]^{3+}$

PrPII [179-201] l97Asp(OAllyl) 98

NH_2-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-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 testcleavage 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] l97Asp(OH) 98a

NH_2-Cys-Val-Asn-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-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 testcleavage 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 $[4M+4H]^{4+}$, 861.1349 $[3M+3H]^{3+}$

PrPII [179-201] l97Asn(NHGlcNAc) 99

NH_2-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_2O (0.1% TFA) in 25 min). Product **99** was obtained in 5% yield. ESI-MS: $Mass_{calc}$ for $C_{117}H_{195}N_{33}O_{43}S$: 2782,381 $[M+H]^+$; $Mass_{obs}$: 557.6782 $[5M+5H]^{5+}$, 696.1077 $[4M+4H]^{4+}$, 928.1411 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] 197Asp(OAllyl) l81Asp(OAllyl) 101

NH_2-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 testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{122}H_{198}N_{32}O_{44}S_2$: 2879,368 $[M+H]^+$; 577.0433 $[5M+5H]^{5+}$, 721.0547 $[4M+4H]^{4+}$, 961.0687 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] 197Asp(OH) l81Asp(OH) 102

NH_2-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 testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{116}H_{190}N_{32}O_{44}S_2$: 2799,306 $[M+H]^+$; 561.0348 $[5M+5H]^{5+}$, 700.7952 $[4M+4H]^{4+}$, 934.0596 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] 197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 103

NH_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-$NHNH_2$

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 H_2O (0.1% TFA) in 25 min). Product **103** was obtained in 12% yield. ESI-MS: $Mass_{calc}$ for $C_{132}H_{218}N_{36}O_{52}S_2$: 3203,496 $[M+H]^+$; 641.6708 $[5M+5H]^{5+}$, 801.8330 $[4M+4H]^{4+}$, 1068.7792 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] l81Asp(OAllyl) 96a

NH_2-Asp(SAcm)-Cys-Val-Asp(OAllyl)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-$NHNH_2$

Starting from 8 µmol **96**, Boc-Asp(OtBu;SAcm)-OH **9** was coupled according to procedure 7 (conditions for manual peptide elongation). A testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{119}H_{195}N_{33}O_{43}S_2$: 2838,353 $[M+H]^+$; 568.6403$[5M+5H]^{5+}$, 710.5454 $[4M+4H]^{4+}$, 947.0598 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] l81Asp(OH) 96b

NH_2-Asp(SAcm)-Cys-Val-Asp(OH)-Ile-Thr-Ile-Lys-Gln-His-Thr-Val-Thr-Thr-Thr-Thr-Lys-Gly-Glu-Asn-Phe-Thr-$NHNH_2$

Starting from 8 µmol **96a**, the allyl ester was cleaved according to procedure 8 (removal of Allyl). A testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{116}H_{191}N_{33}O_{43}S_2$: 2798,321 $[M+H]^+$; 560.6376 $[5M+5H]^{5+}$, 700.5515 $[4M+4H]^{4+}$, 933.7285 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] l81Asn(NHGlcNAc) 106

NH_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-$NHNH_2$

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 H_2O (0.1% TFA) in 25 min). Product **106** was obtained in 9% yield. ESI-MS:

$Mass_{calc}$ 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 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] l97Asp(OAllyl) 98a

NH_2-Asp(SAcm)-Cys-Val-Asn-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 **98**, Boc-Asp(OtBu;SAcm)-OH **9** was coupled according to procedure 7 (conditions for manual peptide elongation). A testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{119}H_{195}N_{33}O_{43}S_2$: 2838,353 $[M+H]^+$; $Mass_{obs}$: 568.8401 $[5M+5H]^{5+}$, 710.5454 $[4M+4H]^{4+}$, 947.0598 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] l97Asp(OH) 98b

NH_2-Asp(SAcm)-Cys-Val-Asn-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 **98a**, the allyl ester was cleaved according to procedure 8 (removal of Allyl). A testcleavage was performed using reagent B. ESI-MS: $Mass_{calc}$ for $C_{116}H_{191}N_{33}O_{43}S_2$: 2798,321 $[M+H]^+$; $Mass_{obs}$: 560.6376 $[5M+5H]^{5+}$, 700.5461 $[4M+4H]^{4+}$, 933.7285 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] l97Asn(NHGlcNAc) 107

NH_2-Asp(SAcm)-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 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_2O (0.1% TFA) in 25 min). Product **107** was obtained in 8% yield. ESI-MS: $Mass_{calc}$ 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 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] 197Asn(NHGlcNAc) 181Asn(NHGlcNAc) thioester 108

NH_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-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: $Mass_{calc}$ for

$C_{140}H_{222}N_{34}O_{54}S_3$: 3340,491 $[M+H]^+$; 678.8711 $[5M+5H]^{5+}$, 836.0992 $[4M+4H]^{4+}$, 1114.8026 $[3M+3H]^{3+}$; ESI-MS: $Mass_{calc}$ for $C_{132}H_{214}N_{34}O_{52}S_2$: 3171,459 $[M+H]^+$; 643.0701 $[5M+5H]^{5+}$, 794.3464 $[4M+4H]^{4+}$, 1058.4524 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] 181Asn(NHGlcNAc) thioester 110

NH_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

1.5 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: $Mass_{calc}$ for $C_{132}H_{209}N_{33}O_{49}S_3$: 3137,412 $[M+H]^+$; 636.5219 $[5M+5H]^{5+}$, 785.0719 $[4M+4H]^{4+}$, 1046.4288 $[3M+3H]^{3+}$; ESI-MS: $Mass_{calc}$ for $C_{124}H_{200}N_{33}O_{47}S_2$: 2968,379 $[M+H]^+$; 602.5219 $[5M+5H]^{5+}$, 743.1570 $[4M+4H]^{4+}$, 990.5410 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-201] 197Asn(NHGlcNAc) thioester 112

NH_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: $Mass_{calc}$ for $C_{132}H_{209}N_{33}O_{49}S_3$: 3137,412 $[M+H]^+$; 636.0476 $[5M+5H]^{5+}$, 785.3240$[4M+4H]^{4+}$, 1047.0970 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-213] 202Asp(SH)197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 113

NH_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-$NHNH_2$

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: $Mass_{calc}$ for $C_{193}H_{323}N_{53}O_{71}S_6$: 4712,170 $[M+H]^+$; 786.5 $[6M+6H]^{6+}$, 943.4 $[5M+5H]^{5+}$, 1179.4 $[4M+4H]^{4+}$, 1571.2 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-213] 202Asp(SH)197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 114

NH_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-Glu-Thr-Asp(SH)-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

1.1 mg **110/111** 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: $Mass_{calc}$ for $C_{193}H_{316}N_{52}O_{68}S_7$: 4675.099 $[M+H]^+$; 780.2 $[6M+6H]^{6+}$, 936.1 $[5M+5H]^{5+}$, 1170.1 $[4M+4H]^{4+}$, 1559.6 $[3M+3H]^{3+}$

PrPII [179Asp(SAcm)-213] 202Asp(SH)197Asn(NHGlcNAc) 181Asn(NHGlcNAc) 114

NH_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-Glu-Thr-Asp(SH)-Val-Lys-Met-Met-Glu-Arg-Val-Val-Glu-Gln-Met-$NHNH_2$

0.8 mg **112**and 0.4 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: $Mass_{calc}$ for $C_{193}H_{316}N_{52}O_{68}S_7$: 4675.099 $[M+H]^+$; 780.3 $[6M+6H]^{6+}$, 936.2 $[5M+5H]^{5+}$, 1170.0 $[4M+4H]^{4+}$, 1559.5 $[3M+3H]^{3+}$

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