# 7. Summary

In the course of this work, three series of glycopeptides were synthesised. These three series were analogous to the MUC1 repeating unit, which consists of twenty amino acids.

In the first section, a series of MUC1 glycopeptides was synthesised, based on new results regarding the amino acid sequence of the repeating unit.

In the second section of this work, glycopeptides with N-glycosylation sites next to O-glycosylation sites were made. For examination of the synthesis, the sequence of the MUC1 repeating unit was chosen as a model.

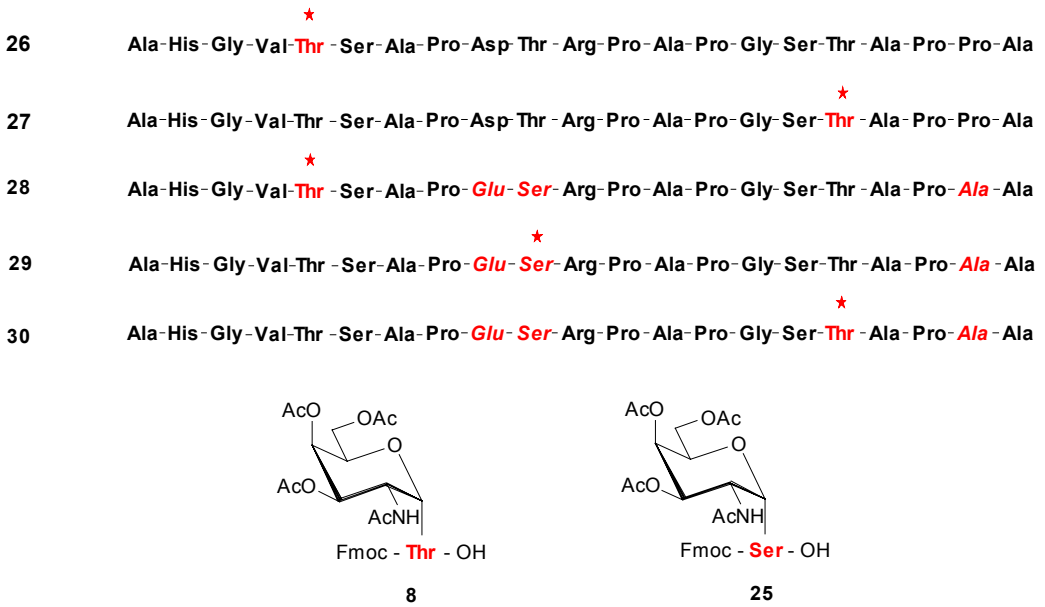
In the third section of this work, glycopeptides bearing the sTF-antigen were synthesised chemoenzymatically for the first time, using the catalysis of the 2,3-sialyltransferase.

The syntheses of the glycopeptides were performed manually, either in a multiple 20-hole peptide synthesiser or on glass columns, the outlets of which were connected via a vacuum manifold. Employment of the glycosyl amino acid building blocks **8**, **25**, **14** and **24** allowed specific introduction of a monosaccharide unit (**8**, **25**) or a disaccharide unit (**14**, **24**) to the growing peptide chain.

All of the glycopeptides synthesised here are intended to be tested in a T-cell stimulation assay, and were presented to the laboratories of Prof. Dr. Joyce Taylor-Papadimitriou (London) and Prof. Dr. Marianna Nuti (Rome) for execution of these tests. The parallel investigations, which had already begun, were to be continued. In addition, Prof. Dr. Hans-Georg Hanisch (Cologne) will also investigate the behaviour of the glycopeptides in the MHC complex.

The following is a summary of the results of the individual sections:

1) The amino acid sequence of the MUC1 repeating unit in cancer cells is subject to polymorphism, whereas the MUC1 amino acid sequence of healthy cells is not. In the cancerous MUC1 repeating units, the amino acids $Asp^{8}Thr^{9}$ are exchanged for $Glu^{8}Ser^{9}$, and $Pro^{19}$ is replaced with $Ala^{19}$, $Thr^{19}$ or $Gln^{19}$ (Hanisch 2000). Bearing this in mind, the glycopeptides **26–30** were synthesised in a manual 20-hole peptide synthesiser. The glycosylation sites were introduced using monosaccharide building blocks **8** and **25**. After the successful synthesis of compounds **26–30**, the MUC1 glycopeptides **36–45**, modified with the disaccharide building blocks **14** and **24**, were produced. The proton shifts of the glycopeptides **26–30** and **36–45** could be assigned, using $^{1}H^{1}H$-TOCSY and $^{1}H^{1}H$-NOESY NMR spectra. The sequences determined using NMR spectroscopy corresponded with the expected glycopeptide sequences.







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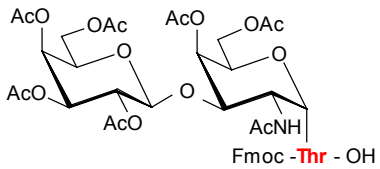


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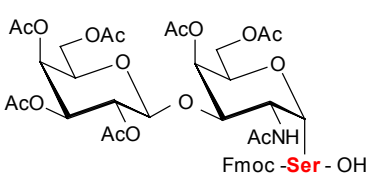


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**Figure 7.1.:** Glycopeptides **26–30** with the Tn monosaccharide building block **8** and **25**, and glycopeptides **36–45** with the TF disaccharide building block **14** and **24**.

Glycopeptides **36–45** were tested using a T-cell stimulation assay in the laboratories of Professor Joyce Taylor-Papadimitriou. In this assay, glycopeptide **38**, which has a glycosylation site at $Ser^5$, resulted in stimulation which in a further test proved relatively reproducible. Based on these results, glycopeptides **46–54** were synthesised for further investigations. All glycopeptides **46–54** are glycosylated at $Ser^5$; individual amino acids were exchanged.

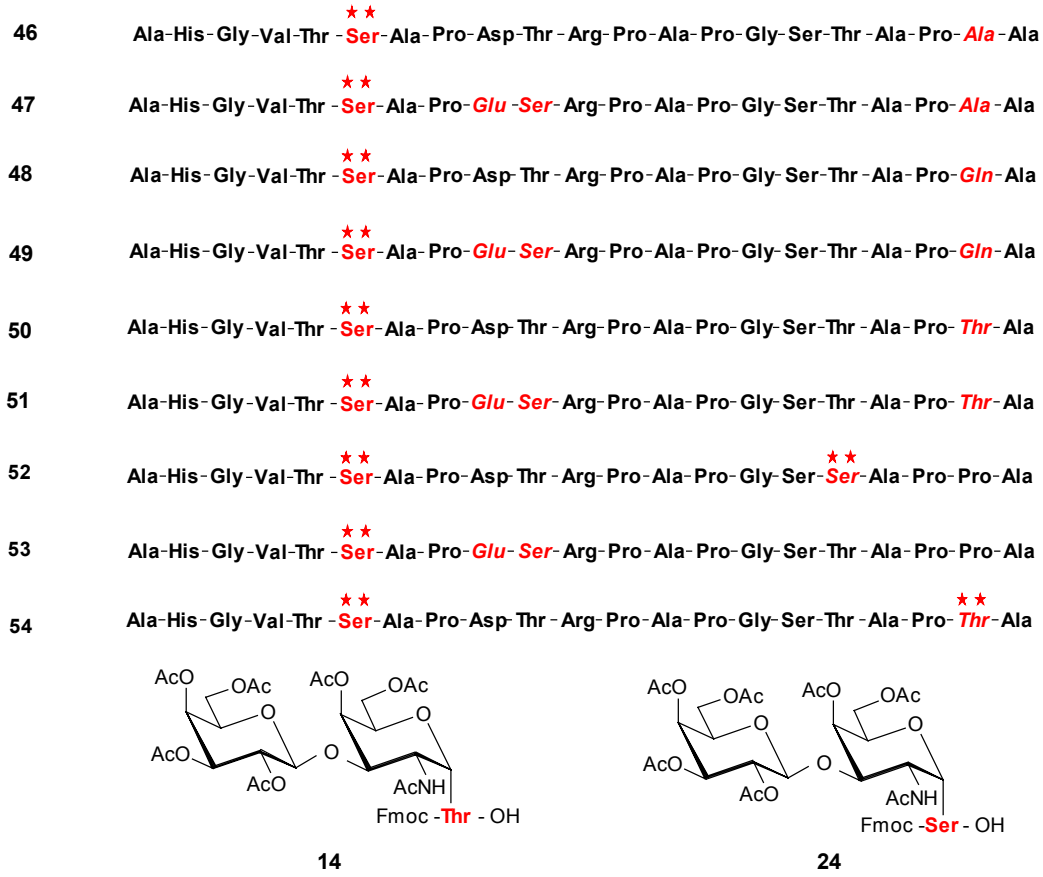


**Figure 7.2.:** Glycopeptides **46–54**

2) Investigations of a protein data bank (Swiss Prot) showed that exchange of Thr for Asn in the DTR motif at the end of the tandem repeating domain in naturally occurring MUC1 glycoproteins is possible. In addition, one finds N-glycosylation sites in MUC1 glycoproteins, between the repeating unit and the transmembrane domain. The synthesis of mixed glycopeptides, which have a peptide chain containing both an N-glycosyl and an O-glycosyl carbohydrate side chain, is therefore interesting. In the course of this work, such compounds (**63–67**), in which the peptide sequence is derived from the MUC1 structure, were made for the first time.

For the syntheses, a new glycosyl amino acid building block was necessary, in order to introduce a corresponding N-glycosidic carbohydrate side chain during the construction of the glycopeptide. The glycosyl amino acid consisting of asparagine and chitobiose was chosen as building block. The research group of Prof. Dr. Meyer already were experienced in the synthesis of this compound.

While the coupling of the O-building blocks could be mostly conducted without any problems, the coupling of the chitobiosyl building block was accompanied by the formation of aspartamide as a side reaction.

The assignation of the proton shifts of glycopeptides **63–67**, with sugar moieties at various glycosylation sites, could be made using the $^{1}H^{1}H$-TOCSY and $^{1}H^{1}H$-NOESY NMR spectra. The proton shifts of the carbohydrate protons of the various sugar residues could be assigned using the $^{1}H^{1}H$-TOCSY spectra.

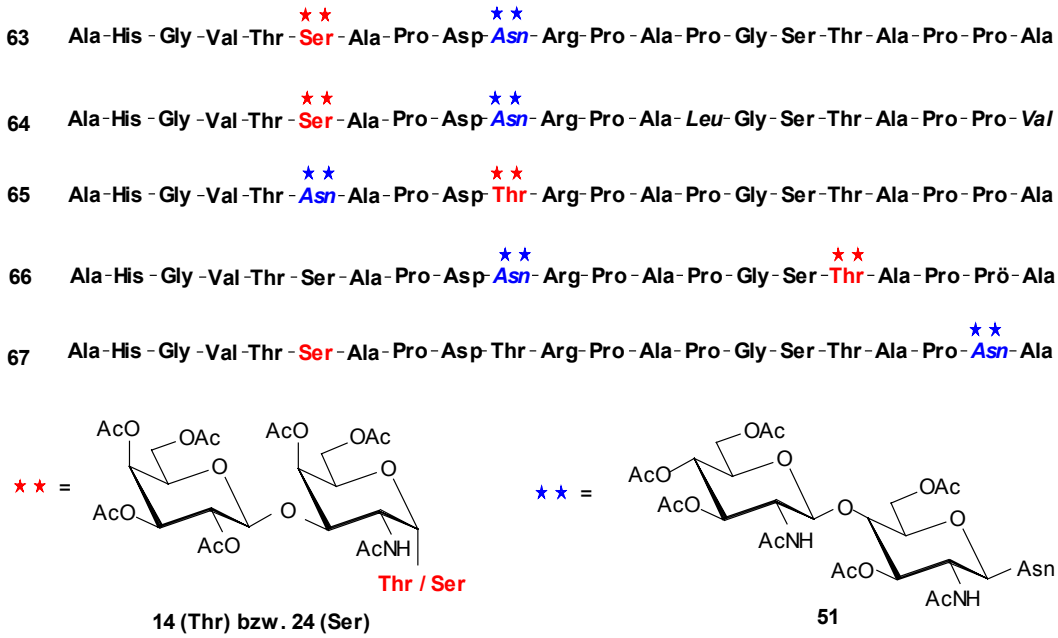


**Figure 7.3.:** Glycopeptides **63–67** with N-glycosyl and O-glycosyl-linked carbohydrate side chains

3) A cancer-associated reduction in expression of a glucosylaminoacidtransferase in carcinoma cell lines leads to termination of the biosynthesis of the sugar side chains of MUC1, so that the Tn monosaccharide and the TF monosaccharide appear in the side chain of the MUC1 glycoprotein preferentially. These shortened sugar chains in turn serve as substrate for specific sialyltransferases and this results in the formation of sialylated structures.

The purely chemical synthesis of a sialylglycosyl amino acid building block for a corresponding glycopeptide synthesis is only possible via many reaction steps. The great sensitivity and the negative charge of the 5-N-acetylneuraminic acid make a suitable protective group combination necessary so that such a building block can be used for a glycopeptide synthesis. A better alternative to organochemical synthesis is the enzymatic transfer of the sialyl group, using CMP-NeuAc under catalysis of a sialyltransferase, onto a complete glycopeptide, which has already been synthesised. In intial investigations, Kihlberg was able to sialylate a synthetic MUC1 glycopeptide using a 2,3-sialyltransferase (Kihlberg 2001). Multiply glycosylated synthetic peptides have to date not yet been enzymatically sialylated.

During this work, Neu5Ac was transfered from CMP-Neu5Ac to a series of multiply glycosylated synthetic peptides bearing the TF antigen, under catalysis of 2,3-sialyltransferase. Thus, compounds **76–83** could be achieved.


**Figure 7.4.:** Enzymatically sialylated glycopeptides **76–83**

The 2,3-sialyltransferase necessary for this was expressed in HI5 insect cells by the research group of Professor Henrik Clausen (Copenhagen), purified by treatment with Amberlite IRA-95 and S-Sephadex, and concentrated by centrifugal filtration. The required glycopeptide substrate was produced in a parallel solid phase synthesis on glass columns, and subsequently sialylated with the 2,3-sialyltransferase. The reaction times of the enzymatic sialylation of the glycopeptides with up to five glycosyl chains were always of a similar magnitude. Retardation of the reaction by the glycosyl side chains already present was not observed. In the case of the multiply sialylated compounds, a correspondingly larger amount of 2,3-sialytransferase and CMP-Neu5Ac was required. An almost quantitative conversion could then always be observed.

In the MALDI-TOF mass spectrometric investigation of the sialylated products **76**–**83**, fragmentation products arose, which resulted from the stepwise cleavage of one or more sialic acid units. In the ESI mass spectra measured as a result of this, uniform molecular peaks could be obtained. Analysis of the samples using reversed phase HPLC on a C-18 column also indicate one uniform product. Upon closer examination of the $^{1}H^{1}H$-TOCSY spectra, however, several of the samples exhibit a weak, second carbohydrate trace. Thus, it is possible that these trace substances, present in small amounts, may be incompletely sialylated side products, which could not be fully separated from the products with the available purification methods.