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Synthetic Glycopeptides for the Development of Tumour-Selective Vaccines

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Abstract: Based on structural information reported for the tumour-associated epithelial mucin MUC1, glycopeptides have been synthesized which contain tumour-associated saccharide antigens, such as the Thomsen-Friedenreich (T), T_N or sialyl T_N antigen, in combination with peptide sequences of the tandem repeat region of MUC1. Solid-phase syntheses have been carried out using *N*-Fmoc protected *O*-glycosyl serine and threonine building blocks and an allylic anchor which is stable to basic and acidic conditions, but can be cleaved under neutral conditions in a palladium(0)-catalysed allyl transfer reaction. In addition, a (2–3)sialyl T antigen threonine building block was prepared by a chemoenzymatic strategy and used in the synthesis of an *N*-terminal glycopeptide antigen of leukosialin (CD43). The proliferation of cytotoxic T cells could be induced using a construct consisting of a MUC1-glycopeptide antigen and a T cell epitope Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: glycopeptides; tumour associated antigens; sialyl Tn-antigen; sialyl T-antigen; MUC1 mucin; solid-phase synthesis; allylic anchor (HYCRON); synthetic antitumour-vaccines

In the year 1902 when Emil Fischer published his first synthesis of a free peptide [1], he was awarded the Nobel prize for his work on purines [2] and carbohydrates [3]. This coincidence reminds us that Emil Fischer was not only the pioneer of peptide chemistry [4] but was the founder of the preparative chemistry of other important classes of primary natural products [5,6]. In his time, analytical methods did not allow the recognition of covalent conjugates of proteins with carbohydrates. Therefore, separate research schools stemmed from Emil Fischer, one of peptide chemistry including people such as Max Bergmann, Emil Abderhalden and Hans Leuchs, and the other concerned with carbohydrate chemistry with people such as Otto Warburg, Carl Freudenberg, Geza Zemplén and Burkhard Helferich. For many years peptides and proteins on the one hand, and carbohydrates on the other, were dealt with as separate classes of natural products in research as well as in text books. Improved separation techniques and analytical methods, however, revealed that most proteins found in mammalians are glycoproteins [7]. The question whether the carbohydrate portions of glycoproteins play particular roles in the processes of biological selectivity was discussed for many years. This was answered by the Austrian physician Karl Landsteiner, a former postdoctoral fellow of Emil Fischer, who discovered the blood group substances in 1901 [8]. These are glycolipids and glycoproteins not only exposed on the surface of erythrocytes, but also on the mucosa and on epithelial cells. They constitute impressive examples of biologically selective carbohydrate structures.

If the glycoproteins of blood groups A and B are compared, it becomes clear that the major portions of these macromolecules, i.e. the whole protein and the main part of the oligosaccharide side chains, are identical in these opposing blood groups.

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Even the so-called tetrasaccharide determinants (Figure 1) are almost identical except for the functional group X in the 2-position of the terminal galactose.

If one considers the consequences of transferring blood from one person to another dependent on the correct group in this position it becomes clear how precise biological selectivity can be written into carbohydrate structures.

This not only holds true for blood group substances, but also for numerous other glycoproteins, in particular, those exposed on the outer cell membranes. Glycoproteins of cell membranes often are ligands involved in the processes of intercellular communication and the organized cooperation between cells in multicellular organisms including cell adhesion, cell differentiation and the regulation of cell growth [9].

About 25 years ago, investigations of cell biology and biochemistry showed that normal cells and tumour cells are quite different in the glycoprotein profile of their outer-cell membranes. Glycoproteins altered in composition and structure in tumour cells in comparison with those of normal cells were described as tumour-associated antigens [10,11]. For example, G. F. Springer and coworkers reported that glycoproteins from epithelial tumour cells with Thomsen-Friedenreich antigen (T antigen) structure **1** are tumour-associated antigens (Scheme 1) [12].

From the cross-reactivity of monoclonal antibodies induced by these compounds Springer *et al.* concluded that these T-antigen glycoproteins are structurally closely related to asialoglycophorin [13].

Glycophorin is the major transmembrane glycoprotein of erythrocytes [14]. On its *N*-terminal portion it carries a number of cryptic T-antigen side chains **2** substituted at O-6 and O-3′ with α-glycosidically bound sialic acid residues (Scheme 2).

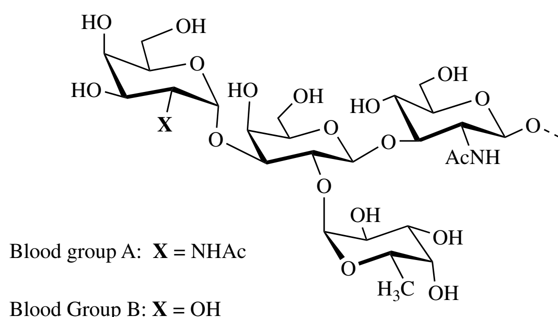
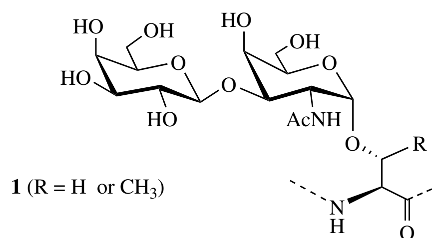


Figure 1 Blood group determinants of blood groups A and B (H form).



Scheme 1

Glycophorin A exists in two blood group specificities identical in terms of their carbohydrate side chains, but different in the 131 amino acid glycoprotein by two amino acid residues. One of these differences concerns the *N*-terminal amino acid, which is serine in (asialo)glycophorin A of M blood group specificity **2a**, but leucine in that of N blood group specificity **2b**.

In the course of a collaboration with tumour-immunologists, the *N*-terminal glycotripeptide **3** of M blood group asialoglycophorin A [15] was synthesized using Fmoc- and 2-(4-pyridyl)ethoxycarbonyl-(Pyoc)-protecting groups in combination with benzyl ester protection. The deprotected *N*-acetylated glycotripeptide **3** of exactly specified structure carrying two tumour-associated T-antigen side chains was coupled to bovine serum albumin by means of a water-soluble carbodiimide in combination with 1-hydroxy-benzotriazole in water at 4 °C and pH 6 to give the T antigen glycopeptide BSA conjugate **4** as a synthetic vaccine (Scheme 3) [15,16].

According to photometric analysis an average of about 30 glycopeptide molecules **3** were coupled per molecule of BSA. This neoglycoprotein antigen [4] was used for the immunization of mice. The monoclonal antibody 82-A6 obtained from this antigen **4** showed affinity to all the epithelial tumour cells investigated. However, control experiments revealed that the antibody also exhibited some affinity to the normal cells of these tissues. It was concluded from these experiments that the T antigen glycopeptide is a tumour-associated, but not a sufficiently tumour-selective, antigen. Obviously, additional structural information is required in order to form a tumour-specific T antigen glycopeptide. Investigations of the specificity of the antibody 82-A6 in greater detail using modified forms of glycophorin A suggested that the required additional information is located in the peptide portion of these glycopeptide antigens.

In a direct ELISA the binding of murine anti T antibody 82-A6 to glycophorins and asialoglycophorins

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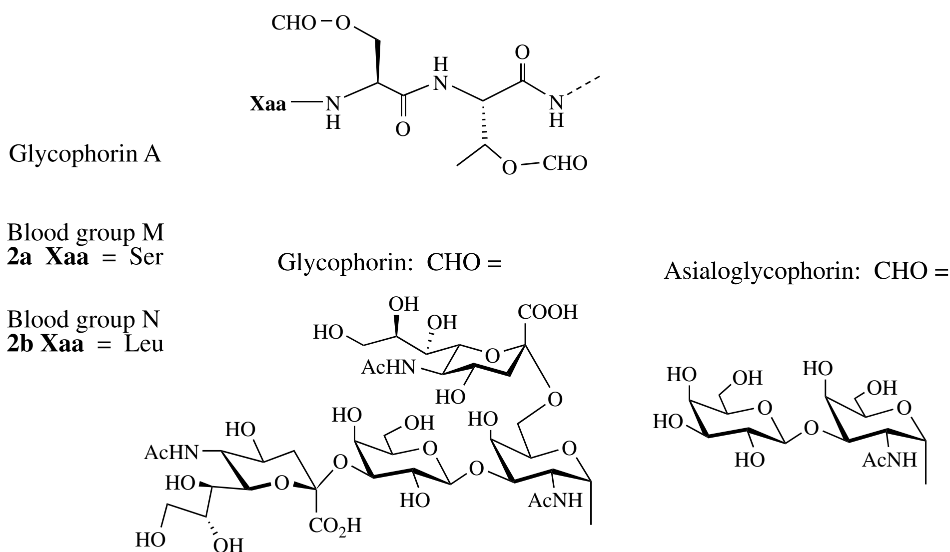
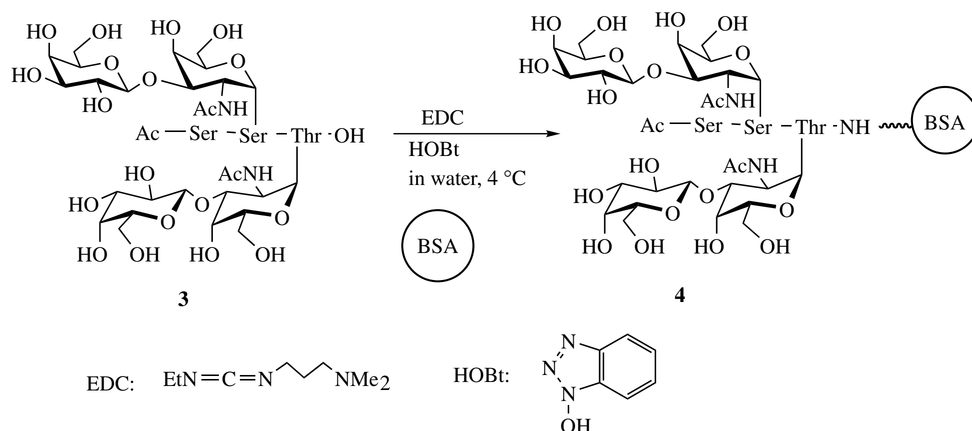


Scheme 2



Scheme 3

was determined using a β-galactosidase-bound anti-mouse antibody which cleaves 4-methylumbelliferyl-β-galactoside. Binding was shown by the fluorescence (450 nm) of the liberated umbelliferone [17].

The antibody 82-A6 does not bind to glycophorins of M and N blood group specificity in which the T antigen disaccharide is covered by the sialic acid residues (Scheme 2). After enzymatic removal of these sialic acids, the obtained asialoglycophorins were strongly recognized by antibody 82-A6. These results demonstrate the specificity of this antibody to the T antigen saccharide (Figure 2).

However, the binding to asialoglycophorin of M blood group specificity corresponding to the inducing glycopeptide antigen **2a** (or **3** and **4**) proved to be distinctly stronger than that to asialoglycophorin of N blood group specificity having a different *N*-terminal amino acid. This difference suggests that not only the T antigen saccharide, but also the amino acid sequence, contribute to the epitope recognized by the antibody. As a consequence, for the construction of a sufficiently tumour-selective glycoprotein antigen, a tumour-associated saccharide antigen like T antigen should be combined with a tumour-selective peptide structure.

In this context, interesting investigations of tumour-associated mucins, in particular, the polymorphic tumour-associated epithelial mucin MUC1, were published during the past decade [19]. MUC1 is a heavily glycosylated membrane glycoprotein containing a domain of numerous tandem repeats

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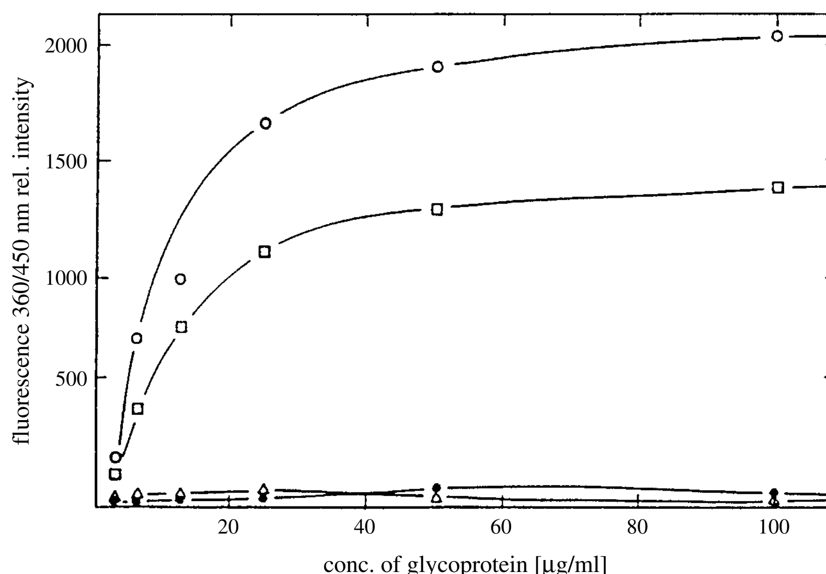
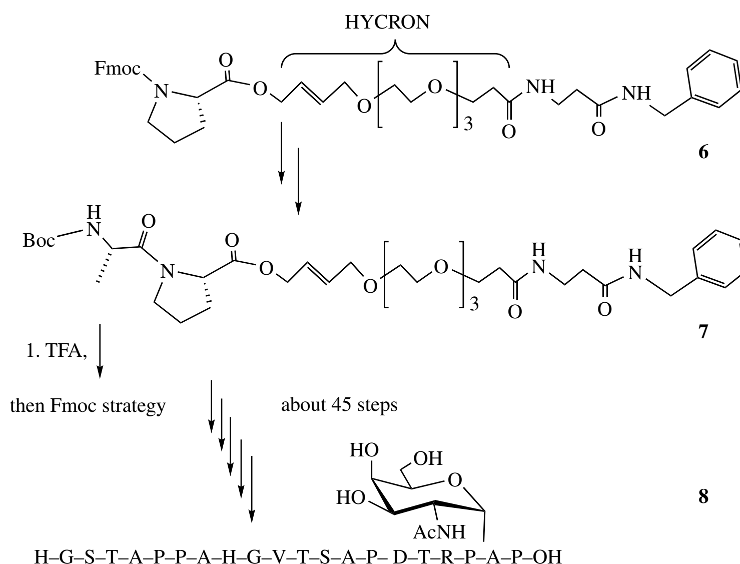


Figure 2 Binding of monoclonal antibody 82-A6 induced with **4** to glycophorin of M (▽) and N (●) and to asialoglycophorin of M (○) and N (□) blood group specificity [17,18].

of the 20 amino acid sequence **5** in its extracellular part.

TAPPAHGVTSAPDTRPAPGS **5**

Most of the O-glycosylation sites are located within the tandem repeat region. The oligosaccharide side chains of MUC1 on normal epithelial cells are large, extended via glucosamine transfer at O-6 of the GalNAc of the T antigen. In contrast, incomplete saccharide side chains have been found in MUC1 on epithelial tumour cells [20]. We conclude that due to these short saccharide side chains, peptide epitopes residing in the tandem repeat region of the protein backbone become accessible to the immune system only in the tumour-associated form of MUC1. Since these epitopes should be masked by the large saccharides of MUC 1 on normal cells, peptide sequences of the tandem repeat region **5** of MUC1 should provide tumour-selective peptide information.



Scheme 4

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Based on this conclusion, we synthesized glycopeptide partial sequences of the tandem repeat of MUC1 with tumour-associated Tn-antigen [21,22], T-antigen [21,23] and sialyl Tn- [24] antigen saccharide side-chains.

As a tool in these solid-phase syntheses the allylic HYCRON anchor [22] was applied (Scheme 4). It is stable to the acids and bases usually applied in peptide synthesis. Thus, Fmoc as well as Boc strategy can be combined with the allylic anchor [22,25]. Morpholine is the preferred base for Fmoc removal from HYCRON anchored peptides.

In addition, the HYCRON anchor is sensitive to diketopiperazine formation on the stage of the resin-linked dipeptide. Therefore, a switch from Fmoc to Boc strategy is recommended for the coupling of the second amino acid as was carried out in the synthesis of tandem repeat glycopeptide **7** containing a Tn-antigen monosaccharide side chain starting from Fmoc-proline linked via the HYCRON anchor to aminomethyl polystyrene **6** [26]. Cleavage of the allylic anchor was achieved under practically neutral conditions by palladium(0)-catalysed allyl-transfer to weakly basic or neutral allyl-trapping nucleophiles such as morpholine, N-methylaniline, dimedone or other N-, C- or S-nucleophiles. Removal of all protecting groups furnished the tandem repeat glycopeptide **8**.

This efficient method was also applied to the synthesis of sialyl Tn antigen glycopeptides with the partial sequence of MUC1. The required sialyl Tn antigen building block was synthesized according to a convergent strategy (Scheme 5). The protected Tn antigen threonine conjugate **9** carrying the Fmoc/OtBu protecting group combination was subjected to a selective removal of the O-acetyl groups by mild transesterification with catalytic sodium methanolate in methanol to give **10** [27,28].

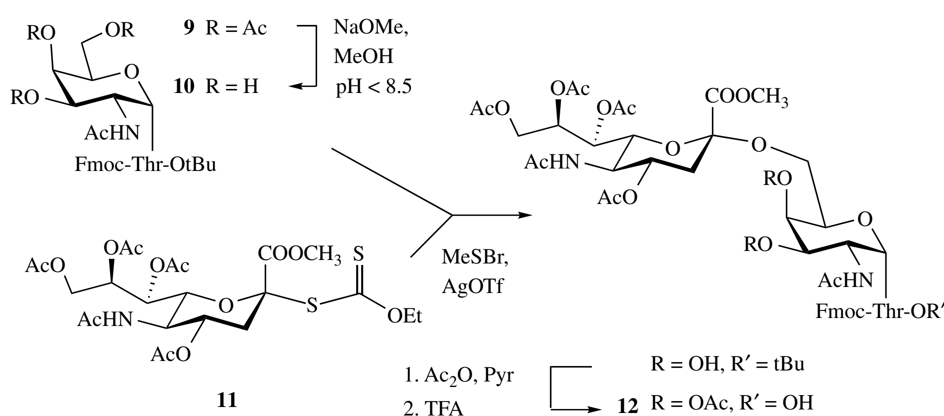
Under these conditions, the base-sensitive Fmoc group remained unaffected. The Tn antigen threonine derivative **10** deblocked in its carbohydrate portion was regioselectively sialylated using the xanthate [29] **11** of the protected sialic acid methyl ester activated with methyl sulfenyl trifluoromethanesulfonate [30] at low temperature in acetonitrile/dichloromethane. After purification the desired sialyl Tn threonine conjugate was O-acetylated in the 3,4-position. Subsequent acidolysis of the tert-butyl ester gave the required Fmoc sialyl-Tn antigen threonine building block **12**.

Solid-phase synthesis of sialyl Tn glycopeptides was performed starting from allylic anchored *C*-terminal amino acids like **6** and applying TBTU [31] as the condensing reagent.

Palladium(0)-catalysed cleavage of the allylic anchor gave, for example, the protected sialyl-Tn-glycoundecapeptide **13** from which the Trt and OtBu groups were smoothly removed by acidolysis to give **14** in an overall yield of 42% [28].

A difficult problem was the removal of the stable methyl ester from the carbohydrate portion of the glycopeptide (Scheme 7). While the O-acetyl groups are readily removed from **14** at pH 10 in aqueous methanol, the methyl ester remained untouched. Its hydrolysis required optimized conditions at pH 11.5 in water and was then achieved without β-elimination of the carbohydrate or epimerization within the peptide backbone to yield the sialyl Tn MUC1 glycopeptide **15** in analytically pure form [27,28].

Of about 20 glycopeptides from MUC1 carrying tumour-associated Tn-, T- or sialyl Tn antigen



Scheme 5

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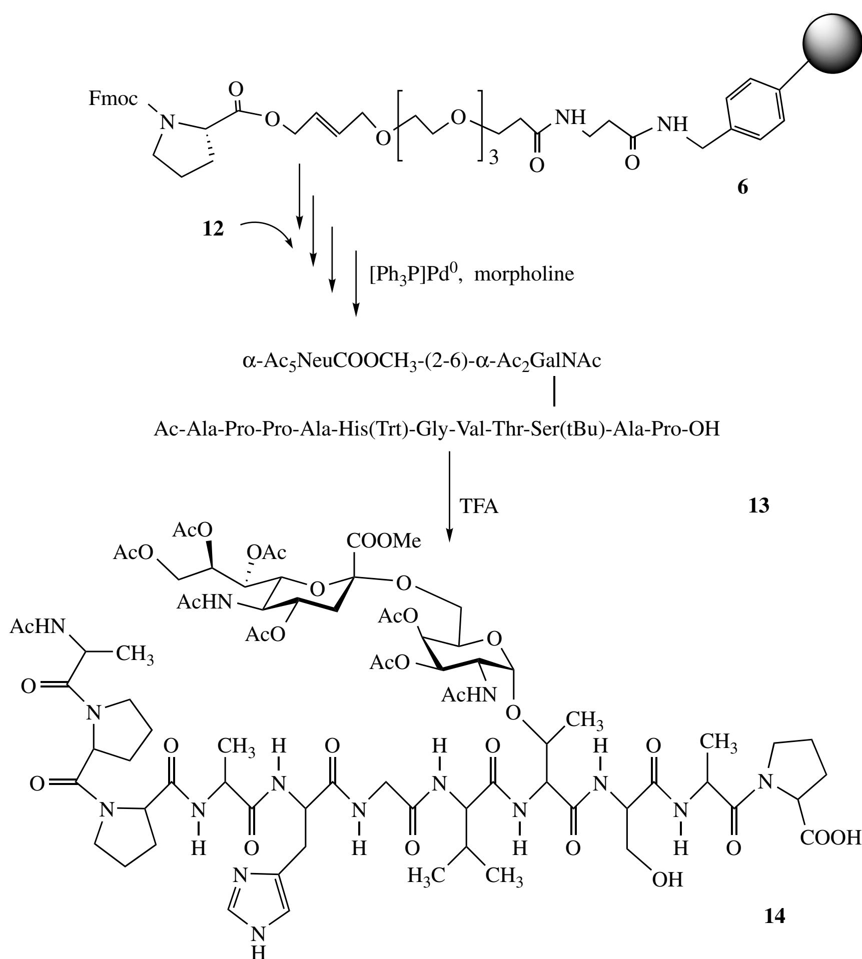

Scheme 6

saccharides only one proved to stimulate lymphocyte proliferation. Because this may not be a result of the desired selectivity, but could indicate a low antigenicity of these 'natural' glycopeptides, an alternative concept was developed. It consisted of the combination of the tumour-associated MUC1 glycopeptides with a known T cell epitope, e.g. from tetanus toxin (Figure 3) [32].

The two portions were conjugated in construct **16** using a flexible oligoethylene glycol spacer, and the peptide motif PDTRPAP was incorporated as the immunodominant peptide domain [33,34].

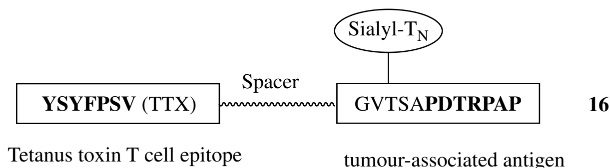

Figure 3 Potential synthetic vaccine consisting of a tumour-associated antigen and a portion of a T cell epitope.

The construction of the conjugate **16** was performed according to a convergent strategy including a condensation of two large fragments, one of them linked to the solid-phase [32]. On the one hand, the sialyl Tn glycododecapeptide sequence **18** of the MUC1 tandem repeat was assembled on solid-phase starting from the allylic HYCRON anchored proline **6** and using a sialyl Tn threonine building block **17** protected as a benzyl ester in its sialic acid portion (Scheme 8).

Parallel to this, conjugate **19** of the spacer amino acid with a tetanus toxin heptapeptide epitope was also assembled via Fmoc strategy on HYCRON resin and released by Pd(0)-catalysed cleavage of the allylic anchor. This fragment was now coupled to the solid-phase-bound MUC1 glycododecapeptide **18** after Fmoc removal and carboxy activation using HATU/HOAT [35] to furnish the T-cell

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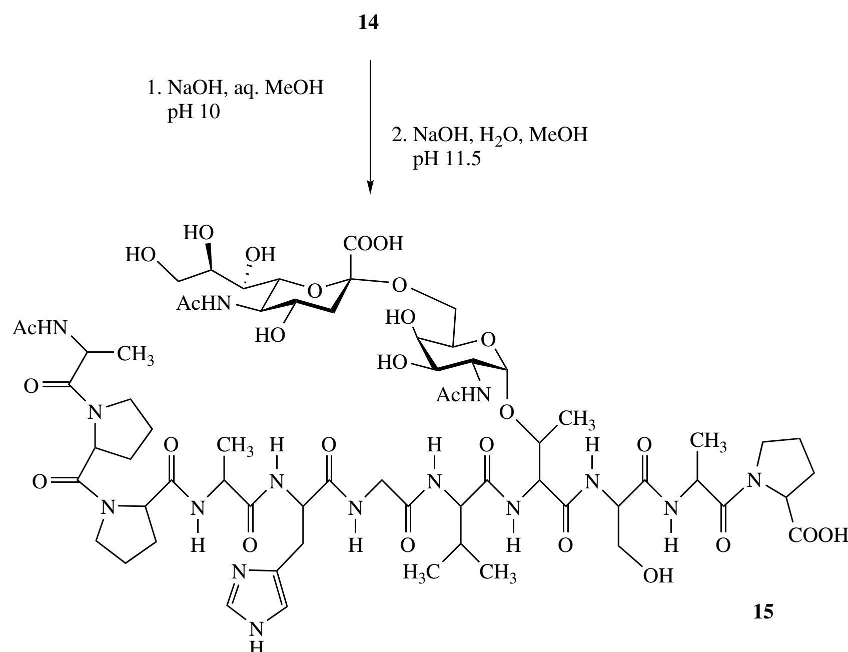

Scheme 7

epitope-spacer-MUC1 glycopeptide antigen conjugate **20**. Palladium(0)-catalysed cleavage and complete deprotection of **20** included the hydrogenolysis of the sialic benzyl ester and gave the construct **21** corresponding to **16** containing the tetanus toxin (TTX) epitope together with a non-immunogenic spacer and the tumour-associated MUC1 glycopeptide antigen [32].

For comparative immunological evaluation, this construct **21**, the corresponding MUC1 sialyl Tn glycododecapeptide, the unglycosylated MUC1 dodecapeptide, the sialyl Tn threonine, and the tetanus toxin-spacer conjugate, all completely deprotected, were administered to cultured peripheral blood lymphocytes (PBL) in concentrations of 0.1–1.0 μg/200 μl. After incubation for 2 days centres of proliferation occurred, and restimulation was carried out every 7 days. After the second restimulation, an ELISA with murine anti-human-IFNγ antibodies showed that the proliferating cells produced interferon-γ. After the fourth restimulation, the rate of proliferation was determined by incorporation of [^{3}H]thymidine. It was shown in this experiment that proliferation of T-cells only occurred in the presence of antigen-presenting cells. Purified T-cells did not proliferate under these conditions. This is considered as proof of an antigen-specific reactivity. Finally the proliferating T-cells were characterized in a fluorescence-activated cell sorter (FACS analysis) using labelled antibodies directed against surface protein clusters. These analyses showed that the conjugate **21** quantitatively induced proliferation of CD3-positive T-cells. Amongst those CD3-positive T cells, 53% were CD8-positive. The other substances, all partial structures of **21**, also induced proliferation of CD3-positive, but almost no proliferation of CD8-positive T-cells. These results justify the anticipation that conjugates of tumour-associated MUC1 glycopeptide antigens with T-cell epitopes can induce a cytotoxic T-cell response considered important for the development of synthetic antitumour vaccines [32].

Further investigations of the tumour-associated MUC1 glycopeptide antigens concern the synthesis of glycopeptide antigen–protein conjugates of exactly specified composition for the induction of antibodies and conformational analysis which show the influence of the glycan on to the conformation of the peptide backbone. It was demonstrated by preliminary NMR experiments and molecular simulations (in cooperation with W. D. Hull, Heidelberg), that the glycododecapeptide obtained from **18** by detachment from the resin and complete deprotection adopted a preferred conformation in water, whereas the corresponding unglycosylated peptide of identical sequence did not prefer any conformation in aqueous solution. This result illustrates that the type of the glycan, as well as the number

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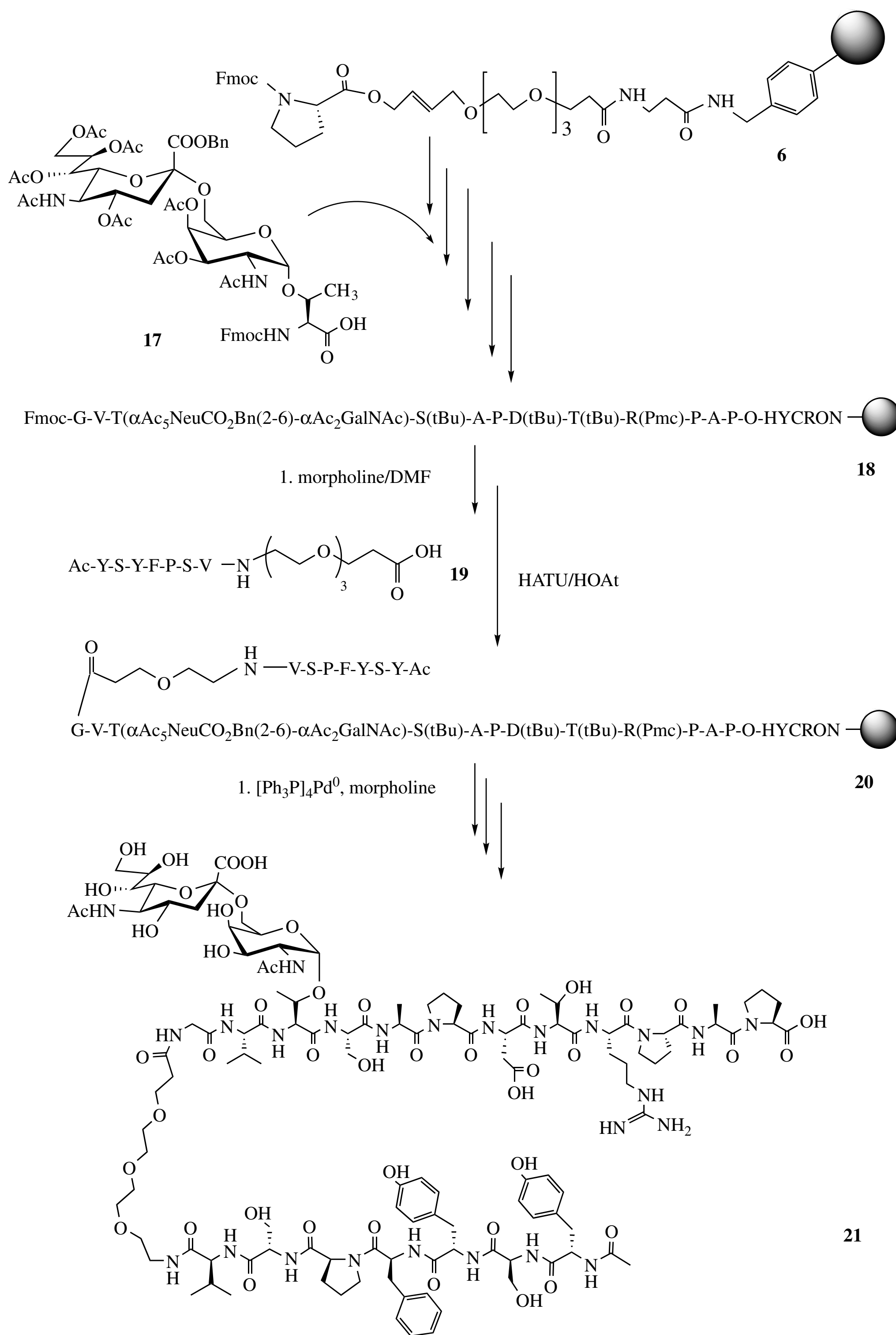


Scheme 8

and position, can influence the conformation of the MUC tandem repeat peptides and, thus, the spatial presentation of the peptide epitopes.

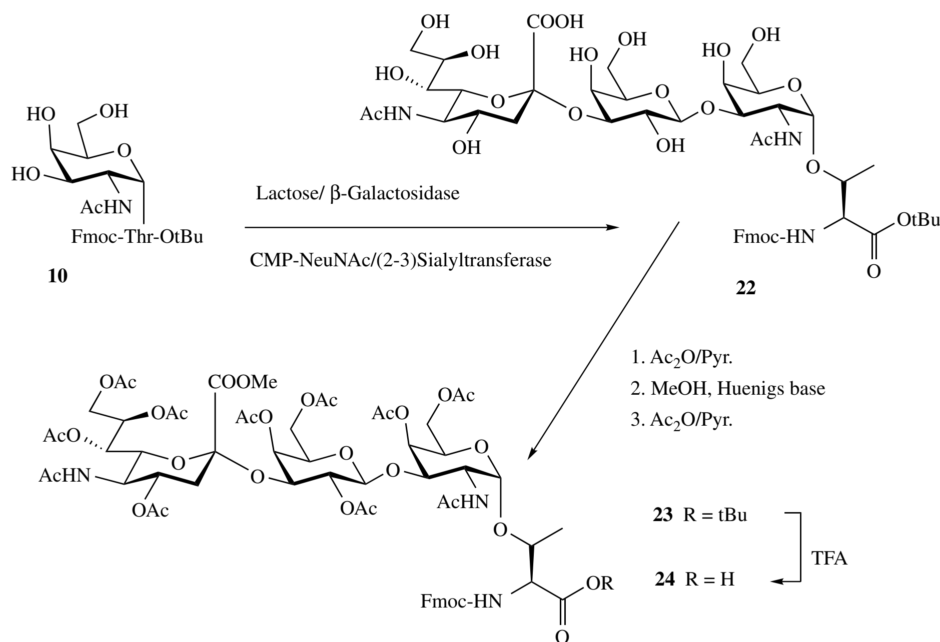
For this reason we are also interested in the synthesis of glycopeptides containing other tumour-associated saccharide antigens. Among them, the (2–3)sialyl T antigen is an important candidate. The sialyl T threonine building was synthesized in a chemoenzymatic strategy starting from the selectively deblocked Tn antigen precursor **10** (Scheme 9) [36].

The sialyl T threonine conjugate **22** was formed by an enzymatic reaction cascade consisting of a regio- and stereoselective transfer of galactose from lactose catalysed by a galactosidase and an immediate enzymatic sialylation of the T antigen intermediate [37]. In order to convert **22** into a building block applicable to solid phase synthesis, the functional groups of its carbohydrate portion must be protected. This was achieved by peracetylation and trapping the intermediate mixed anhydride of sialic acid and acetic acid with methanol/Huenigs base. Since some O-acetyl groups were lost during the trapping methyl ester formation, a second acetylation was carried out to give the completely protected sialyl T threonine **23**. Acidolytic cleavage of its tert-butyl ester yielded the desired Fmoc sialyl T threonine building block **24** [36].

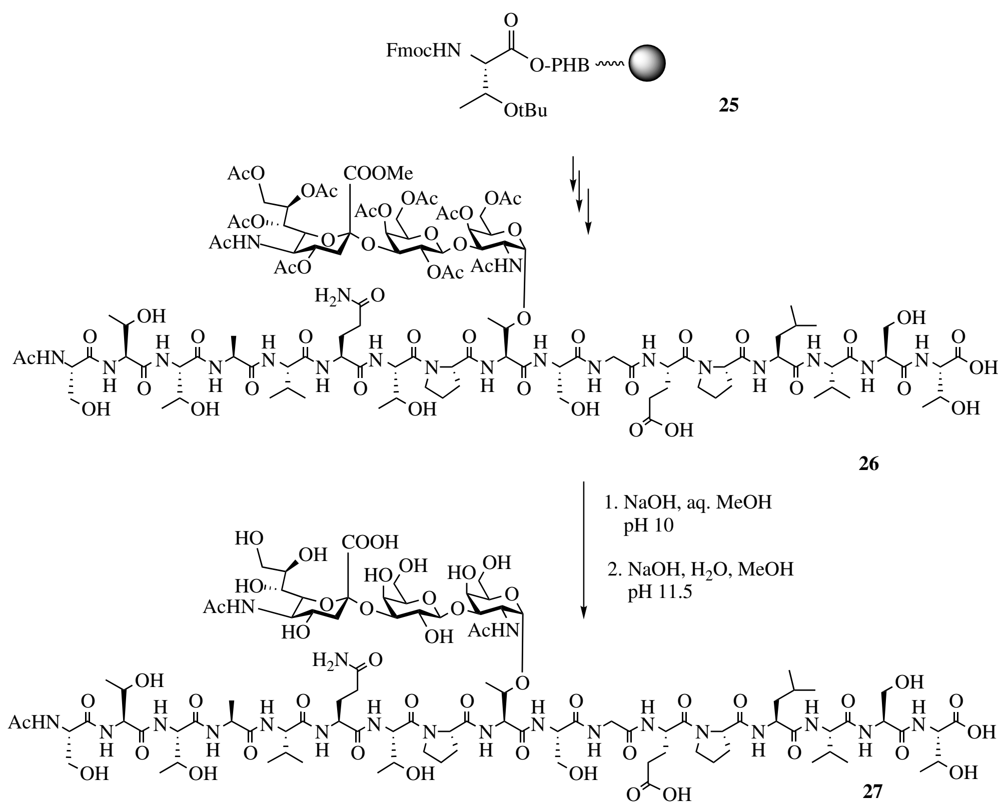
Solid-phase synthesis of *N*-terminal glycopeptides of leukosialin [38] were carried out starting from Fmoc threonine **25** linked to the polymer support through the acid sensitive Wang linker [39]. Fmoc strategy and HBTU [31] or HATU [35] were applied to assemble the glycopeptide (Scheme 10).

After acidolytic cleavage of the Wang anchor and simultaneous removal of the acid-labile side-chain protecting groups, the glycoheptadecapeptide **26** was isolated in an overall yield of 43% (Scheme 10). The critical removal of the protecting groups from the carbohydrate side chain was achieved as described above (Scheme 7) to furnish the free *N*-terminal glycoheptadecapeptide **27** of leukosialin with the tumour-associated (2–3)sialyl T antigen side-chain typical for acute myeloid leukaemia. Investigations of the effect of **27** on T cell proliferation, of its preferred conformation in water and the induction of antibodies are to be carried out, as outlined for the series of MUC1 glycopeptides.

Emil Fischer was the pioneer in both carbohydrate and peptide chemistry. The results obtained with modern methods of molecular biological and chemical analyses have revealed that glycoproteins composed of both covalently linked protein and saccharide portions play major functions in biological selectivity. Cooperative effects of both portions of glycoproteins have been found in cell adhesion phenomena and in the formation of selective epitopes. The chemical synthesis of glycopeptides now makes complex conjugates of this type available which are useful model compounds for the



Scheme 9



Scheme 10

investigation of these cooperative effects, for example, in the development of sufficiently selective antitumour vaccines.

Acknowledgement

I would like to heartily thank my co-workers who have pushed ahead the syntheses of MUC antigen glycopeptides with commitment and numerous ideas. They are: Nicole Bèzay, Peter Braun, Constanze Brocke, Sebastian Dziadek, Stefanie Keil, Michael Leuck, Beate Liebe and Oliver Seitz. My special thanks go to the Deutsche Forschugsgemeinschaft and to the Stiftung Rheinland-Pfalz fuer Innovation for their generous support of this work.

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