

Synthetic Antitumor Vaccines Through Coupling of Mucin Glycopeptide Antigens to Proteins

Markus Glaffig and Horst Kunz

Abstract The requirements for coupling reactions of carbohydrate molecules very much depend upon the biological recognition processes that should be investigated and upon the target structures of the desired carbohydrate ligand. If the carbohydrate conjugate itself is the recognized ligand, as for example, the binding site of a P-selectin ligand comprising sialyl-LewisX and a specific peptide sequence, the natural glycoside bond must be installed. A stereoselective and regioselective block glycosylation between a sialyl-LewisX trichloroacetimidate and a partially deprotected Thomsen–Friedenreich antigen derivative was developed to achieve this aim. In contrast, the coupling reactions by which glycopeptides from tumor-associated glycoproteins are conjugated to immune stimulating components in order to afford efficient vaccines can entail artificial linkages as long as they do not interfere with the immune reactions. For example, the coupling of glycophorin glycopeptides to bovine serum albumin was successfully achieved by carboxylic activation with a water-soluble carbodiimide in the presence of a supernucleophilic additive. This conjugation method is only recommendable if the glycopeptide does not contain several carboxylic and/or amino functions. The photochemically or radical initiator promoted thiol-ene coupling succeeded in couplings of MUC1 glycopeptide antigens to bovine serum albumin, however, is accompanied by oxidative disulfide formation. The conjugation of glycopeptide antigens from the tandem repeat region of the tumor-associated mucin MUC1 to bovine serum albumin or tetanus toxoid is efficiently accomplished using diethyl squarate as the coupling reagent. The intermediate squaric monoamide esters can be isolated and characterized, and then applied to a mild connecting process to the carrier proteins. The MUC1 glycopeptide-tetanus toxoid conjugates proved to be particularly useful vaccines. They induce extraordinarily strong immune responses in mice. The induced antibodies are prevailingly of the IgG1 isotype and show efficient binding to the glycoproteins exposed on epithelial tumor cells.

M. Glaffig · H. Kunz (✉)

Institut Für Organische Chemie, Johannes Gutenberg-Universität Mainz,
55099 Mainz, Germany
e-mail: hokunz@uni-mainz.de

1 Introduction

Since the discovery of the human blood group substances by Karl Landsteiner in 1901 [1], researchers have always been strongly interested in the question which functions do carbohydrates have in natural glycoconjugates, in particular in glycoproteins and glycolipids. For investigations of the role of the saccharide portions of natural glycoconjugates, model compounds are considered helpful in which a carbohydrate is linked to a suitable component in a well-known format. From interactions of these model conjugates with natural receptors or enzymes, conclusions can hopefully be drawn which shine light on the recognition processes amounting to the biological selectivity. Efficient coupling reactions often are a crucial prerequisite for the synthesis of these model glycoconjugates. The choice of the coupling method distinctly depends upon the nature of the functional molecule to which the carbohydrate is to be conjugated and upon the role, the carbohydrate does play in the considered biological process.

If the glycan merely influences the physicochemical properties of the glycoconjugate or protects it from degradation, an arsenal of coupling reactions can be exploited for forming a mimic of the natural prototype. These coupling processes can involve artificial linker structures. The same often applies to glycoconjugates in which the carbohydrate constitutes the solely recognized ligand, as for example, a blood group antigen [2], a bacterial cell wall saccharide antigen [3], or as the ligand of a lectin [4–6]. A number of efficient ligation reactions have been introduced during the past decade which can be applied to the decoration of peptides or proteins with carbohydrates through unnatural linkage structures, as for example, by thiol-ene couplings [7], cross-metathesis reactions [8], 1,3-dipolar cycloaddition (click) reactions [9], or Diels–Alder cycloaddition reactions with inverse electron demand [10]. The situation, however, is completely different if the carbohydrate constitutes only a part of the structure recognized by a receptor, enzyme or antibody, and the bound epitope comprises of both, carbohydrate and backbone structures to which the carbohydrate is conjugated. This also includes glycoprotein epitopes in which the carbohydrate exerts distinct influence onto the conformation of a recognized epitope even if this is a pure peptide structure [11–16]. In these cases, the artificial linker disarranges the recognized epitope, and observed effects may result in misleading conclusions.

Epitopes involving both peptide and carbohydrate structures are known from ligands of selectins [17] and tumor-associated mucin antigens [18]. In syntheses of model compounds representing these types of glycopeptide epitopes, the linkages between the carbohydrates and the peptide backbone should closely correspond to the nature prototype.

2 Glycan Coupling Through Glycosylation—Glycopeptide Ligands of Selectins

Selectins are important carbohydrate recognizing receptors involved in the early phase of recruitment of leukocytes into inflamed tissues [19]. P- and E-selectins are expressed on the apical surface of endothelial cells of the blood vessels within the inflamed area, while L-selectin is exposed on the leukocyte. The tetrasaccharide sialyl LewisX has been described as a ligand to these selectins [20]. Initial interaction between P-selectin and its glycoprotein ligands occurs immediately after activation of P-selectin through inflammatory cytokines and results in rolling of the leukocytes. The expression of E-selectin requires several hours since the cells need to synthesize this glycoprotein after activation. Its interaction with the E-selectin ligands on the leukocytes results in a closer attraction of the rolling leukocytes to the endothelial cells [21]. The selective inhibition of these cell adhesion processes certainly is important for the treatment of inflammatory diseases and also for the prevention of tumor cell metastasis [22]. It was revealed that sialyl LewisX is a required, but only moderate ligand to P- and E-selectin. For an intensive binding, the cooperation of peptide segments of the glycoprotein ligands carrying the sialyl LewisX is mandatory. In the case of the P-selectin glycoprotein ligand 1 (PSGL-1) the *N*-terminal peptide, a sequence containing *O*-sulfated tyrosine residues (Fig. 1) was identified as an important ligand structure [23].

In fact, it was found that synthetic sialyl-LewisX glycopeptides with peptide sequences containing charged amino acids bind distinctly stronger to P-selectin than glycopeptides lacking charged groups [24, 25]. A sialyl-LewisX-RGD-peptide, for example, inhibited the binding of HL-60 tumor cell to an immobilized human P-selectin-IgG [24] with an IC_{50} value of 26 μ mol. In this glycopeptide, the sialyl-LewisX tetrasaccharide was *N*-glycosidically coupled directly to *N*-4 of an asparagine. Thus, the distance between the saccharide ligand and the charged units did not correspond to the natural prototype (Fig. 1).

In order to obtain more active inhibitors of P-selectin, sialyl-LewisX trifluoroacetimidate **1** and the *N*-fluorenylmethoxycarbonyl-(Fmoc)-protected T-antigen threonine conjugate **2** [26], selectively deblocked in 4- and 6-position of the galactosamine unit were ligated by the activation of the trichloroacetimidate

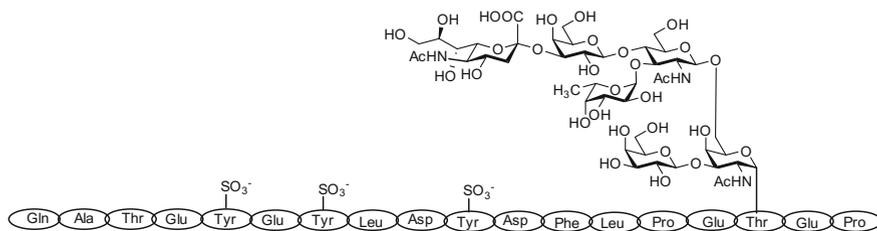


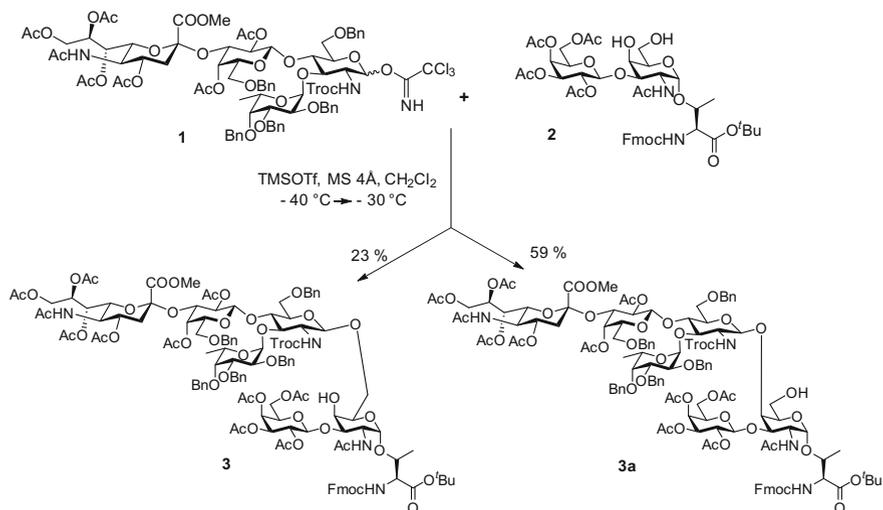
Fig. 1 P-selectin binding region of human P-selectin glycoprotein ligand 1 (Ref. [23] and lit. cited therein.)

with trimethylsilyl-trifluoromethanesulfonate (TMS-triflate) at low temperature. This coupling proceeded with high overall yield and high stereoselectivity but unexpected regioselectivity. The desired product of glycosylation at the primary 6-OH group **3** was the minor product. Fortunately, it was separable from the undesired regioisomer **3a** by flash-chromatography [27] and, thus, applicable to the solid-phase synthesis of a PSGL-1 binding domain glycopeptide (Scheme 1). In conjugate **3**, the natural linkage between sialyl-LewisX as a part of the ligand structure and the peptide backbone represented by the threonine is established, and thus the natural distance and orientation to the binding sites within a peptide sequence are guaranteed.

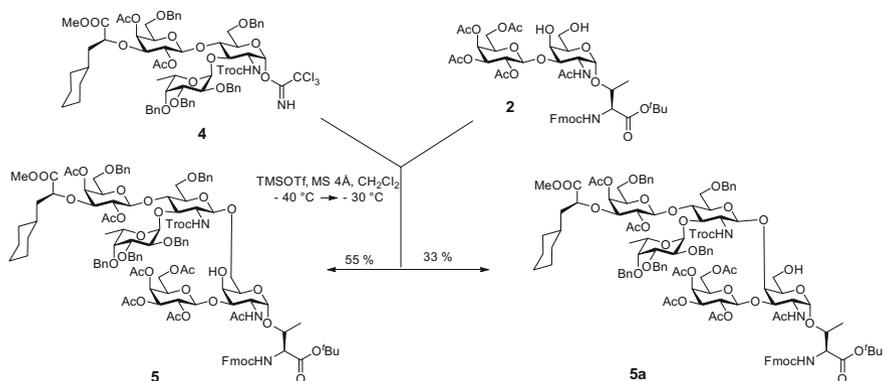
Alternatively, the mimic **4** of sialyl-LewisX containing cyclohexyl-lactic acid as the surrogate of sialic acid [28] was coupled to the partially deprotected T-antigen-threonine building block **2** [26].

After activation of the pseudo-tetrasaccharide trichloroacetimidate **4** with TMS-triflate, the β -glycoside bond to the acceptor was formed with high stereoselectivity and excellent overall yield. Again, the regioselective differentiation between the primary 6- and the secondary 4-hydroxy groups of the T-antigen threonine derivative was only moderate. However, in this case, the desired compound **5** was the major product. Flash-chromatographic separation gave the desired regioisomer **5** of the glycosyl amino acid in a yield of 55%, while the unexpected regioisomer **5a** was isolated in a yield of 33% (Scheme 2) [29].

The building block **5** after selective cleavage of the *tert*-butyl ester was introduced into the solid-phase synthesis of the PSGL-1 glycopeptide recognition domain. Acidolytic detachment of the assembled glycopeptide from the resin and



Scheme 1 Coupling of sialyl-LewisX to a T-antigen-threonine to install the natural β -glycoside linkage occurring in the PSGL-1 recognition domain [27]



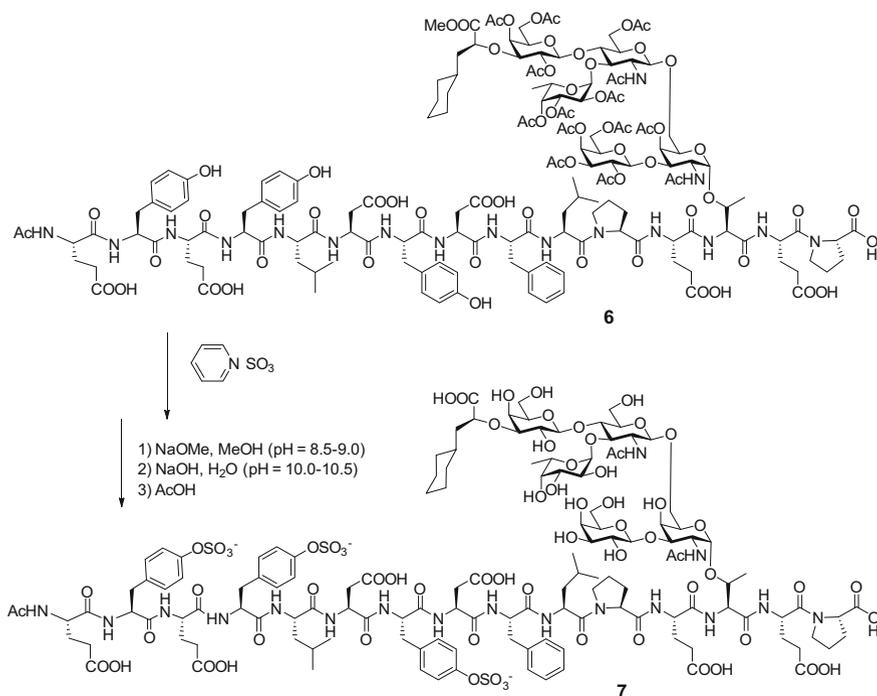
Scheme 2 Coupling of saccharide building blocks by stereoselective glycosylation to give the pseudo-sialyl-LewisX glycosyl threonine **5** [28]

concomitant removal of all acid-sensitive protecting groups from the amino acid side chains afforded the PSGL-1 glycopeptide binding sequence **6** still protected in the carbohydrate portion. Treatment of **6** with pyridine-SO₃ complex in pyridine/dimethylformamide, first at 0 °C, then at room temperature for 18 h, afforded the glycopeptide *O*-sulfated in all three tyrosine residues. After subsequent careful removal of the carbohydrate protecting groups under mild basic conditions preventing β-elimination of the carbohydrate, the sulfated PSGL-1 glycopeptide binding domain **7** was isolated by preparative RP-HPLC in pure form (Scheme 3). This compound inhibited the binding of murine granulocytes to a murine P-selectin-IgG fusion protein with an IC₅₀ of 20 μmol. The corresponding binding of human granulocytes was inhibited by **7** even more efficiently (IC₅₀ 5 μmol). This is not surprising since **7** represents the peptide sequence of human PSGL-1.

The potentiation of the binding effects of both, the pseudo-tetrasaccharide and the anionic centers, was only achieved with an inhibitor which contains the binding groups in optimal distance and steric arrangement. The coupling of the saccharide ligand to the peptide backbone in the natural structure is considered the prerequisite for this efficiency. This requirement would also be fulfilled for conjugates in which the whole glycopeptide recognition site is linked to a carrier molecule, for example, a protein, a dendrimer or a polymer.

3 Glycophorin-Derived Vaccines Containing Nature-Like Linkages

Small molecules and endogenous structures often are not sufficiently immunogenic in order to elicit an appropriate immune response. To obtain a vaccine, these compounds need to be coupled to immune stimulating components. A most general



Scheme 3 Tyrosine *O*-sulfation and deprotection of the PSGL-1 glycopeptide recognition domain [29]

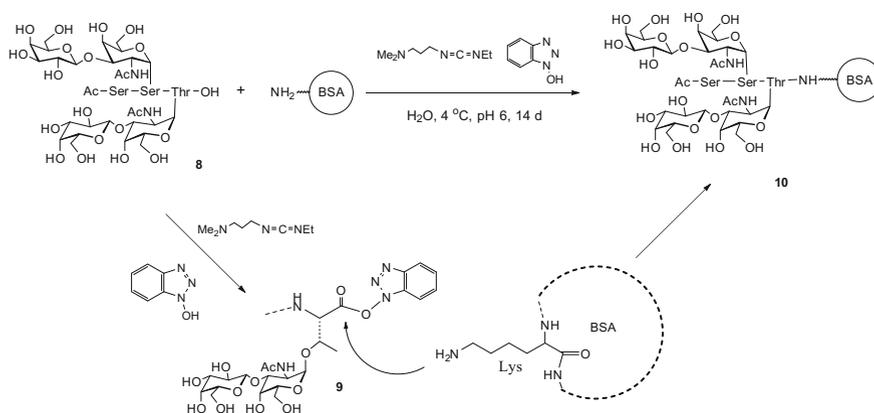
way for the construction of a vaccine consists of the conjugation to a carrier protein which provides T-cell epitopes that induce T-cell activation via major histocompatibility complex (MHC) pathways.

Inspired by reports of Springer [30] on the Thomsen-Friedenreich (T)-antigen as a tumor-associated carbohydrate antigen, we started the chemical synthesis of T- and Tn-antigen carrying glycopeptides in the early 80s. Springer and his coworkers had identified tumor-associated T-antigen containing glycoproteins on the membranes of epithelial tumor cells, while these molecules were absent on epithelial cells of normal tissues. The authors isolated these tumor-associated T-antigen and Tn-antigen glycoproteins from tumor cells and induced antibodies against these molecules. From cross reactivity of the obtained antibodies, they concluded that the tumor-associated T- and Tn-antigen glycoproteins should be structurally related to asialoglycophorin, the sialidase-treated form of glycophorin which is the major transmembrane glycoprotein on the red blood cells. Glycophorin occurs in two blood group forms M and N, which are decorated with the same *O*-glycan pattern, but are different in only two of the 132 amino acids (position 1 and 5). One of the differences concerns the N-terminal amino acid which serine in M-blood group and leucine in N-blood group glycophorin [30].

With the aim of constructing a vaccine against the tumor-associated epithelial glycoproteins, we synthesized the *N*-terminal glyco-tripeptide of M-blood group asialoglycophorin **8** [31]. The coupling of this glycopeptide to bovine serum albumin (BSA) as the carrier protein in water was achieved using the water-soluble *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC) and 1-hydroxy-benzotriazole (HOBt, Scheme 4) [32].

This coupling process has become a reliable method for linking antigens to proteins. Instead of HOBt, *N*-hydroxy-succinimide (HO-Su or NHS) can be used as an additive for the conjugation of (glyco)peptides [33] as well as of small molecules, as for example, the designer opioid fentanyl [34]. The procedure takes profit from a remarkable chemoselectivity. In water at pH 6, the carboxy group of **8** reacts with EDC to furnish the corresponding *O*-acyl isourea which preferentially is attacked by the super-nucleophile HOBt to form the active ester **9** (Scheme 4). This active ester obviously is sufficiently resistant to hydrolysis, and therefore prevalently undergoes aminolysis by the amino functions of the lysine side chains of BSA to afford the glycopeptide-protein conjugate **10**.

By immunizing mice with blood group M-asialoglycophorin-BSA vaccine **10** and cloning, a monoclonal antibody (82-A6, IgM subclass) was obtained [35]. Monoclonal antibody 82-A6 reacted with normal and tumor epithelial cells showing its binding to the T-antigen disaccharide. However, the monoclonal antibody 82-A6 exhibited distinctly higher affinity towards M-blood group asialoglycophorin having the identical *N*-terminal tripeptide sequence Ser-Ser(T-antigen)-Thr(T-antigen) as **10** than to N-blood group asialoglycophorin with the *N*-terminal sequence Leu-Ser(T-antigen)-Thr(T-antigen) (Fig. 2) [33b]. It appeared amazing that an antibody induced with a synthetic vaccine presenting only the small *N*-terminal part of the huge M-blood asialogroup glycophorin (132 amino acids) differentiates between the two M- and N-blood group asialoglycophorins which carry the



Scheme 4 Coupling of a T-antigen glycopeptide to bovine serum albumin in water through carboxy activation with a water-soluble carbodiimide/1-hydroxy-benzotriazole [31]

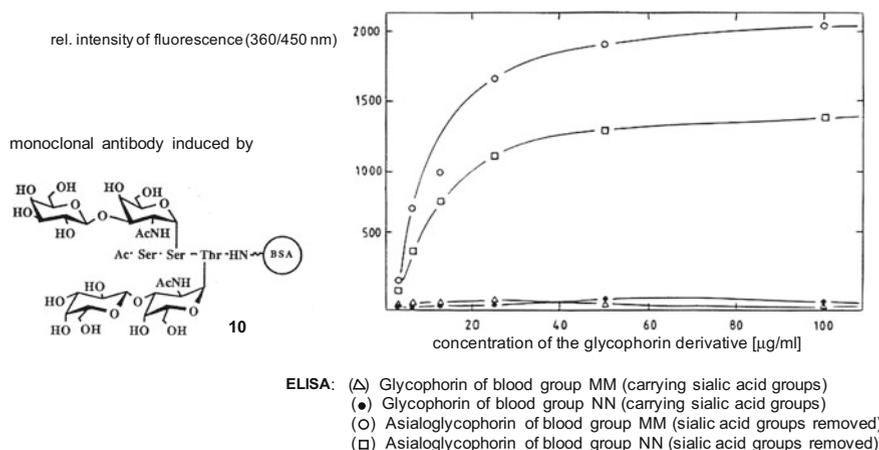


Fig. 2 Recognition of M- and N-blood group asialoglycophorin by monoclonal IgM antibody induced in mice by vaccine 10

identical glycan pattern (Fig. 2). This result suggested that not only the recognized saccharide (T-antigen) but also the amino acid sequence contributes to the recognized epitope. Nature obviously does not make differences between classes of natural products as they are organized in chapters of scientific text books, if selective recognition by the immune system is concerned.

It must also be concluded from the observed differentiation between M- and N-blood group asialoglycophorin by antibody 82-A6 induced through the glycopeptide vaccine that the tumor-associated carbohydrate antigen in a vaccine is not sufficient for the induction of tumor-selective antisera. It needs to be combined with a tumor-relevant peptide sequence in order to form a tumor-typical glycopeptide antigen. The peptide sequence of glycopeptide 8 obviously did not meet these criteria.

Biochemical and molecularbiological analyses of membrane glycoproteins from carcinoma cells, in particular, reported by the group of J Taylor-Papadimitriou [36], have revealed that the tumor-associated mucin MUC1 is a characteristic membrane glycoprotein occurring on many epithelial tumors.

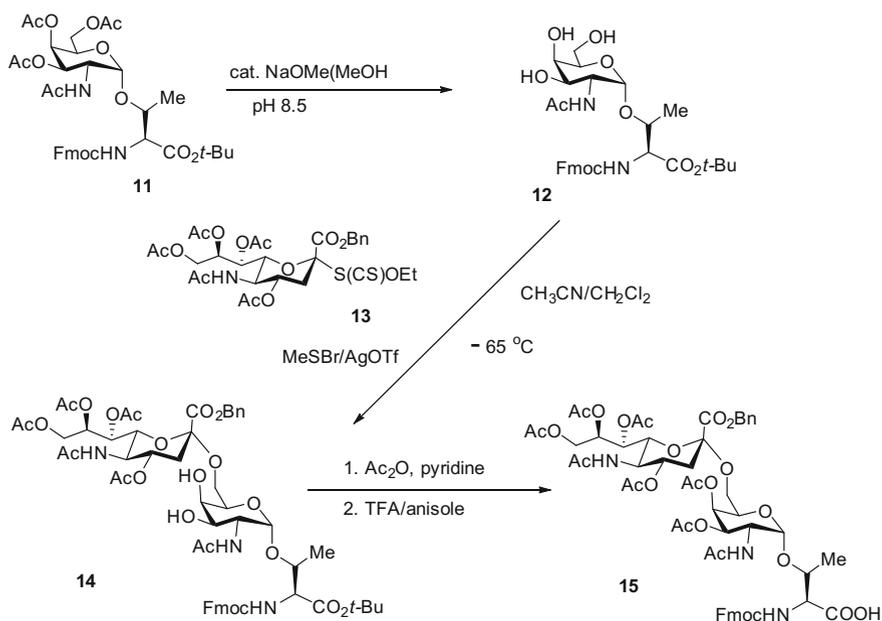
4 The Tumor-Associated Mucin MUC1—A Promising Target for the Development of Antitumor Vaccines and Synthesis of Tumor-Associated MUC1 Glycopeptide Antigens

Mucin MUC1 is expressed on many epithelial tissues [37]. It is a large membrane-bound glycoprotein. In its extracellular portion, it contains an extended domain comprising a variable number (20–125) of tandem repeats of the amino acid

from tumor cells. Because of the biological micro-heterogeneity, MUC1 molecules isolated from tumor cell membranes can carry long oligosaccharides typical for normal cells next to short, tumor-associated glycans even on a single protein chain. Therefore, sufficiently pure tumor-typical MUC1 glycopeptide antigens must be built up by synthesis.

Solid-phase synthesis of glycopeptides (SPPS) using glycosylated amino acid building blocks nowadays is a powerful, flexible method for the construction of exactly specified MUC1 glycopeptide antigens [18, 42]. The required Fmoc-protected glycosyl serine and threonine derivatives are obtained by stereo- and regioselective extension of the saccharide portion of the corresponding galactosamine (Tn-antigen) conjugates, as for example, **11** [43]. Due to the acetamido group and to the *O*-acyl protection, the glycoside bond of glycosylated amino acids and peptides are sufficiently stable to acids (carbonyl oxygens are prevailingly protonated thus creating a Faraday cage that protects the saccharide bonds [44]) the *tert*-butyl ester of **11**, as well as those of other Fmoc glycosyl amino acids, can selectively be cleaved using a cocktail of trifluoroacetic acid (TFA)/triisopropylsilane(TIS)/water (10:1:1) or TFA/anisole. The extension of the saccharide portion, however, requires methods which do not affect the Fmoc- as well as the *tert*-butyl ester protection nor the glycoside bond (Scheme 5).

In this sense, the selective removal of the *O*-acetyl groups from **11** was achieved by transesterification in dry methanol at a pH of 8.5 to give the key substrate **12** for

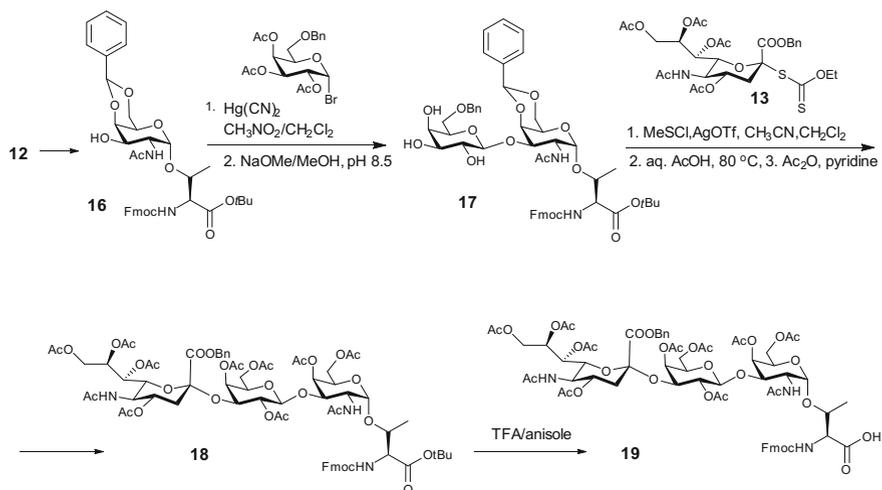


Scheme 5 Synthesis of Fmoc-protected glycosyl amino acid building blocks [18, 42]: Sialyl-Tn threonine

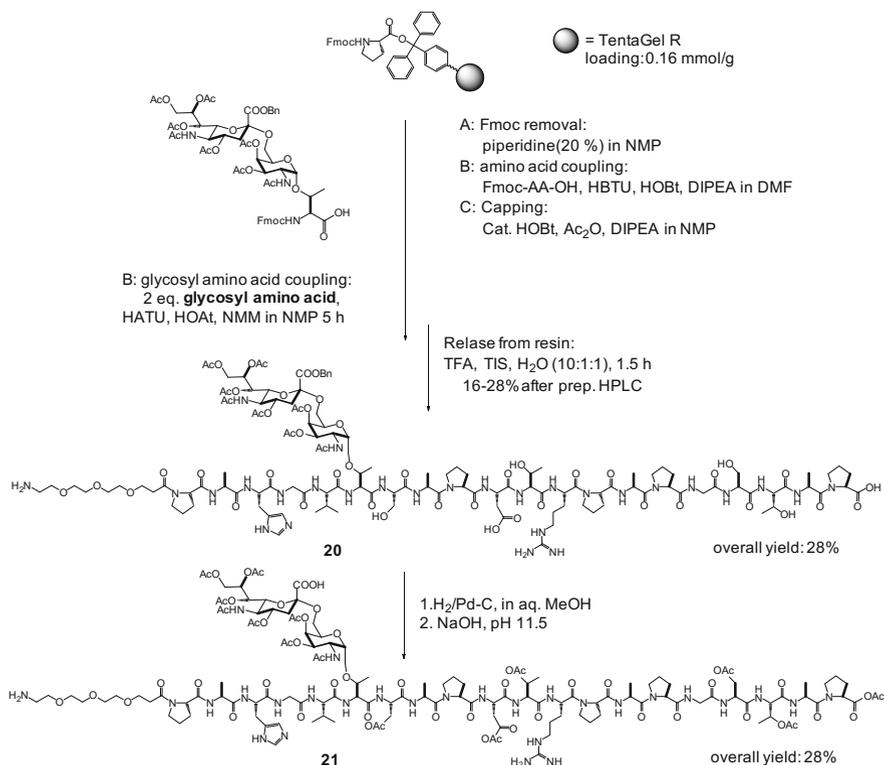
further glycosylation reactions [43, 45]. Regio- and stereoselective sialylation at O-6 of **12** using the sialyl ester xanthate **13** afforded the sialyl-Tn antigen-threonine derivative **14** which after acetylation and acidolytic removal of the *tert*-butyl ester furnished sialyl-Tn building block **15** applicable to solid-phase glycopeptide syntheses (Scheme 5). Further extensions of the carbohydrate to give T-antigen and sialyl-T-antigen building blocks are displayed in Refs. [18, 42]. Acid-catalyzed introduction of a 4,6-benzylidene protection to furnish **16** followed by galactosylation under Helferich conditions and careful methanolysis of the *O*-acetal groups resulted in the formation of the T-antigen structure **17** (Scheme 6). If Fmoc protection is lost under these conditions, it can selectively be reintroduced using *O*-Fmoc-hydroxy-succinimide.

Regioselective sialylation to give the 2,3-sialyl-T antigen threonine derivative **18** was achieved under the conditions described above [43]. *O*-Acetylation and final acidolysis of the *tert*-butyl ester yielded the Fmoc-sialyl-T-threonine building block **19** [46].

The solid-phase synthesis of the MUC1 glycopeptide antigens are usually conducted using Tentagel-resins equipped with trityl- or 2-chlorotrityl linkers according to the Fmoc strategy. Example **20**, in which the sequence is *N*-terminally extended with a triethylene glycol spacer and the tumor-associated carbohydrate antigen sialyl-Tn is incorporated at threonine-6 is shown in Scheme 7 [47]. The trityl anchor prevents the formation of a diketopiperazine on the level of the resin-linked dipeptide. The coupling of the glycosyl amino acid, because of its demanding preparations applied in only slight excess, was carried out manually using the more reactive *O*-(7-azabenzotrazolyl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) [48] instead of *O*-benzotrazolyl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU). After detachment from resin with



Scheme 6 Synthesis of core 1 di- and trisaccharide threonine building blocks



Scheme 7 Solid-phase synthesis of tumor-associated MUC1 glycopeptide antigens [47]

concomitant acidolytic removal of the side chain protection and purification by preparative HPLC glycopeptide **20** was obtained. Hydrogenation of the sialic benzyl ester and careful alkaline hydrolysis of the *O*-acetyl functions at pH 11.5 afforded after purification by preparative HPLC the MUC1 glycopeptide antigen **21** in a 30 mg scale.

The glycopeptide antigens, as for example **21**, represent endogenous structures and therefore, are of insufficient immunogenicity. In order to gain an efficient vaccine, these compounds must be conjugated to immune stimulating components. Most frequently, proteins serve as immune stimulants because they contain several T-cell activating peptide epitopes.

5 Thiol-Ene Coupling for the Formation of Glycopeptide Antigen-Protein Conjugates

The construction of vaccines demands coupling reactions which do not form immunogenic linker structures, as for example, electron-poor homo- or hetero-aromatic systems. Thioether linkages are considered immunologically silent

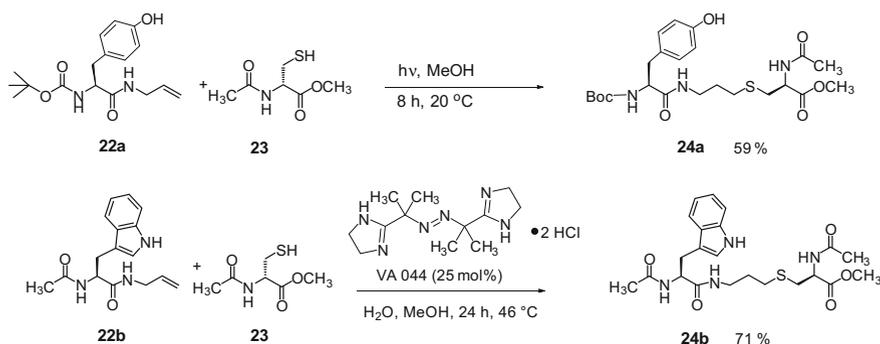
structures. Given the great number of functional groups in both, the deprotected glycopeptide antigen and the carrier protein to which the glycopeptide should be linked, a biorthogonal thioether formation is highly desirable. Thioether formation via heterobifunctional linkers, as for example *N*-succinimidyl-4-(maleimidomethyl)cyclohexane carboxylate [49], has frequently been applied for conjugation reactions. However, the immunologically less critical radical-type addition of thiol to nonactivated double bonds was not used for the coupling to proteins up to 2007, although the reaction was known since hundred years [50]. In order to prove whether sensitive amino acid derivatives, as for example, tyrosine **22a** or tryptophane **22b** are affected by photochemically or radical-type initiated thiol radical addition, model reaction was performed [51] (Scheme 8).

The thiol radicals generated from methyl *N*-acetyl cysteinate **23** either photochemically or by initiation with VA 044 as the initiator reacted with the amino acid *N*-allylamides **22** to furnish the thioether-linked conjugates **24**. The disulfide of **23** was the only side product. The electron-rich aromatic rings and the α -CH positions were not affected.

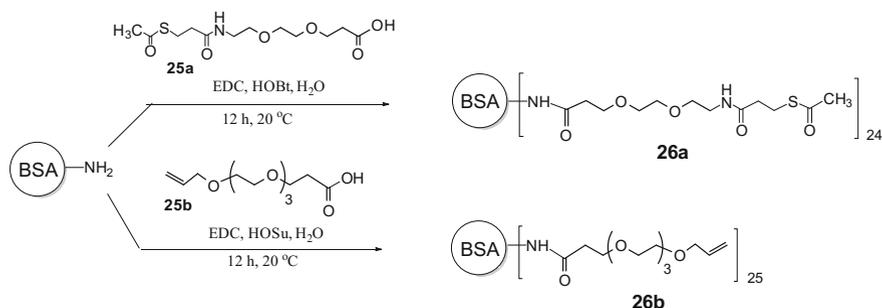
After these promising results of the model reactions bovine serum albumin (BSA) as the carrier protein was decorated with functional oligoethylene glycol spacer molecules **25** terminating either in *S*-acetylthio- or allyl ether functions. These reactions (Scheme 9) were carried out at room temperature through acylation at the lysine amino functions using the water-soluble carbodiimide in combination with HOBt or *N*-hydroxy-succinimide (HOSu) under weakly acidic conditions as described above (Scheme 4) for the glycophorin glycopeptide vaccine.

MALDI mass spectra gave evidence that in the conjugates **26** on average 25 of the 59 lysine residues of BSA were acylated with the functional spacers [51].

The protein molecules **26** decorated with functional side chains were subjected to radical-type thiol-ene coupling with MUC1 glycopeptide antigens. On the one hand, the MUC1 glycopeptide **27** carrying a T-antigen side chain was prepared on solid-phase as described above and *N*-terminally extended with a mono-allylamido-succinoyl group. On the other hand, the *S*-acetyl protections were removed from the



Scheme 8 Radical addition of *N*-acetyl cysteine methyl ester to *N*-protected tyrosine and tryptophane *N*-allylamide

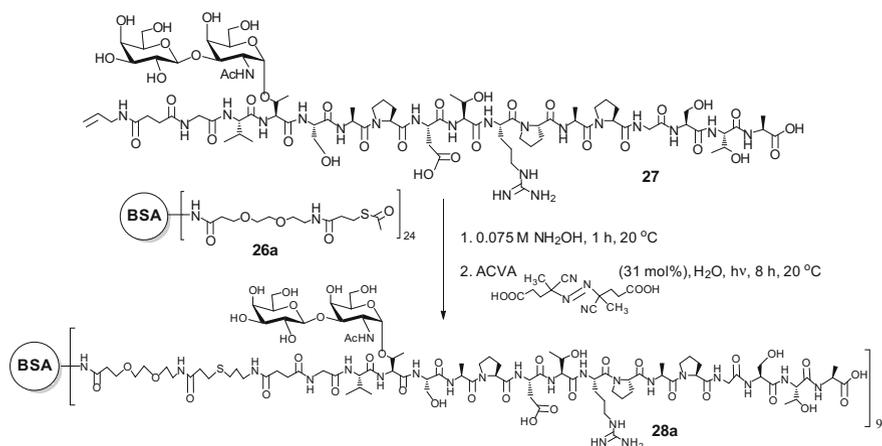


Scheme 9 Decoration of BSA with thiol- and allyl ether functionalized spacer molecules [51]

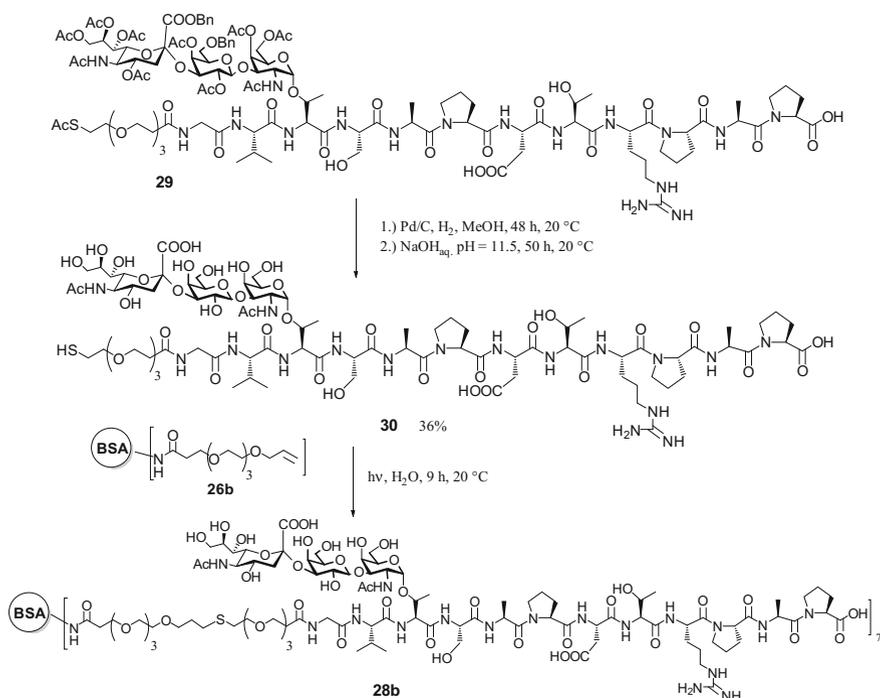
thiol-functionalized BSA **26a** using hydroxylamine solution under argon atmosphere. The set-free thiols were activated in water with the radical initiator ACVA (Scheme 10) under irradiation ($\lambda = 254$ nm) in the presence of **27**. Thus, the two components were linked to each other in the thiol-ene coupling to give the neoglycoprotein conjugate **28a**. According to its MALDI-TOF mass spectrum, conjugate **28a** carried on average 9 molecules glycopeptide per molecule protein.

The yield of the thiol-ene coupling amounts to about 35% [51]. Longer reaction time probably is recommendable.

The alternative coupling between the protein-bound olefin **26b** and the glycopeptide antigen equipped with the *N*-terminal thiol-functionalized spacer is displayed in Scheme 11. Glycopeptide **29** was assembled on solid-phase. The complex 2,3-sialyl-T antigen threonine building block **19** (Scheme 6) was introduced at position Thr-3. Finally, an *S*-acetylated triethylene glycol spacer acid was



Scheme 10 Thiol-ene coupling of a glycopeptide antigen to thiol-functionalized BSA [51]



Scheme 11 Thiol-ene coupling of a glycopeptide antigen to olefin-decorated BSA [51]

condensed at the *N*-terminus before the glycopeptide **29** was detached from the resin. Removal of the sialic benzyl ester by hydrogenolysis also effected some desulfurization and, thus, reduced the yield of the desired glycopeptide **30** which was isolated after careful saponification at pH 11.5. It certainly would be more appropriate to remove the benzyl ester in this saponification step. Olefine-decorated BSA **26b** and glycopeptide antigen **30** were subjected to thiol-ene ligation by photochemical activation in water (Scheme 11).

This thiol-ene coupling with reverse arrangement of the functional groups appeared slightly less efficient. The number of glycopeptide antigens linked to a protein molecule was considered not optimal for the creation of an efficient vaccine. Oxidation to give the disulfide of glycopeptide **30** was observed as the major side reaction. Its amount increased with extended reaction time. The great number of different functional groups of the glycopeptides interferes with these coupling reactions proceeding via thyl radicals. These factors are obviously less important in corresponding coupling reactions of carbohydrate ligands to proteins [52].

As the BSA conjugates of glycopeptide antigens exhibited only moderate immunogenicity, in further experiments they were not used as vaccines but served as coating material for probing antisera in ELISA analyses.

6 Coupling of Glycopeptides to Proteins Using Diethyl Squarate

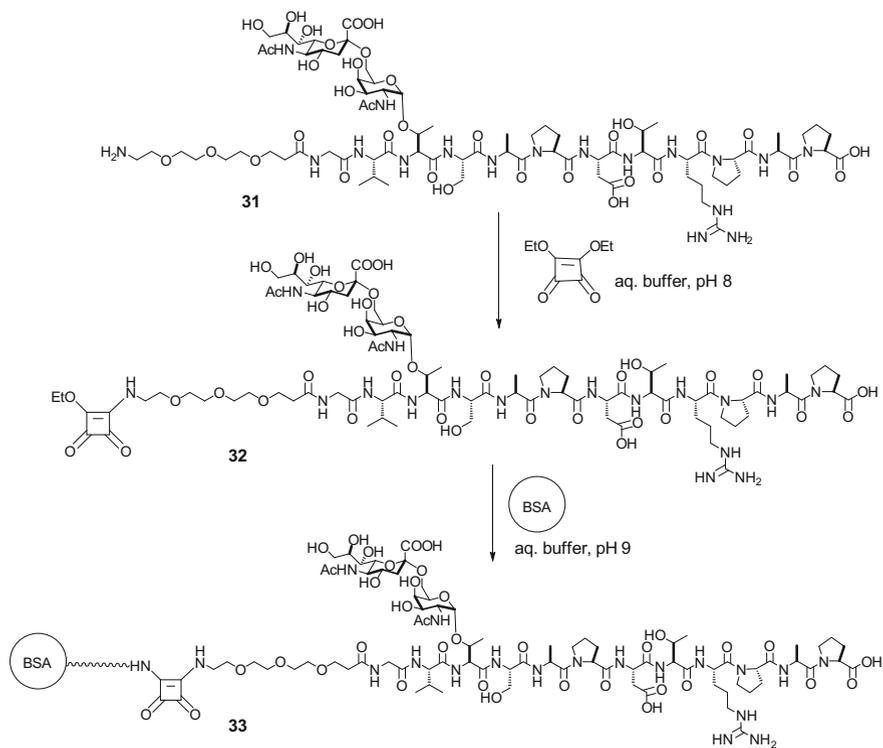
Regarding the limited practicability of the photo-induced thio-ene coupling for the construction of glycopeptide-protein vaccines, the condensation reactions promoted by water-soluble carbodiimides, as for example EDC (see Schemes 4 and 9), may be considered useful alternatives. But orienting investigations with MUC1 tandem repeat peptides revealed that the activation of unprotected oligopeptides of this type with EDC and hydroxyl-benzotriazol (HOBt) or *N*-hydroxy-succinimide in dimethylformamide (DMF) or water gives the expected C-terminal active esters only in a mixture with a number of other products. Obviously, aspartic side chain carboxylic groups were also activated and, in particular, aspartimide rearrangement took place [53].

In contrast to these experiences, the differentiated reactivity of squaric diesters towards amines [54] appeared a promising tool for exploitation in glycopeptide-protein conjugate formation.

In fact, the sialyl-Tn glycopeptide MUC1 sequence **31** synthesized on solid-phase and completely deprotected reacted with diethyl squarate in water at pH 8 to selectively furnish the glycopeptide squaric monoamide derivative **32** [55]. The compound can be purified by chromatography and characterized by NMR spectroscopy. In water at pH 9.0, it underwent further aminolysis by the amino functions of BSA lysine side chains to yield the BSA-glycopeptide conjugate **33** (Scheme 12) [55]. According to its MALDI-TOF spectrum, the conjugate contained on average 6 molecules glycopeptide per molecule BSA. Optimization of this coupling showed that the coupling rate is higher at pH 9.5, so that the second steps in further squarate couplings were conducted under these conditions.

The coupling via preformed squarate monoamides is also very valuable for the synthesis of complex, fully synthetic vaccines as was demonstrated for vaccines in which the tumor-associated MUC1 glycopeptide as the B-cell epitope was combined with three T-cell epitopes (Scheme 13) [56].

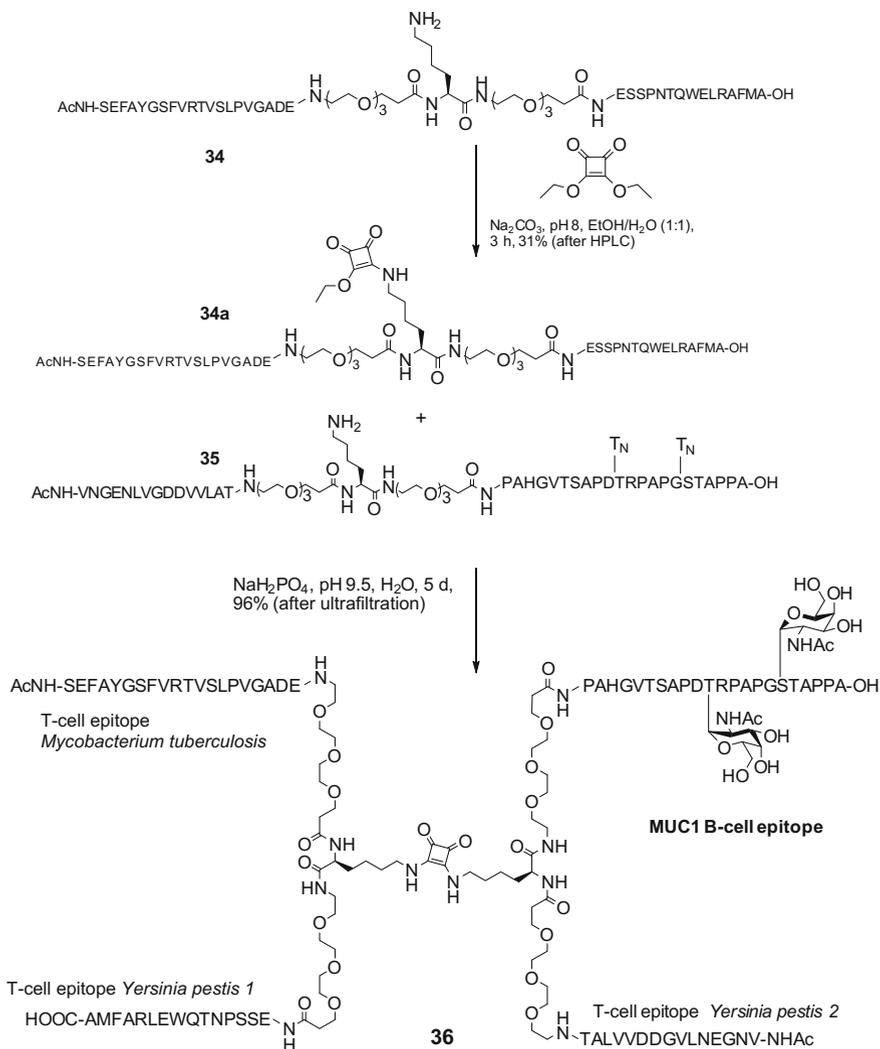
In a linear solid-phase synthesis, the combination **34** of the T-cell epitope peptide from *Yersinia pestis* 1 with that from *Mycobacterium tuberculosis* is constructed. These components are connected by two triethylene glycol spacer molecules separated by a central lysine. The 4-amino group of this lysine was transformed into the squaric monoamide ester **34a** in water/ethanol at pH 8. This monoamide was then reacted with an analogously synthesized conjugate **35** of the MUC1 glycopeptide with another T-cell epitope from *Yersinia pestis* 2 also connected to each other by two spacers linked through a lysine in the center. The coupling between the two large conjugates was achieved in high yield by squaric



Scheme 12 Coupling of a glycopeptide antigen to BSA through squaric acid amide formation

diamide formation in water at pH 9.5 (Scheme 13) [56]. It is noteworthy that the vaccine **36** isolated after ultrafiltration was pure, and its structure was confirmed by high-resolution ESI mass spectra and 2D-COSY- HSQC- and HMBC NMR spectra.

Immunization of mice with vaccine **36** without application of any external immune stimulating adjuvant elicited significant immune responses of IgG antibodies. The antisera were much stronger than those induced by the Eastern part of **36** which is equal to conjugate **35** [56].



Scheme 13 A fully synthetic MUC1 glycopeptide vaccine in which the MUC1 B-cell epitope is covalently combined with three T-cell epitopes via squaric diamide coupling [56]

7 Glycopeptide-Tetanus Toxoid Vaccines

The fully synthetic vaccines as well as the glycopeptide conjugates with bovine serum albumin induced significant immune responses. However, overcoming the natural tolerance of the immune system towards glycopeptides as endogenous structures demands vaccines which elicit very strong immune reactions. Synthetic vaccines in which mucin glycopeptides are combined with T-cell epitopes from

tetanus toxoid had shown promising immunological properties [57–59]. The use of the tetanus toxoid itself was expected even more advantageous. Tetanus toxoid is frequently applied for vaccination in human medicine since many years. As a consequence, vaccine candidates consisting of MUC1 glycopeptide antigens and tetanus toxoid could also be used in human medicine as antitumor vaccines provided they are sufficiently effective and tumor selective. Tetanus toxoid is a much larger protein (molar weight ~ 150 kDa) than BSA, and it is very expensive. As a rule, only small amounts of this carrier are available for academic studies.

Relying on the encouraging results of the coupling reaction with the aid of diethyl squarate, the MUC1 glycopeptide antigen **21** containing the tumor-associated sialyl-Tn antigen and the *N*-terminal spacer amino acid were treated with diethyl squarate in water/ethanol at pH 8 for 1.5 h (Scheme 14). After neutralization with 1 N acetic acid, the solution was lyophilized, and the product was purified by semi-preparative HPLC to give the squaric monoamide ester **37** in high yield. This compound was characterized by high-resolution ESI mass spectrometry and by NMR spectroscopy (COSY, HSQC). The thus isolated compound was dissolved together with tetanus toxoid in aqueous sodium phosphate buffer of pH 9.5 and stirred for 3 days. After ultrafiltration through a 30 kDa membrane using deionized water and lyophilization, the MUC1-glycopeptide-tetanus toxoid conjugate **38** was isolated as a colorless amorphous substance [47]. The loading of the protein with glycopeptide antigens could not be measured by MALDI-TOF mass spectrometry. Therefore, the loading was estimated by comparative ELISA tests using an antiserum of a mouse which had been immunized with fully synthetic vaccine containing the MUC1 glycopeptide antigen **31** (see Ref. [60]). Tetanus toxoid conjugate **38** and the corresponding BSA conjugate **39** (Fig. 4) served as coats in these comparative ELISAs. The bound antibodies were photometrically determined using a biotinylated secondary anti-mouse antibody and treatment with streptavidin-horseradish peroxidase via catalyzed oxidation to give a staining (see below). It was estimated from these experiments that the tetanus toxoid vaccine **38** carries on average at least 20 molecules of the glycopeptide antigen per molecule tetanus toxoid [47]. Gravimetric estimation indicated a larger loading of on average 44 molecules glycopeptide. Because of the hydrophilicity of tetanus toxoid and its conjugate **38**, this estimation certainly is less accurate.

The immunization of mice with MUC1 glycopeptide-tetanus toxoid vaccine **38** actually resulted in very strong immune reactions of the vaccinated mice. Ten

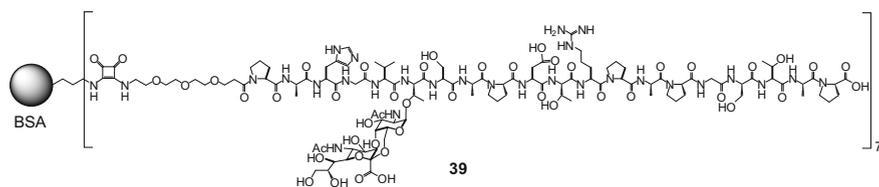


Fig. 4 BSA conjugate **39** of the MUC1 glycopeptide **21** used as coating material in ELISA analyses of antisera induced by vaccine **38**

BALB/c mice were vaccinated with 20 μg of vaccine **38** three times at intervals of 21 days. Five days after the third immunization, blood was drawn from the tail vein of each mouse. The obtained antisera were subjected to ELISA analyses in which the BSA conjugate **39** (Fig. 4) of the glycopeptide **21** served as the coating material placed in the wells of the micro-titer plates.

After washing with water, the antisera induced by vaccine **38** were added to the wells in a dilution series (Fig. 5). Incubation at 37 °C lasted for 1 h. After thoroughly washing with buffer solution the biotinylated sheep-anti-mouse antibody was added in order to determine the induced mouse antibodies which are bound to the BSA conjugate **39**. Coordination of biotin to streptavidin which is linked to the horseradish peroxidase (HPO) allowed for the quantitative determination of the bound antibodies through photometric analysis of the HPO-catalyzed oxidation of the colorless di-ammonium 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonate) (ABTS) by hydrogen peroxide to give a green radical cation. The optical density of this color in relation to the dilution of the mouse antiserum affords the titer of antibodies induced by **38** which recognize the tumor-associated MUC1 antigen presented in the BSA conjugate **39** (Fig. 5) [47].

All ten mice showed dramatic immune responses certainly strong enough to override the natural tolerance of the immune system towards the endogenous MUC1 glycopeptide structure. End point titers (10% remaining absorption) of up to 1 million were observed. For a serum diluted by a factor of 10^6 still, 10% of the original serum antibody binding was recorded. The recognition of the glycopeptide structure in conjugate **39** is structure-selective. This was demonstrated by the complete neutralization of this binding after addition of a few μg of the glycopeptide antigen **21** to the antiserum [47].

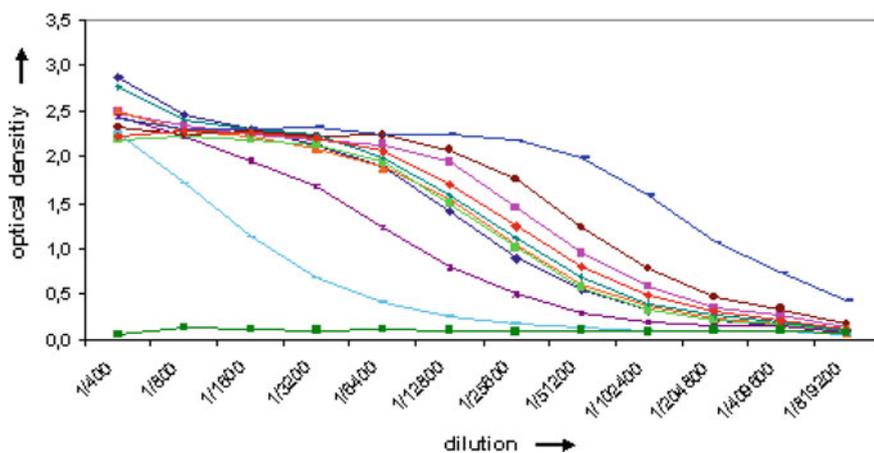


Fig. 5 ELISA analyses of antisera of ten mice immunized with MUC1 glycopeptide-tetanus toxoid vaccine **38**. The BSA conjugate **39** served as the coating material [47]; *green bottom line* negative control

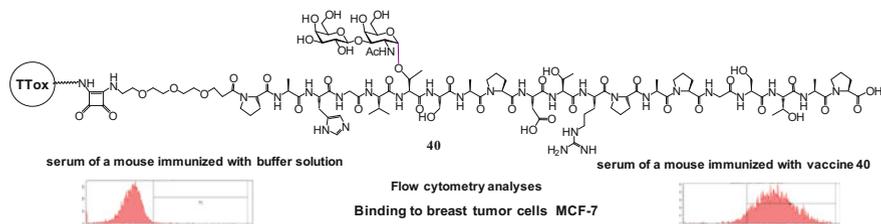
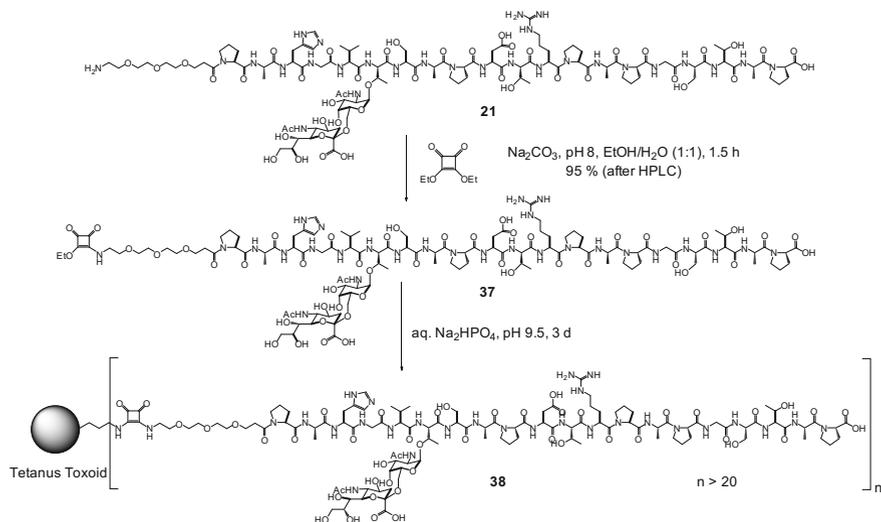


Fig. 6 Antiserum induced in mice by T-antigen MUC1 glycopeptide-tetanus toxoid vaccine **40** and its binding to MCF-7 breast tumor cells (picture on the right) [61]

In a second example of MUC1 glycopeptide-tetanus toxoid vaccine (Fig. 6) the sialyl-Tn antigen saccharide was substituted for the Thomsen-Friedenreich antigen (T-antigen). Vaccine **40** was prepared by solid-phase synthesis, deprotection, and coupling reactions in complete analogy to the construction of vaccine **38** [61]. Immunization of BALB/c mice with vaccine **40** also resulted in very strong immune responses, and the induced antiserum exhibited strong and selective recognition of the corresponding BSA conjugate which contains the T-antigen glycopeptide present in vaccine **40**. The induced antisera showed end point titers of about 800,000. The antiserum of one of the mice vaccinated with **40** was used for the investigation of its binding to breast tumor cells of cell line MCF-7. To this end, the MCF-7 tumor cells were incubated with the antiserum of this mouse, and after washing the antibodies bound to the tumor cells were detected with fluorescent-labeled goat-anti-mouse antibodies. The thus treated cells were passed through a flow cytometer in which all cells are counted by scattering a Laser beam, but analyzed according to their fluorescence. For comparison, the MCF-7 tumor cells were also incubated with the serum of a mouse which was just treated with buffer solution instead of a vaccine. The serum of this mouse showed no binding to the tumor cells (Fig. 6, left picture), whereas the 1000-fold diluted antiserum of the mouse vaccinated with vaccine **40** showed almost complete recognition of the membrane molecules present on the MCF-7 tumor cells [61].

Addition of the glycopeptide contained in the vaccine **40** to the elicited antiserum abolished this binding to the tumor cells to a large extent. This neutralization of the binding again gives evidence that the antibodies elicited by the synthetic vaccines actually bind to tumor-associated mucin MUC1 molecules exposed on the surface of the epithelial tumor cells.

In the synthesis of a third MUC1 glycopeptide antigen of this series, the tumor-associated sialyl-Tn antigen was introduced with the serine of the GSTA region of the tandem repeat, and the sequence was C-terminally extended by two amino acids in order to complete the STAPPA motif [62]. It had been found that glycosylation in this region favored a helix-type conformation of this segment which was considered characteristic for tumor-associated MUC1 [11]. The coupling of this glycopeptide antigen to tetanus toxoid was achieved via formation of the squaric monoamide ester **61** and its reaction with tetanus toxoid in aqueous sodium



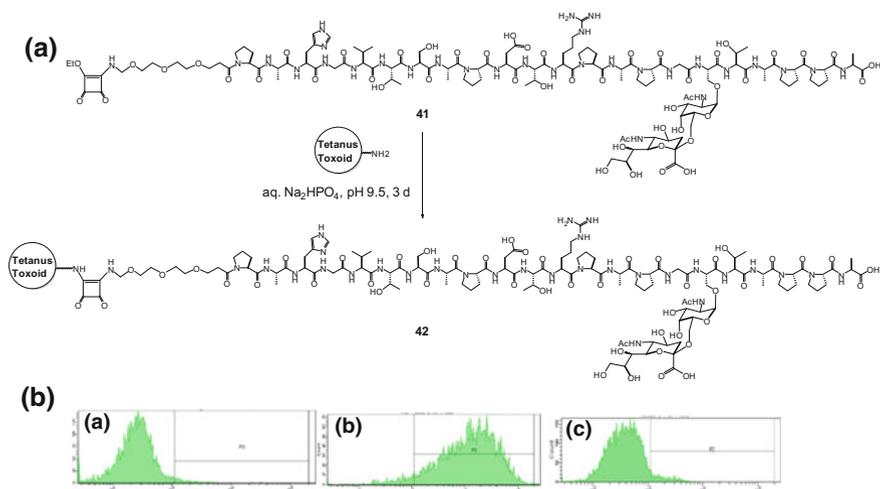
Scheme 14 Coupling of a tumor-associated MUC1 glycopeptide antigen to tetanus toxoid through squaric acid diamide formation [47]

phosphate buffer solution at pH 9.5 (Scheme 15A). Ultrafiltration and lyophilization afforded the MUC1 glycopeptide-tetanus toxoid vaccine **42** in which the glycosylation position is shifted and the STAPPA motif is completed in comparison to the vaccine **38** described above (Scheme 14).

Immunization of mice with this vaccine again elicited very strong immune responses in all animals. End point titers amounted to 500,000 and higher. The antiserum was investigated concerning the binding to the MCF-7 breast tumor cells (Scheme 15B). In comparison to the serum of a mouse which just was vaccinated with buffer solution (Scheme 15Ba), the antibodies in the antiserum of a mouse vaccinated with vaccine **42** showed more than 97% binding of the tumor cells (Scheme 15Bb). In contrast, antibodies induced against tetanus toxoid itself (Scheme 15Bc) exhibited no binding to the MCL-7 tumor cells.

ELISA with isotype-specific secondary antibodies revealed that prevalingly IgG1 antibodies had been induced (Fig. 7A) giving evidence that the switch to IgG-producing B-cells within an adaptive immune response had occurred.

In addition to probing the antibodies concerning their recognition of tumor cells in cell cultures (Scheme 15B), their capability of selective binding to tumor cells in tumor tissues was investigated [62]. Figure 7B shows two examples of mammary carcinoma tissue sections fixed with formalin and embedded in paraffin in a light microscope (magnification 1/100). On the one hand, the tissues were treated with an isotyping IgG antibody (Fig. 7B, a and c) and on the other hand with the antiserum of a mouse immunized with vaccine **42** (Fig. 7B, b and d). The antibodies bound to the tumor tissues were detected with a biotinylated secondary antibody. Its adhesion to the bound mouse antibodies was visualized with a streptavidin-horseradish



Scheme 15 **A** Formation of a MUC1 glycopeptide-tetanus toxoid vaccine (**42**) with glycosylation in the STAPPA sequence [62]; **B** Binding of antisera to MCF-7 breast tumor cells measured by flow cytometry: **a** serum of a mouse immunized with buffer solution (control); **b** antiserum of a mouse vaccinated with **42**; antiserum of a mouse vaccinated with tetanus toxoid

peroxidase conjugate that catalyzes the oxidation of 3-amino-9-ethyl-carbazole to a rose-colored dye.

While the tumor tissues in the early phase (G1 phase, Fig. 7B, a) after treatment with the antiserum of the mouse vaccinated with **42** indicated only weak binding (Fig. 7B, b) of the antibodies induced by the synthetic vaccine, the advanced tumor (Fig. 7B, c) after incubation with the antiserum elicited by vaccine **42** displayed very strong binding of the antibodies induced by vaccine **42** (Fig. 7B, d).

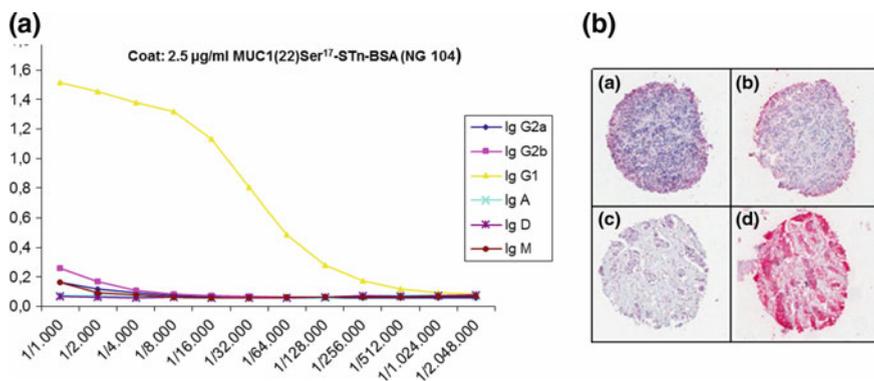


Fig. 7 **a** Antibody isotypes induced in mice through vaccination with MUC1 glycopeptide-tetanus toxoid vaccine **42**. **b** Recognition of tumor cells in mammary carcinoma tissue sections by the antiserum induced through vaccine **42** [62] in a light microscope: early stage tumor: *a*, prior to and *b*, after treatment with the antiserum; advanced tumor *c*, prior to and *d*, after treatment with the antiserum

In view of the very promising results achieved in vaccinations with the synthetic vaccine **42**, hybridomas were generated by fusing spleen cells from one of the immunized mice with murine myeloma cells. The experiments resulted in the production of a monoclonal antibody GGSK-1/30 which strongly binds to the MUC1 glycopeptide epitope presented in the vaccine **42** [63]. Flow cytometry experiments showed that monoclonal antibody GGSK-1/30 does bind with high rates to mammary carcinoma cells of cell lines T47-D and MCF-7 (~95%) and also recognize pancreas tumor cells PANC-1 with a rate of >73%, but does not recognize normal human mammary epithelial cells (HMEC) in analogous investigations. This selectivity in the binding to tumor cells was confirmed in fluorescence microscopy studies of MCF-7 and T47-D tumor cells and normal HMEC cells after treatment with monoclonal antibody GGSK-1/30 and subsequent staining with a fluorescent-labeled (Alexa Fluor 488) goat-anti-mouse secondary IgG1 antibody (Fig. 8).

The MCF-7 tumor cells (Fig. 8a) distinctly differ in their habitus from the normal epithelial cells HMEC (Fig. 8d). After incubation, these tumor cells with monoclonal antibody GGSK-1/30 and staining with the secondary antibody, their membranes brightly shine in green fluorescence (Fig. 8b) indicating the intense binding of mAb GGSK-1/30 to the molecules exposed on the membranes of the tumor cells. Since the same treatment of normal HMEC cells did not result in any effect (no fluorescence at all), the blue 4',6-diamidino-2-phenylindole (DAPI) was added as a second fluorescence dye which colorizes the nuclei (Fig. 8e). Addition of DAPI to the MCF-7 cells already labeled with GGSK-1/30 and the Alexa Fluor 488-linked secondary antibody shows both, the green fluorescence of the membranes and the blue fluorescence of the nuclei (Fig. 8c).

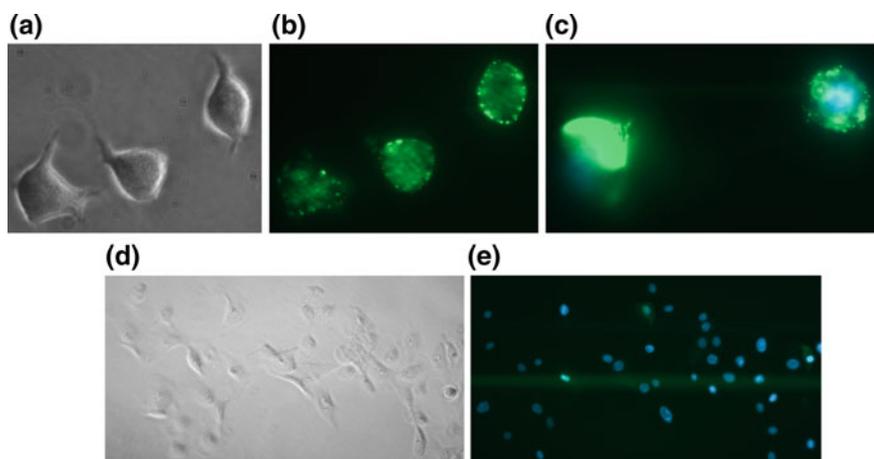


Fig. 8 Fluorescence microscopy of MCF-7 breast tumor cells (a) and normal human epithelial cells HMEC (d). b Staining of MCF-7 with monoclonal antibody GGSK-1/30 and an Alexa Fluor 488-labeled secondary antibody. c Same treatment as in b and addition of blue fluorescent DAPI visualizing the nuclei. e HMEC cells after treatment with monoclonal antibody GGSK-1/30 and an Alexa Fluor 488-labeled secondary antibody and addition of DAPI

These experiments give evidence that the monoclonal antibody induced in mice through the tumor-associated MUC1 glycopeptide-tetanus toxoid vaccine **42** completely differentiated between epithelial tumor cells and normal epithelial cells. This result is of particular importance because a strong immune response induced by the synthetic vaccine **42** which can overcome the natural tolerance against the endogenous structure should not cause severe autoimmune reactions.

8 Conclusion

Coupling reactions, in which the carbohydrate should be linked to a partner molecule to give a biological ligand structure comprising both components, are most demanding since the natural linkage needs to be installed. In a stereo- and regioselective glycosylation of a Thomsen-Friedenreich antigen with an sialyl-LewisX trichloroacetimidate as the glycosyl donor the natural β -glycoside connection was achieved. The obtained hexasaccharide-threonine building block was introduced into the synthesis of the natural glycopeptide binding site of the P-selectin glycoprotein ligand-1 (PSGL-1) which exhibited much stronger binding to P-selection than sialyl-LewisX itself.

Fortunately, for the construction of glycopeptide vaccines containing endogenous glycopeptide antigens, less strict requirements are applicable as far as the target antigen structure is appropriately presented to the immune system in the framework of the prepared vaccine. The carboxy-terminal activation of a glycopeptide by a water-soluble carbodiimide was realized in the preparation of vaccines comprising *N*-terminal glycophorin glycopeptides and bovine serum albumin. However, it can only be applied to molecules which have only one carboxy group of enhanced reactivity. More generally applicable ligation with proteins is achieved by radical-induced thiol-ene coupling reactions and conjugation reactions based on the differentiated electrophilic reactivity of squaric acid diesters. In particular, the latter have the advantage that amino functions of exposed reactivity on the glycopeptide selectively react with squaric diester in aqueous solutions at pH 8 to give the squaric monoamide ester which can be isolated and purified. It is then applicable to a mild coupling reaction in which it is subject to aminolysis by amino groups of lysine side chains of the protein. According to this strategy, MUC1 glycopeptide-tetanus toxoid vaccines are accessible, which induce very strong immune responses in mice. These immune reactions have the potential to override the natural tolerance of the immune system towards endogenous structures. The induced antibodies selectively recognize epithelial tumor cells both in cell culture and in tumor tissues, as was demonstrated for mamma carcinomas. Since a monoclonal IgG1 antibody generated from one of the immunized mice completely differentiated between normal and tumor epithelial cells encouraging preconditions are fulfilled for the development of an active vaccination of patients against their tumor diseases.

References

1. Landsteiner K (1901) Ueber Agglutinationserscheinungen normalen menschlichen Blutes. *Wiener Kl. Wochenschrift* 14:1132–1134
2. Lemieux RU (1989) The origin of the specificity in the recognition of oligosaccharides by proteins. *Chem Soc Rev* 18:347–374
3. Bundle DA, Nitz M, Wu Y, Sadowska JM (2008) A uniquely small, protective carbohydrate epitope may yield a conjugate vaccine for candida albicans. *ACS Symp Ser* 989:163–183
4. Etzler ME, Kabat EA (1970) Purification and characterization of a lectin (plant hemagglutinin) with blood group A specificity from Dolichos biflorus. *Biochemistry* 9:869–877
5. Lis H, Sharon N (1998) Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chem Rev* 98:637–674
6. Simanek EE, McGarvey GJ, Jablonowski JA, Wong C-H (1998) Selectin-Carbohydrate interactions: from natural ligands to designed mimics. *Chem Rev* 98:833–882
7. Dondoni A, Massi A, Nanni P, Roda A (2009) A new ligation strategy for peptide and protein glycosylation: photoinduced thiol-ene coupling. *Chem Eur J* 15:11444–11449
8. Lin YA, Chalker JM, Davis BG (2009) Olefin metathesis for site-selective protein modification. *ChemBioChem* 10, 959–969
9. Lee DJ, Sung-Hyun Yang S-H, Williams GM, Brimble MA (2012) Synthesis of multivalent neoglyconjugates of MUC1 by the conjugation of carbohydrate-centered, triazole-linked glycoclusters to MUC1 peptides using click chemistry. *J Org Chem* 77:7564–7571
10. Doll F, Buntz A, Späte A-K, Scharf VF, Timper A, Schimpf W, Hauck CR, Zumbusch A, Wittmann V (2016) Visualization of protein-specific glycosylation inside living cells. *Angew Chem Int Ed* 55:2262–2266
11. Braun P, Davies GM, Price MR, Williams PM, Tendler SPJ, Kunz H (1998) Effects of glycosylation on fragments of tumor associated human epithelial mucin MUC1. *Bioorg Med Chem* 6:1531–1545
12. Coltart DM, Royyuru AK, Willaims LJ, Glunz PW, Sames D, Kuduk SD, Schwarz JB, Chen X-T, Danishefsky SJ, Live DH (2002) Principles of mucin architecture: structural studies on synthetic glycopeptides bearing clustered Mono-, Di-, Tri-, and hexasaccharide glycodomains. *J Am Chem Soc* 124:9833–9844
13. Corzana F, Busto JH, Garzia de Luis M, Jimenez-Barbero J, Avenoza A, Peregrina M (2009) The nature and sequence of the amino acid aglycone strongly modulates the conformation and dynamics effects of tn antigen's clusters. *Chem Eur J* 15:3863–3874
14. Kuhn A, Kunz H (2007) Saccharide-induced peptide conformation in glycopeptides of the recognition region of li-cadherin. *Angew Chem Int Ed* 46:454–458
15. Hashimoto R, Fujitani N, Takegawa Y, Kurogochi M, Matsushita T, Naruchi K, Ohyabu N, Hinou H, Gao XD, Manri N, Satake H, Kaneko A, Sakamoto T, Nishimura S-I (2011) An efficient approach for the characterization of mucin-type glycopeptides: the effect of O-glycosylation on the conformation of synthetic mucin peptides. *Chem Eur J* 17:2393–2404
16. Bogert A, Heimbürg-Molinario J, Song X, Lasanjak Y, Ju T, Liu M, Thompson P, Raghupati G, Barany G, Smith DF, Cummings RD, Live D (2012) Deciphering structural elements of mucin glycoprotein recognition. *ACS Chem Biol* 7:1031–1039
17. Lijun X, Ramachandran V, McDaniel JM, Nguyen KN, Cummings RD, McEver RP (2003) N-terminal residues in murine P-selectin glycoprotein ligand-1 required for binding to murine P-selectin. *Blood* 101:552–559
18. Gaidzik N, Westerlind U, Kunz H (2013) The development of synthetic antitumor vaccines from mucin glycopeptide antigens. *Chem Soc Rev* 42:4421–4442
19. Angiari S, Constantin G (2013) Selectins and their ligands as potential immunotherapeutic targets in neurological diseases. *Immunotherapy* 5:1207–1220
20. Phillips ML, Nudelman E, Gaeta FC, Perez M, Singhal AK, Hakomori S, Paulson JC (1990) ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand. *Sialyl-Lex. Science* 250:1130–1132

21. Springer A (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301–314
22. Coupland LA, Parish CR (2014) Platelets, selectins, and the control of tumor metastasis. *Semin Oncol* 41:422–434
23. Leppänen A, Mehtal P, Ouyang Y-B, Ju T, Helin J, Moore KL, van Die I, Canfield WM, McEver RP, Cummings RD (1999) A novel glycosulfopeptide binds to p-selectin and inhibits leukocyte adhesion to P-selectin. *J Biol Chem* 274:24838–24848
24. Sprengard U, Kretzschmar G, Bartnik E, Hüls C, Kunz H (1995) Synthesis of an RGD Sialyl-LewisX glycoconjugate: a new highly active ligand for P-selectin. *Angew Chem Int Ed* 34:990–993
25. Koeller KM, Smith MEB, Wong C-H (2000) Tyrosine sulfation on a PSGL-1 glycopeptide influences the reactivity of glycosyltransferases responsible for synthesis of the attached O-Glycan. *J Am Chem Soc* 122:742–743
26. Brocke C, Kunz H (2004) Synthetic tumor-associated glycopeptide antigens from the tandem repeat of epithelial mucin MUC4. *Synthesis*, 525–542
27. Baumann K, Kowalczyk D, Kunz H (2008) Total synthesis of the glycopeptide recognition domain of the P-Selectin glycoprotein ligand 1. *Angew Chem Int Ed* 47:3445–3449
28. Kolb HC, Ernst B (1997) Development of tools for the design of selectin antagonists. *Chem Eur J* 3:1571–1578
29. Baumann K, Kowalczyk D, Gutjahr T, Pieczyk M, Jones C, Wild MK, Vestweber D, Kunz H (2009) Sulfated and non-sulfated glycopeptide recognition domain of P-selectin glycoprotein ligand 1 and their binding to P- and E-selectin. *Angew Chem Int Ed* 48:3174–3178
30. Review: Springer GF (1984) Tn and T, general carcinoma autoantigens. *Science* 224, 1198–1206
31. Kunz H, Birnbach S (1986) Synthesis of O-glycopeptides of the tumor-associated Tn- and T-antigen type and their binding to bovine serum albumin. *Angew Chem Int Ed* 25:360–362
32. König W, Geiger R (1970) Eine neue methode zur synthese von peptiden: aktivierung der carboxylgruppe mit dicyclohexylcarbodiimid unter zusatz von 1-hydroxy-benzotriazol. *Chem Ber* 103:788–798
33. Kunz H, von dem Bruch K (1994) Neoglycoproteins from synthetic glycopeptides. *Methods Enzymol* 247:3–30
34. Bremer PT, Kimishima A, Schlosburg JE, Zhou B, Collins KC, Janda KD (2016) Combatting synthetic designer opioids: a conjugate vaccine ablates lethal doses of fentanyl class drugs. *Angew Chem Int Ed* 55:3772–3775
35. (a) Dippold W, Steinborn A, Meyer zum Büschenfelde K-H (1999) The role of the thomsen-friedenreich antigen as a tumor-associated molecule. *Environ. Health Persp* 88, 255–257. (b) Steinborn A (1990) Dissertation: definition von Proliferations- und Differenzierungsmolekülen auf menschlichen Tumorzellen. Universität Mainz, p. 73
36. Review: Beatson RE, Taylor-Papadimitriou J, Burchell JM (2010) MUC1 Immunotherapy. *Immunotherapy* 2, 305–327
37. Zotter S, Hageman PC, Lossnitzer A, van den Tweel J, Hilkens J, Mooi WJ, Hilgers J (1988) Monoclonal antibodies to epithelial sialomucins recognize epitopes at different cellular sites in adenolymphomas of the parotid gland. *Int J Cancer Suppl* 3, 38–44
38. Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani EN, Wilson D (1990) Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J Biol Chem* 265, 15286–15293
39. Burchell JM, Mungul A, Taylor-Papadimitiou J (2001) O-Linked glycosylation in the mammary gland: changes that occur during malignancy. *J Mammary Gland Biol Neoplasia* 6:355–364
40. Hanisch F-G, Peter-Katalinic J, Egge H, Dabrowski U, Uhlenbruck G (1990) Structures of acidic O-linked poly lactosaminoglycans on human skim milk mucins. *Glycoconjugate J* 7:525–543

41. Brockhausen I, Yang JM, Burchell J, Whitehouse C, Taylor-Papadimitriou J (1995) Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. *Eur J Biochem* 233:607–617
42. Review: Becker T, Dziadek S, Wittrock S, Kunz H (2006) Synthetic glycopeptides from the mucin family as potential tools in cancer immunotherapy. *Curr Cancer Drug Targets* 6, 491–517
43. Liebe B, Kunz H (1997) Solid-phase synthesis of a Sialyl-Tn glyoundecapeptide of the MUC1 repeating unit. *Helv Chim Acta* 80:1473–1482
44. Kunz H, Unverzagt C (1988) Protective group dependent stability of intersaccharide bonds. Synthesis of fucosyl chitobiose glycopeptides. *Angew Chem Int Ed* 27:1697–1699
45. Sjölin P, Elofsson M, Kihlberg J (1996) Removal of acyl protective groups from glycopeptides. Base does not epimerize peptide stereocenters, and β -elimination is slow. *J Org Chem* 61:560–565
46. Dziadek S, Brocke C, Kunz H (2004) Biomimetic synthesis of the tumor-associated (2,3)-sialyl-T antigen and its incorporation into glycopeptide antigens from the mucins MUC1 and MUC4. *Chem Eur J* 10:4150–4162
47. Kaiser A, Gaidzik N, Westerlind U, Kowalczyk D, Hobel A, Schmitt E, Kunz H (2009) A synthetic vaccine consisting of a tumor-associated Sialyl-Tn-MUC1 tandem repeat glycopeptide and tetanus toxoid: induction of a strong and highly selective immune response. *Angew Chem Int Ed* 48:7551–7555
48. Carpino LA (1993) 1-Hydroxy-benzotriazole. An efficient peptide coupling additive. *J Am Chem Soc* 115:4397–4398
49. Yoshitake S, Imagawa M, Ishikawa E, Niitsu Y, Urushizaki I, Nishiura M, Kanazawa R, Kurosaki H, Tachibana S, Nakazawa N, Ogawa H (1982) Mild and efficient conjugation of rabbit Fab' and horseradish peroxidase using a maleimide compound and its use for enzyme immunoassay. *J Biochem* 92:1413–1424
50. Posner T (1905) Beiträge zur Kenntniss der ungesättigten Verbindungen. II. Ueber die Addition von Mercaptanen an ungesättigte Kohlenwasserstoffe. *Ber Dtsch Chem Ges* 38: 646–657
51. Wittrock S, Becker T, Kunz H (2007) Synthetic vaccines of tumor-associated glycopeptide antigens by immune-compatible thioether linkage to bovine serum albumin. *Angew Chem Int Ed* 46:5226–5230
52. Dondoni A, Massi A, Nanni P, Roda A (2009) A new ligation strategy for peptide and protein glycosylation: photoinduced thiol–ene coupling. *Chem Eur J* 15:11444–11449
53. Glaffig M, Kunz H (2016) unpublished experiments
54. Tietze LF, Arlt M, Beller M, Glüsenkamp K-H, Jäde E, Rajewski MF (1991) Squaric acid diethyl ester: a new coupling reagent for the formation of drug bio-polymer conjugates. *Chem Ber* 124:1215–1221
55. Dziadek S, Kowalczyk D, Kunz H (2005) Synthetic vaccines consisting of tumor-associated MUC1 glycopeptide antigens and bovine serum albumin. *Angew Chem Int Ed* 44:7624–7630
56. Palitzsch B, Hartmann S, Stergiou N, Glaffig M, Schmitt E, Kunz H (2014) A fully synthetic four-component antitumor vaccine consisting of a mucin glycopeptide antigen combined with three different T-helper cell epitopes. *Angew Chem Int Ed* 53:14245–14249
57. Keil S, Claus C, Dippold W, Kunz H (2001) Towards the development of antitumor vaccines: a synthetic conjugate of a tumor-associated MUC1 glycopeptide antigen and a tetanus toxin epitope. *Angew Chem Int Ed* 40:366–369
58. Wilkinson BL, Day S, Malins LR, Apostolopoulos V, Payne RJ (2011) Self-adjuvanting multicomponent cancer vaccine candidates combining per-glycosylated MUC1 glycopeptides and the Toll-like receptor 2 agonist Pam₃CysSer. *Angew Chem Int Ed* 50, 1635–1639
59. Cai H, Chen M-S, Sun Z-Y, Zhao Y-F, Kunz H, Li Y-M (2013) Self-adjuvanting synthetic antitumor vaccine from MUC1 glycopeptides conjugated to T-cell epitopes from tetanus toxoid. *Angew Chem Int Ed* 52:6106–6110

60. Dziadek S, Hobel A, Schmitt E, Kunz H (2005) A fully synthetic vaccine consisting of a tumor-associated glycopeptide antigen and a T-cell epitope for the induction of a highly specific humoral immune response. *Angew Chem Int Ed* 44:7630–7635
61. Hoffmann-Röder A, Kaiser A, Wagner S, Gaidzik N, Kowalczyk D, Westerlind U, Gerlitzki B, Schmitt E, Kunz H (2010) Synthetic antitumor vaccines from tetanus toxoid conjugates of MUC1 glycopeptides with the Thomsen-Friedenreich antigen and a fluorine substituted analogue. *Angew Chem Int Ed* 49:8498–8503
62. Gaidzik N, Kaiser A, Kowalczyk D, Westerlind U, Gerlitzki B, Sinn HP, Schmitt E, Kunz H (2011) Synthetic antitumor vaccines containing MUC1 glycopeptides with two immunodominant domains—induction of a strong immune response against breast tumor tissues. *Angew Chem Int Ed* 50:99778–99981
63. Palitzsch B, Gaidzik N, Stergiou N, Stahn S, Hartmann S, Gerlitzki B, Teusch N, Flemming P, Schmitt E, Kunz H (2016) A synthetic glycopeptide vaccine for the induction of a monoclonal antibody that differentiates between normal and tumor mammary cells and enables the diagnosis of human pancreatic cancer. *Angew Chem Int Ed* 55:2894–2898



<http://www.springer.com/978-3-319-65586-4>

Coupling and Decoupling of Diverse Molecular Units in
Glycosciences

Witczak, Z.J.; Bielski, R. (Eds.)

2018, XIV, 335 p. 244 illus., 81 illus. in color., Hardcover

ISBN: 978-3-319-65586-4