Antitumor Agents

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Synthetic Vaccines Consisting of Tumor-Associated MUC1 Glycopeptide Antigens and Bovine Serum Albumin**

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The identification of cell-surface components that occur only on tumor but not on healthy cells is essential for a selective immunological attack on tumor tissue. About 30 years ago, Springer[1] described membrane glycoproteins containing the Thomsen–Friedenreich T antigen and the monosaccharide Tn antigen side chains as tumor-associated antigens on epithelial tumors. These T and Tn glycoproteins were described to be structurally related to the N-terminal region of asialoglycophorin. Based on this structural information, we synthesized a vaccine consisting of N-terminal glycopeptides from asialoglycophorin of M blood group specificity conjugated with bovine serum albumin (BSA)[2] and employed it in the immunization of mice. Although the monoclonal antibody (82-A6) obtained from these experiments showed affinity to epithelial tumor cells, the selectivity for tumor cells was not sufficient.[3] However, the antibody 82-A6 was capable of distinguishing clearly between asialoglycophorin of M and N

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blood groups,[3] indicating that not only the saccharide, but also the peptide sequence, contributed to the recognized epitope. From this observation we inferred that apart from tumor-associated saccharides, tumor-selective peptide structural elements are required to render an antigen sufficiently tumor-selective.

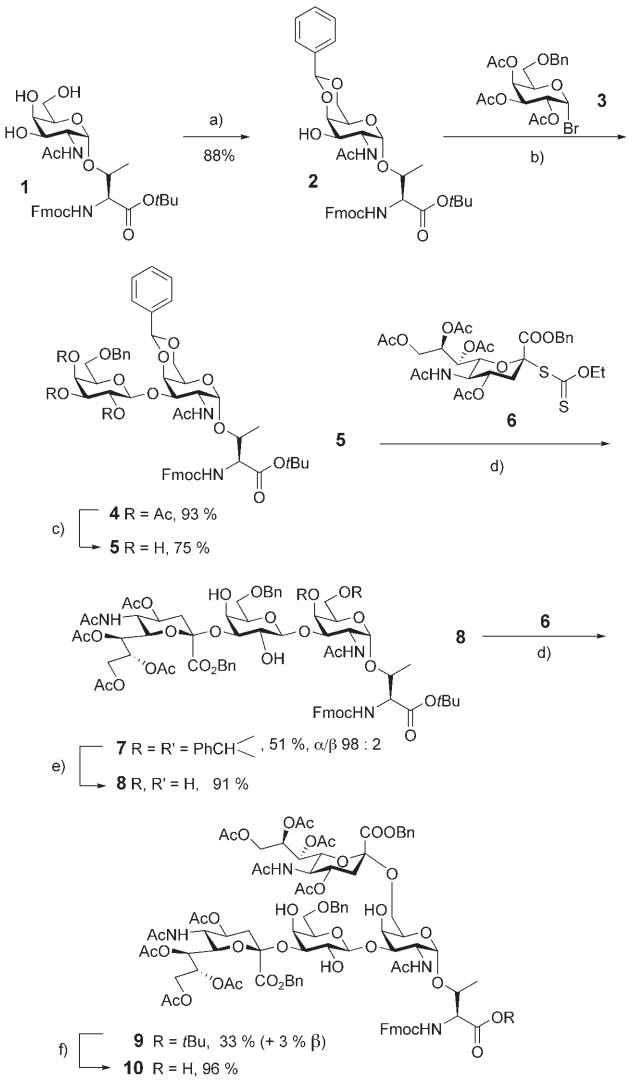
Structural leads for the design of tumor-selective glycopeptide antigens are obtained from analyses of the tumor-associated epithelial mucin MUC1,[4] which is extensively over-expressed on tumor cells. The extracellular portion of MUC1 contains numerous repeating units of the amino acid sequence HGVTSAPDTRPAPGSTAPPA.[5] Most *O*-glycosylation sites are located within these tandem repeats. Owing to the down-regulation of a glucosaminyl transferase (C-2GnT-1) and the concomitant overexpression of sialyl transferases,[6] MUC1 on tumor cells carries short, prematurely sialylated saccharide side chains. Antibodies induced with MUC1 isolated from tumor tissues[4,5] were used to identify the peptide motif PDTRPAP as an immundominant epitope within the MUC1 tandem repeat.[7] The specificity of these anti-MUC1 antibodies was verified with synthetic Tn- and T-antigen glycopeptides.[8,9] Moreover, saturation transfer difference NMR analyses revealed the conformation of a Tn antigen pentapeptide from MUC1 bound to a monoclonal antibody.[10]

In contrast to such studies, which proved valuable for analytical purposes, immunizations with MUC1 extracted from tumor tissues as a vaccine appear not to be promising, because MUC1 from biological isolates generally displays microheterogeneity and contains structural elements that also occur on healthy cells.

For this reason we focused our attention on the development of anticancer vaccines based on defined synthetic Tn, T, and sialyl–Tn glycopeptides[11,12] from MUC1.[13] A construct, in which a glycopeptide sequence from the MUC1 tandem repeat carrying a sialyl–Tn side chain is conjugated through a polar spacer amino acid with a partial T-cell epitope from tetanus toxoid, was used to induce the proliferation of cytotoxic T cells in cell cultures of peripheral blood lymphocytes (PBLs).[14] This effect occurred exclusively in the presence of antigen-presenting cells. Apart from cytotoxic T-cell response, strong T-helper-cell-dependent antibody production is of crucial importance for the development of an efficient antitumor vaccine and requires the use of glycopeptide antigens conjugated with carrier proteins such as BSA or KLH (keyhole limpet hemocyanine).

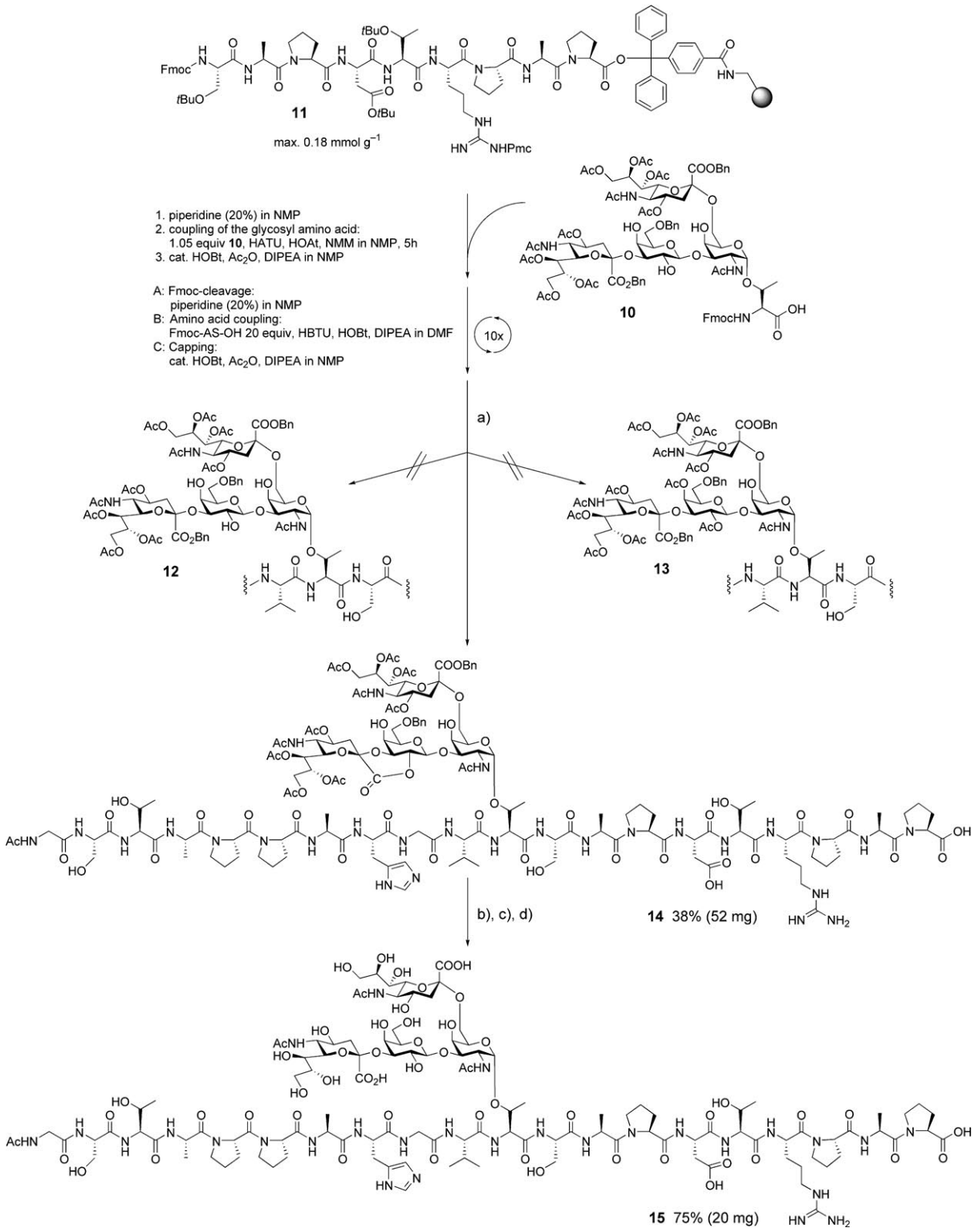
Herein we describe the synthesis of a series of glycopeptide antigens that bear the sialyl-Tn, (2,6)-sialyl-T, (2,3)-sialyl-T, or the glycophorin (2,3-2,6-bissialyl)-T antigens and contain the partial or complete tandem repeat sequence of MUC1. We also report methods to couple these tumor-selective cell surface structures to carrier proteins or to bind them to microtiter plates. The synthetic strategy relies on the synthesis of the corresponding *O*-glycosyl amino acids, followed by sequential solid-phase glycopeptide synthesis and subsequent conjugation to proteins or immobilization.

A range *O*-glycosyl amino acids were synthesized according to a biomimetic strategy[15] starting from the same precursor. Scheme 1 illustrates the preparation of the most



Scheme 1. a) α,α-Dimethoxytoluene, cat. *p*-TsOH, CH_3CN, 3 h; b) $Hg(CN)_2$, CH_2Cl_2/CH_3NO_2 (2:3), 4-Å molecular sieves; c) 1. NaOMe, MeOH, pH 8.5–9, 8 h; 2. Fmoc-OSu, dioxane/H_2O (1:1), NMM; d) MeSBr, AgOTf, CH_3CN/CH_2Cl_2 (2:1), −68 °C, 3-Å molecular sieves; e) 80% AcOH (aq), 80 °C; f) CF_3COOH, anisole (10:1), 2 h.

complex of the tumor-associated MUC1 saccharide antigens, the (2,3-2,6-bissialyl)-T–threonine conjugate. The *N*-Fmoc protected Tn antigen threonine *tert*-butyl ester **1**[16] deprotected at its glycan moiety served as common starting material for the synthesis of all tumor-associated mucin carbohydrate antigens. The base and acid sensitivity of the Fmoc group and the *tert*-butyl ester must be carefully taken into account in all protecting-group manipulations and glycosylation reactions. The precursor **1** was treated with α,α-dimethoxy toluene in acetonitrile in the presence of catalytic *p*-toluenesulfonic acid at pH 4 to give 4,6-benzylidene acetal **2**. Although activation of a glycosyl trichloroacetimidate[17] with trimethylsilyl trifluormethanesulfonate for 3-β-galactosylation is usually very efficient, in this case the benzylidine acetal and *tert*-butyl ester were cleaved or, after addition of sufficient amounts of molecular sieves, predominantly gave the corresponding orthoester.[18] The most successful route involved activation



Scheme 2. a) TFA, TIS, H_2O (15:0.9:0.9); b) H_2, Pd/C (10%), MeOH; c) NaOMe/MeOH, pH 9.5; d) NaOH (aq), pH 11.5, 1 h; then AcOH. DIPEA = diisopropylethylamine.

of the 6-*O*-benzyl protected galactosyl bromide **3**[19] with mercury cyanide (according to the procedure of Helferich and Wedemeyer[20]) in nitromethane/dichloromethane which stereoselectively furnished the T-antigen–threonine conjugate **4** in high yield.

The selective *O*-deacetylation of **4** proved to be particularly challenging. On a 1-g scale, use of catalytic NaOMe in methanol under careful adjustment of the pH value to 8.5 furnished **5** in 62% yield. On a larger scale (5 g) and at pH 8.6–9, not only **5** (39%) but also the *N*-deprotected

conjugate (46%) were formed. Subsequent *N*-acylation of the latter with 9-fluorenyl-methoxycarbonyl-*N*-hydroxy-succinimide gave more of the desired product (36%), resulting in an overall yield of 75% of **5**. Sialylation of **5** with xanthate **6** of the peracetylated sialic acid benzyl ester[14] activated with methylsulfenyl triflate[21] gave **7** with nearly complete regio- and stereoselectivity. After selective cleavage of the benzylidene acetal with aqueous acetic acid (80% *v*/*v*) at 80 °C[22] to afford **8**, a second sialylation step under identical conditions gave the bissialyl-T-antigen–threonine conjugate **9**. Although the conversion remained incomplete after 3.5 h, **9** (33%) as well as some of the corresponding β-anomer (3%) were isolated by preparative HPLC.[23] Acidolysis of the *tert*-butyl ester with trifluoroacetic acid/anisole (10:1) furnished the Fmoc-protected bissialyl-T–threonine building block **10**,[24] which was incorporated into the solid-phase synthesis without further *O*-acetylation.[25] Of the other tumor-associated glycosyl amino acid antigens, the Tn antigen **1**, T antigen **4**, and 2,3-sialyl-T antigen **7** are directly accessible by the synthetic route shown in Scheme 1, while the sialyl-Tn antigen is obtained by regioselective sialylation of **1**[14,16] and the 2,6-sialyl-T antigen through regioselective sialylation after cleavage of the benzylidene acetal in **4**.[15,26]

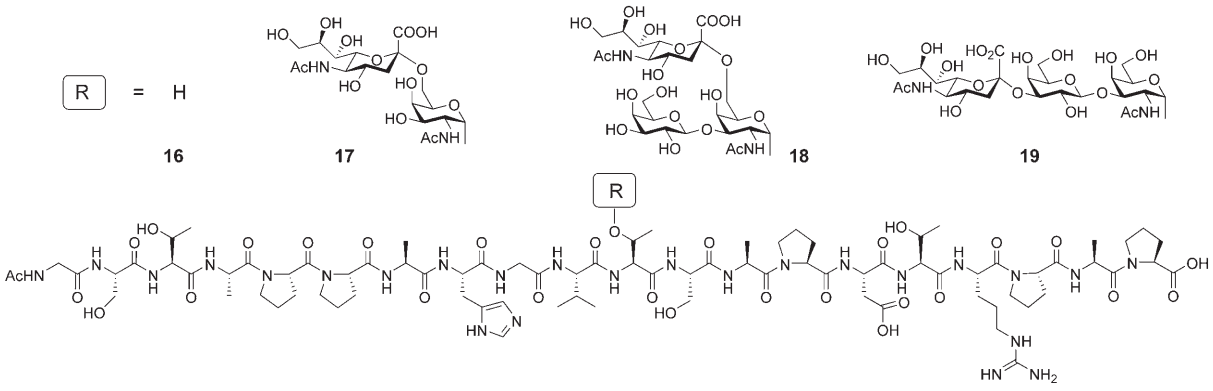
These building blocks were all subsequently applied in the solid-phase glycopeptide synthesis of various MUC1 tandem repeat glycopeptides, as illustrated in Scheme 2 for the bissialyl-T antigen MUC1 glycopeptide. Fmoc-proline was coupled through a trityl linker[27] to a Rapp-Tentagel polymer[28] and used to assemble the protected nonapeptide **11** from MUC1 according to the Fmoc strategy.[29] After cleavage of the Fmoc group with piperidine/*N*-methylpyrrolidone (NMP) (1:4), the *O*-glycosyl amino acid **10** (1.05 equiv) was coupled through activation with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt),[30] and *N*-methylmorpholine (NMM) in NMP. All non-glycosylated Fmoc-protected amino acids were coupled with *O*-(1H-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt),[31] and Hünig base (*i*Pr$_2$NEt) in DMF. The remaining free amino components were capped with acetic anhydride/HOBt/*i*Pr$_2$NEt after each coupling cycle.[31c]

After completion of the peptide sequence, the trityl linker and the acid-labile side-chain protecting groups were cleaved simultaneously with trifluoroacetic acid (TFA), triisopropylsilane (TIS), and small amounts of water.[32] This cleavage procedure liberated neither the expected glycoeicosapeptide **12** with unaltered carbohydrate side chain nor the completely *O*-acetylated analogue **13** formed through the capping reactions. Instead, glycopeptide **14**,[33] which bears a lactone structure formed between the 2,3-linked sialic acid and the 2-OH group of the galactose moiety, was isolated in an overall yield of 38% after approximately 40 steps. Subsequent hydrogenolysis of the benzyl ester and benzyl ether, transesterification with NaOMe/methanol (pH 9.5), opening of the lactone with aqueous NaOH (pH 11.5), neutralization with acetic acid, lyophilization, and purification by semipreparative HPLC furnished the desired MUC1 tandem repeat glycopeptide **15**[34] in 75% yield (based on **14**).

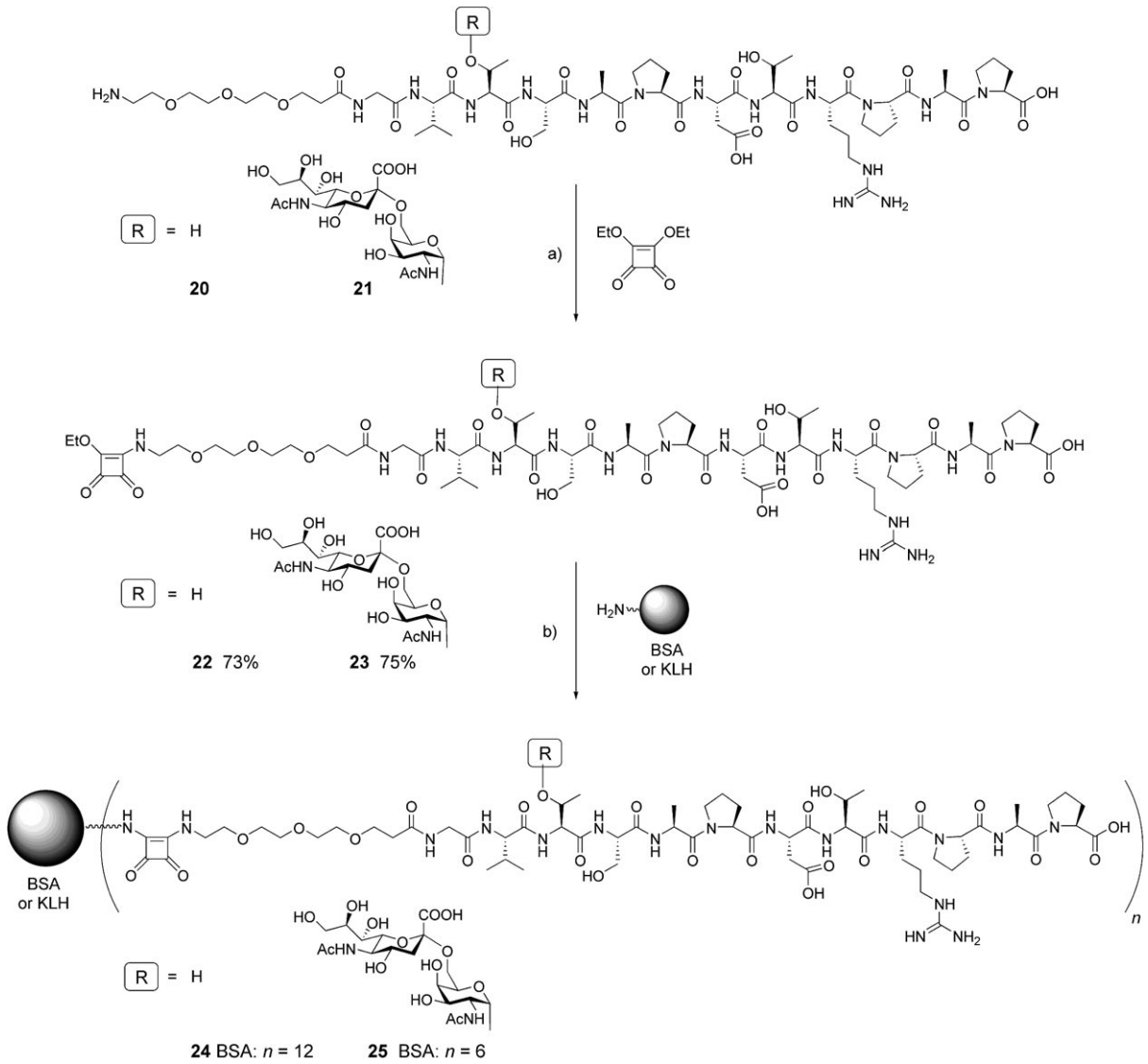
In analogous procedures, the MUC1 eicosapeptide **16** as well as the complete tandem repeat glycopeptides from MUC1 carrying the other important tumor-associated sialyl saccharide antigens, sialyl-Tn (**17**), 2,6-sialyl-T (**18**), and 2,3-sialyl-T (**19**), were synthesized in significantly higher overall yields (Scheme 3). No formation of a lactone was observed during the acidolysis of the trityl linker in any of these syntheses.

According to the procedure illustrated in Scheme 2, the dodecapeptide **20** equipped with a triethylene glycol spacer with an amino group[14] as well as the analogous sialyl-Tn dodecapeptide **21** from MUC1 were synthesized to serve as model compounds for conjugation with bovine serum albumin (BSA) and for immobilization on microtiter plates (Scheme 4).[35] For this purpose, the N-terminal Fmoc group was removed from the resin-bound peptide and all acid-labile side-chain protecting groups were cleaved during the acidolysis of the trityl anchor. The synthesis of sialyl-Tn glycopeptide **21** required the additional removal of the carbohydrate-protecting groups by hydrogenolysis and transesterification catalyzed by NaOMe after cleavage from the resin.

To conjugate the synthetic MUC1 peptides and glycopeptides with proteins, recourse was made to the procedure described by Tietze et al.[36] for the conjugation of a lactosamine phenyl glycoside. The amino groups of the spacers of **20**



Scheme 3. Eicosapeptide **16** and the complete tandem repeat glycopeptides from MUC1 carrying sialyl-Tn (**17**), 2,6-sialyl-T (**18**), and 2,3-sialyl-T (**19**).



Scheme 4. a) Ethanol/water (1:1), Na_2CO_3, pH 8.0, 2 h, preparative HPLC; b) sodium borate (0.07 M), $NaHCO_3$ (0.035 M), pH 9, 24 h, gel filtration chromatography (Sephadex-G25 PD10 column), dialysis against H_2O.

and **21** were converted into the corresponding squaric monoamides **22** and **23**[37] by using diethyl squarate (3,4-diethoxy-3-cyclobutene-1,2-dione) in ethanol/water at pH 8. After purification by preparative HPLC, the activated peptide structures were coupled to BSA in a sodium borate buffer at pH 9.

Conjugation of peptide **22** resulted in a coupling yield of approximately 60% within 24 h and furnished vaccine **24** with an average degree of incorporation of 12 peptide molecules per molecule of protein as determined by MALDI-TOF MS. In the case of glycopeptide **23** the coupling to furnish **25** proceeded more slowly, and led to the incorporation of only half as many antigen molecules. Furthermore, the glycopeptide antigen equipped with the spacer was covalently immobilized on microtiter plates[38] functionalized with a secondary amine **26** (Scheme 5). The covalent linkage of the MUC1 antigen to the microtiter plates (see **27**)[39] prevents the loss of material in ELISA tests when washing with water, which had previously caused difficulties while working with glycopeptide antigens.

The immobilized and conjugated MUC1 glycopeptide antigens not only provide vaccines with a highly defined structure representing tumor-selective surface antigens but also provide tools for the verification of the structural selectivity of an immune response. The initial results are promising[40] and encourage further exploration of the entire spectrum of structures characterized by the synthetic glycopeptide antigens such as **17**, **18**, **19**, **21**, **23**, and **25** for the development of antitumor vaccines.

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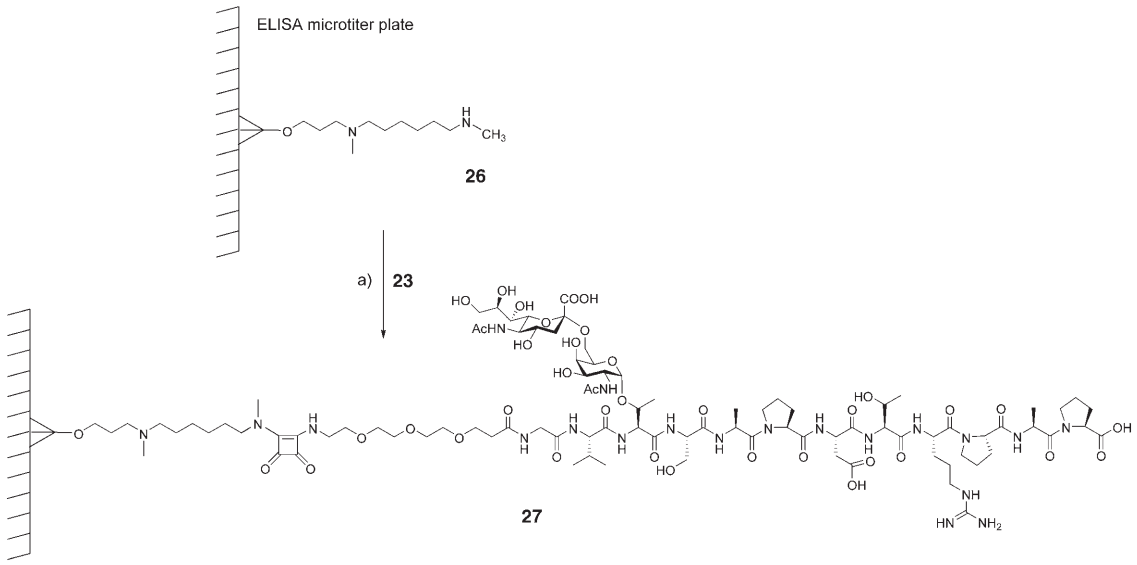

Scheme 5. a) sodium borate (0.07 M), $NaHCO_3$ (0.035 M), pH 9.

Keywords: antigens · antitumor agents · carbohydrates · glycopeptides · synthetic vaccines

[1] G. F. Springer, *Science* **1984**, *224*, 1198.

[2] H. Kunz, S. Birnbach, *Angew. Chem.* **1986**, *98*, 354; *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 360.

[3] a) W. Dippold, A. Steinborn, K.-H. Meyer zum Büschenfelde, *Environ. Health Perspect.* **1990**, *88*, 255; W. Dippold, A. Steinborn, S. Birnbach, H. Kunz, unpublished results; A. Steinborn, PhD thesis, Universität Mainz, **1990**; b) S. Dziadek, H. Kunz, *Chem. Rec.* **2004**, *4*, 308.

[4] a) S. Gendler, C. A. Lancaster, J. Taylor-Papadimitriou, T. Duhig, N. Peal, J. Burchell, L. Pemberton, E. N. Lalani, P. Wilson, *J. Biol. Chem.* **1990**, *265*, 15286; b) for a short overview, see: J. Taylor-Papadimitriou, J. M. Burchell, D. W. Miles, M. Dalziel, *Biochim. Biophys. Acta* **1999**, *1455*, 301.

[5] D. M. Swallow, S. J. Gendler, B. Griffith, G. Corney, J. Taylor-Papadimitriou, *Nature* **1987**, *328*, 82.

[6] a) I. Brockhausen, *Biochim. Biophys. Acta* **1999**, *1473*, 67; I. Brockhausen, *Biochem. Soc. Trans.* **2003**, *31*, 318; b) K. O. Lloyd, J. Burchell, V. Kundryashov, B. W. Yin, J. Taylor-Papadimitriou, *J. Biol. Chem.* **1996**, *271*, 33325; c) J. M. Burchell, A. Mungul, J. Taylor-Papadimitriou, *J. Mammary Gland. Biol. Neoplasia* **2001**, *6*, 355.

[7] a) J. Burchell, J. Taylor-Papadimitriou, M. Boshell, S. Gendler, T. Duhig, *Int. J. Cancer* **1989**, *44*, 691; b) M. R. Price, F. Hudecz, C. O'Sullivan, R. W. Baldwin, P. M. Edwards, S. B. J. Tendler, *Mol. Immunol.* **1990**, *62*, 795.

[8] a) G. F. Hanisch, S. Müller, *Glycobiology* **2000**, *10*, 439; b) S. Müller, F. G. Hanisch, *J. Biol. Chem.* **2002**, *277*, 26103; c) U. Karsten, N. Serttas, H. Paulsen, A. Danielczyk, S. Goletz, *Glycobiology* **2004**, *14*, 681.

[9] S. von Mensdorff-Pouilly, F. G. M. Snijdewint, A. A. Verstraeten, R. H. M. Verheijen, P. Kenemans, *Int. J. Biol. Markers* **2000**, *15*, 343.

[10] H. Möller, N. Serttas, H. Paulsen, J. M. Burchell, J. Taylor-Papadimitriou, B. Meyer, *Eur. J. Biochem.* **2002**, *269*, 1444.

[11] a) O. Seitz, H. Kunz, *Angew. Chem.* **1995**, *107*, 901; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 803; b) M. Leuck, H. Kunz, *J. Prakt. Chem.* **1997**, *339*, 332; c) P. Braun, G. M. Davies, M. R. Price, P. M. Williams, S. B. J. Tendler, H. Kunz, *Bioorg. Med. Chem.* **1998**, *6*, 1531.

[12] B. Liebe, H. Kunz, *Angew. Chem.* **1997**, *109*, 629; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 618.

[13] Other groups also synthesized mucin glycopeptides with Tn, T, and sialyl-Tn antigen saccharides: a) E. Meinjohanns, M. Meldal, A. Schleyer, H. Paulsen, K. Bock, *J. Chem. Soc. Perkin Trans.* 1 **1996**, 985; b) S. D. Kuduk, J. B. Schwarz, X.-T. Chen, P. W. Glunz, D. Sames, G. Ragapunti, P. O. Livingston, S. J. Danishefsky, *J. Am. Chem. Soc.* **1998**, *120*, 12474; c) J. B. Schwarz, S. D. Kuduk, X.-T. Chen, D. Sames, P. W. Glunz, S. J. Danishefsky, *J. Am. Chem. Soc.* **1999**, *121*, 2662; d) M. A. Reddish, L. Jackson, R. R. Koganti, D. Qui, W. Hong, B. M. Longenecker, *Glycoconjugate J.* **1997**, *14*, 549.

[14] S. Keil, C. Claus, W. Dippold, H. Kunz, *Angew. Chem.* **2001**, *113*, 379; *Angew. Chem. Int. Ed.* **2001**, *40*, 366.

[15] a) C. Brocke, H. Kunz, *Synlett* **2003**, 2052; b) S. Dziadek, H. Kunz, *Synlett* **2003**, 1623.

[16] B. Liebe, H. Kunz, *Helv. Chem. Acta.* **1997**, *80*, 1473.

[17] a) R. R. Schmidt, J. Michel, *Angew. Chem.* **1980**, *92*, 763; *Angew. Chem. Int. Ed. Engl.* **1980**, *19*, 731; b) R. R. Schmidt, *Angew. Chem.* **1986**, *98*, 213; *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 212.

[18] T. Reipen, H. Kunz, *Synthesis* **2003**, 2487.

[19] M. Lergenmüller, Y. Ito, T. Ogawa, *Tetrahedron* **1988**, *44*, 1381.

[20] B. Helferich, F. Wedemeyer, *Justus Liebigs Ann. Chem.* **1949**, *563*, 139.

[21] F. Dasgupta, P. Garegg, *Carbohydr. Res.* **1988**, *177*, C13.

[22] N. Mathieux, H. Paulsen, M. Meldal, K. Bock, *J. Chem. Soc. Perkin Trans.* 1 **1997**, 2359.

[23] **9**: $[\alpha]_D^{22} = 16.6$ ($c = 1$, $CHCl_3$); MALDI-TOF MS (dhb, positive): m/z: 1975.2 $[M+Na]^+$, 1991.2 $[M+K]^+$; HR ESI-TOF MS (positive): calcd: 1973.7271; found: 1973.7257; ^1H NMR (400 MHz, $CDCl_3$, COSY, TOSY, HMQC, HMBC): $\delta = 4.81$ ($J_{1,2} = 3.9$ Hz; 1-H), 3.32 (d, 1H, $J_{3',4'} = 2.7$ Hz; 4′-H), 2,71 (dd, 1H, $J_{3eq'',3ax''} = 12.9$ Hz, $J_{3eq'',4''} = 4.7$ Hz; 3″-H_{eq}), 2.54 ppm (dd, 1H, $J_{3eq''',3ax'''} = 12.7$ Hz, $J_{3eq''',4'''} = 4.7$ Hz; 3‴-H_{eq}).

[24] **10**: $[\alpha]_D^{22} = 20.3$ ($c = 1$, $CHCl_3$); HR ESI-TOF MS (positive): calcd: 1917.6645; found: 1917.6743 $[M+Na]^+$.

[25] The removal of *O*-acetyl groups, in particular those on the 4-OH groups of galactose moieties in sialyl-Lewis^x glycopeptides (M. Rösch, H. Herzner, W. Dippold, M. Wild, D. Vestweber, H.

Kunz, *Angew. Chem.* **2001**, *113*, 3954; *Angew. Chem. Int. Ed.* **2001**, *40*, 3836) and in sialyl-T glycopeptides[15] can be difficult.
[26] C. Brocke, H. Kunz, *Synthesis* **2004**, 525.
[27] J. M. J. Frechét, K. E. Haque, *Tetrahedron Lett.* **1975**, *16*, 3055.
[28] E. Bayer, W. Rapp, *Chem. Pept. Proteins* **1986**, *3*, 3.
[29] L. A. Carpino, G. Y. Han, *J. Am. Chem. Soc.* **1970**, *92*, 5748.
[30] L. A. Carpino, D. Jonescu, A. El-Falham, *J. Org. Chem.* **1996**, *61*, 2460.
[31] a) R. Knorr, A. Trzeciak, W. Bannwarth, D. Gillessen, *Tetrahedron Lett.* **1989**, *30*, 1927; b) V. Dourtoglou, B. Gross, V. Lambropoulou, C. Ziondrou, *Synthesis* **1984**, 572; c) W. König, R. Geiger, *Chem. Ber.* **1970**, *103*, 788.
[32] D. A. Pearson, M. Blanchette, M. L. Baker, C. A. Guidon, *Tetrahedron Lett.* **1989**, *30*, 2739.
[33] **14**: MALDI-TOF MS (dhb, positive): *m*/*z*: 3376.0 $[M+H]^+$, 3398.0 $[M+Na]^+$, 3414.3 $[M+K]^+$; ^{1}H NMR (600 MHz, [D$_6$]DMSO, COSY, TOCSY, HMQC, HMBC): $\delta = 4.76$ (2′-H), 4.21 (4′-H), 4.01 ppm (3′-H).
[34] **15**: 19.4 mg; $[\alpha]_D^{22} = -117.4$ ($c = 1$, H$_2$O); MALDI-TOF MS (dhb, positive): *m*/*z*: 2876.6 $[M+H]^+$; ^{1}H NMR (600 MHz, [D$_6$]DMSO, COSY, HMQC, HMBC): $\delta = 4.79$ (d, 1H, $J_{1,2} = 2.1$ Hz, 1-H), 2.66 ppm (dd, 1H, $J_{3eq',3ax'} = 13.7$ Hz, $J_{3eq,4'} = 6.7$ Hz; 3″-H$_{eq}$); ^{13}C NMR (150.9 MHz, [D$_6$]DMSO, HMQC, HMBC): $\delta = 104.5$ (C1′), 98.8 (C1), 98.4 (C2″), 98.1 ppm (C2‴).
[35] According to our previous experience, the incorporation of a flexible, hydrophilic spacer is important for immunological evaluation of synthetic vaccines.[14]
[36] L. F. Tietze, C. Schröder, S. Gabius, U. Brinck, A. Goerlach-Graw, H.-J. Gabius, *Bioconjugate Chem.* **1991**, *2*, 148.
[37] **23**: $[\alpha]_D^{26} = -68.4$ ($c = 1$, H$_2$O); MALDI-TOF MS (dhb, positive): *m*/*z*: 1991.4 $[M+H]^+$; ^{1}H NMR (400 MHz, CD$_3$OD, COSY, HMQC): $\delta = 4.97$ (d, 1H, $J_{1,2} = 3.5$ Hz; 1-H), 4.76–4.64 (m, 3H; CH$_2$O-squarate, H$_\alpha$-Arg), 2.66 ppm (dd, 1H, $J_{3eq',3ax'} = 12.7$ Hz, $J_{3eq',4'} = 4.7$ Hz; 3′-H$_{eq}$).
[38] A. Bergh, B.-G. Magnusson, J. Ohlsson, U. Wellmar, U. J. Milsson, *Glycoconjugate J.* **2005**, *18*, 615.
[39] **27**: For carbohydrate detection with phenol/H$_2$SO$_4$, see reference [2].
[40] See the following Communication in this issue: S. Dziadek, A. Hobel, E. Schmitt, H. Kunz, *Angew. Chem.* **2005**, *117*, 7803; *Angew. Chem. Int. Ed.* **2005**, *44*, 7630.

