Antitumor Vaccines

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A Synthetic Vaccine Consisting of a Tumor-Associated Sialyl-T_N-MUC1 Tandem-Repeat Glycopeptide and Tetanus Toxoid: Induction of a Strong and Highly Selective Immune Response**

*Anton Kaiser, Nikola Gaidzik, Ulrika Westerlind, Danuta Kowalczyk, Alexandra Hobel, Edgar Schmitt, and Horst Kunz**

Dedicated to Professor Herbert Meier on the occasion of his 70th birthday

The development of an antitumor vaccine has to meet three important criteria: The induced immune response has to be strong enough to override the tolerance against tumor associated self antigens such as glycolipids and glycoproteins, which are structures present on normal cells;[1] the presented antigen must be tumor specific, strongly expressed, and different from related structures on normal cells;[2] and the immune response must result in the formation of highly selective antisera,[3] so that an autoimmune reaction against healthy tissue is not triggered. The membrane-bound tumor-associated glycoprotein MUC1[4] could meet these last two criteria. It is expressed on almost all epithelial tissues and highly overexpressed on tumor tissue.[5] The glycan pattern from MUC1 on tumor cells is distinctly altered in comparison to that of normal cells.[2,5] This alteration is a result of a changed activity of glycosyltransferases in the biosynthesis of the mucins in epithelial tumor cells: the glucosaminetransferase GnT2 is strongly down-regulated, whereas sialyltransferases are up-regulated.[5,6] Because of this changed enzyme activity, in particular the T_N antigen and the sialyl-T_N antigen are characteristic tumor-associated saccharide antigens and have been found on epithelial tumor tissue.[4–7] As a result of the modified enzyme activity, the average saccharide length of MUC1 is much shorter on epithelial tumor cells than on normal epithelial cells. Hence, not only the saccharide,[8] but also the peptide backbone from the extracellular tandem-repeat region contribute to the tumor specific glycopeptide epitopes.[9]

Fully synthetic vaccines, consisting of MUC1 glycopeptides linked through an oligoethylene glycol spacer to an ovalbumin (OVA) T-cell epitope were shown to induce highly selective antisera in transgenic mice.[10,11] These sera are virtually monoclonal. The antibodies recognize the tumor-associated MUC1 glycopeptide selectively; neither the identical non-glycosylated peptide sequence, nor the tumor-associated saccharide antigen linked to a different peptide sequence, for example MUC4, are recognized.[12] The humoral immune response is specific after the third immunization, and of the IgG type. Unfortunately, it is effectual for only every third mouse. Therefore, to overcome the barrier for an application in humans, we linked the sialyl-T_N-MUC1 glycopeptide antigen to tetanus toxoid as a carrier protein. Tetanus toxoid conjugates have been applied to humans in several vaccines, in which naturally occurring viral or bacterial structures have been attached to the toxoid.[13]

The tumor-associated MUC1 tandem-repeat glycopeptide antigen was synthesized on Tentagel resin[14] **1**, which is functionalized with a trityl-anchored[15] Fmoc-protected proline (Scheme 1). Coupling of the Fmoc-protected amino acids (10 equiv) was carried out using HBTU/HOBt,[16] and then the glycosylated Fmoc-protected threonine building block **2** (2 equiv) was coupled using a HATU/HOAt[17] procedure as described previously.[10,11] After assembly of the full MUC1 tandem-repeat sequence **3** on the resin (Scheme 1), the glycopeptide **3** carrying the sialyl-T_N-antigen side chain was subjected to replacement of the Fmoc group with an *N*-acetyl group, and then detachment from the resin using a mixture of trifluoracetic acid (TFA)/triisopropylsilane (TIS)/H_2O. After chromatographic purification the NeuNAc benzyl ester was cleaved by hydrogenolysis, and the *O*-acetyl groups were removed by treatment with catalytic amounts of NaOMe in methanol at pH 9.5. The sialyl-T_N-MUC1 glycopeptide antigen **4a** was obtained in 65% yield after preparative HPLC methods. The MUC1 glycopeptides having T_N-antigen (**4b**), one sialyl-T_N-antigen, and one T_N-antigen side chain (**4c**), one sialyl-T_N-antigen and two T_N-antigen side chains (**4d**), as well as one 2,6-sialyl-T-antigen[18] (**4e**), one 2,3-sialyl-T-antigen[19] (**4f**), and one T-antigen side chain (**4g**), as well as the nonglycosylated MUC1 eicosapeptide **4h** were synthesized using an analogous procedure (Scheme 2).

To obtain the vaccine, which contains the tumor-associated sialyl-T_N-MUC1 glycopeptide antigen **4a** as the imprinting antigen, the resin-bound peptide **3** was functionalized with the Fmoc-protected triethylene glycol spacer **5**[20] to give **6** (Scheme 3). Removal of the Fmoc group, acidic cleavage from the resin, and simultaneous removal of all acid-sensitive protecting groups delivered the spacer–glycopeptide antigen

[*] A. Kaiser, N. Gaidzik, Dr. U. Westerlind, D. Kowalczyk, Prof. Dr. H. Kunz
Johannes Gutenberg-Universität Main
Institut für Organische Chemie
Duesbergweg 10–14, 55128 Mainz (Germany)
Fax: (+49) 6131-392-4786
E-mail: hokunz@uni-mainz.de

A. Hobel, Prof. Dr. E. Schmitt
Johannes Gutenberg-Universität Mainz, Institut für Immunologie
Obere Zahlbacher Straße 67, 55101 Mainz (Germany)

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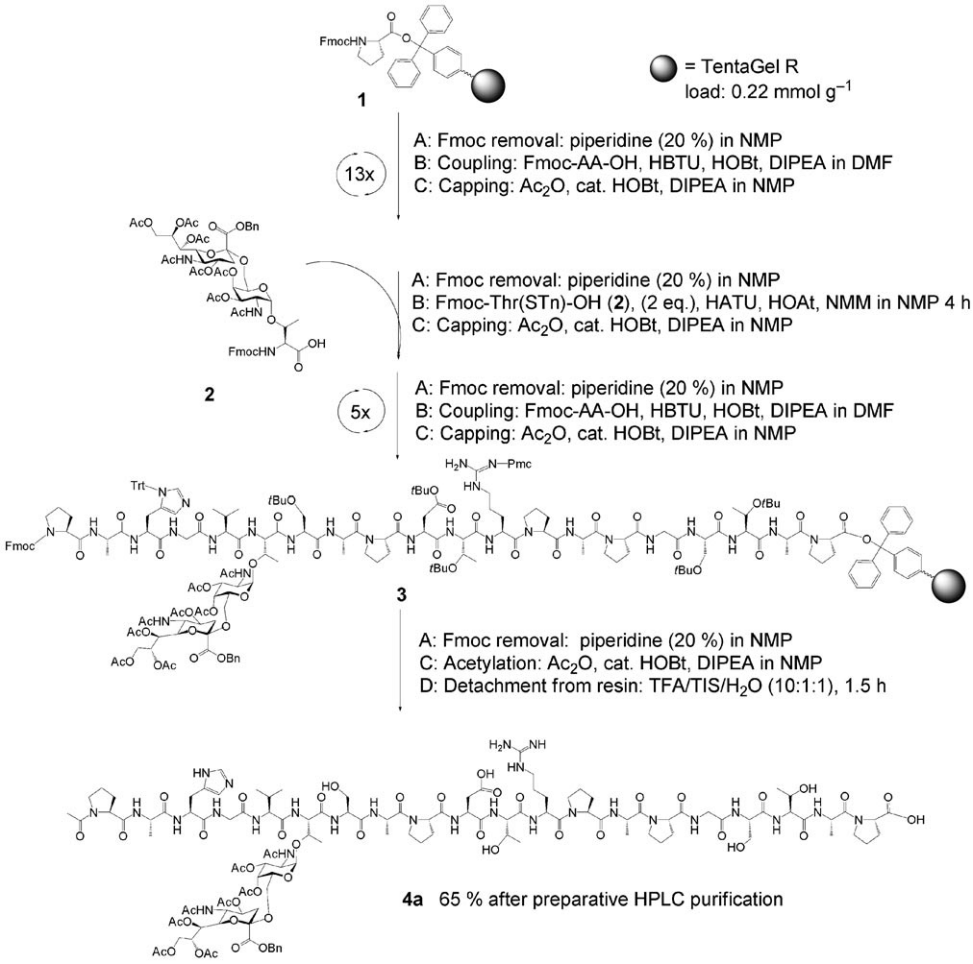
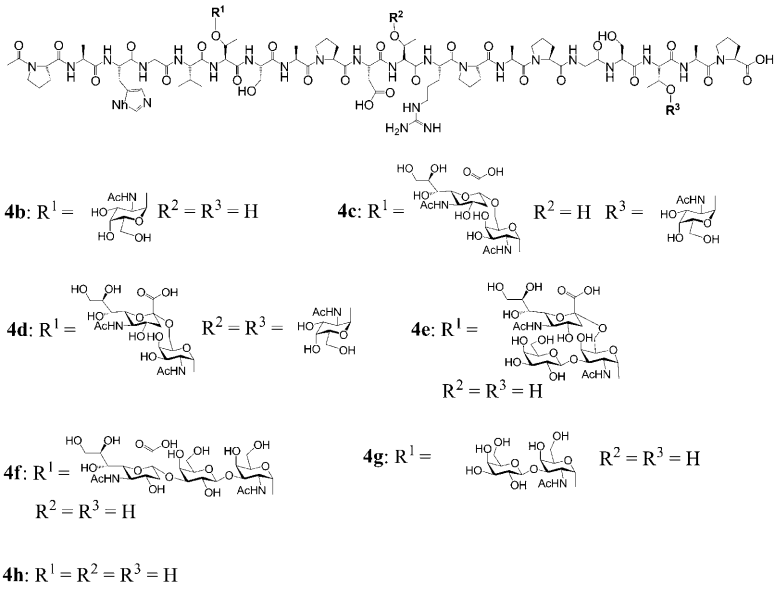


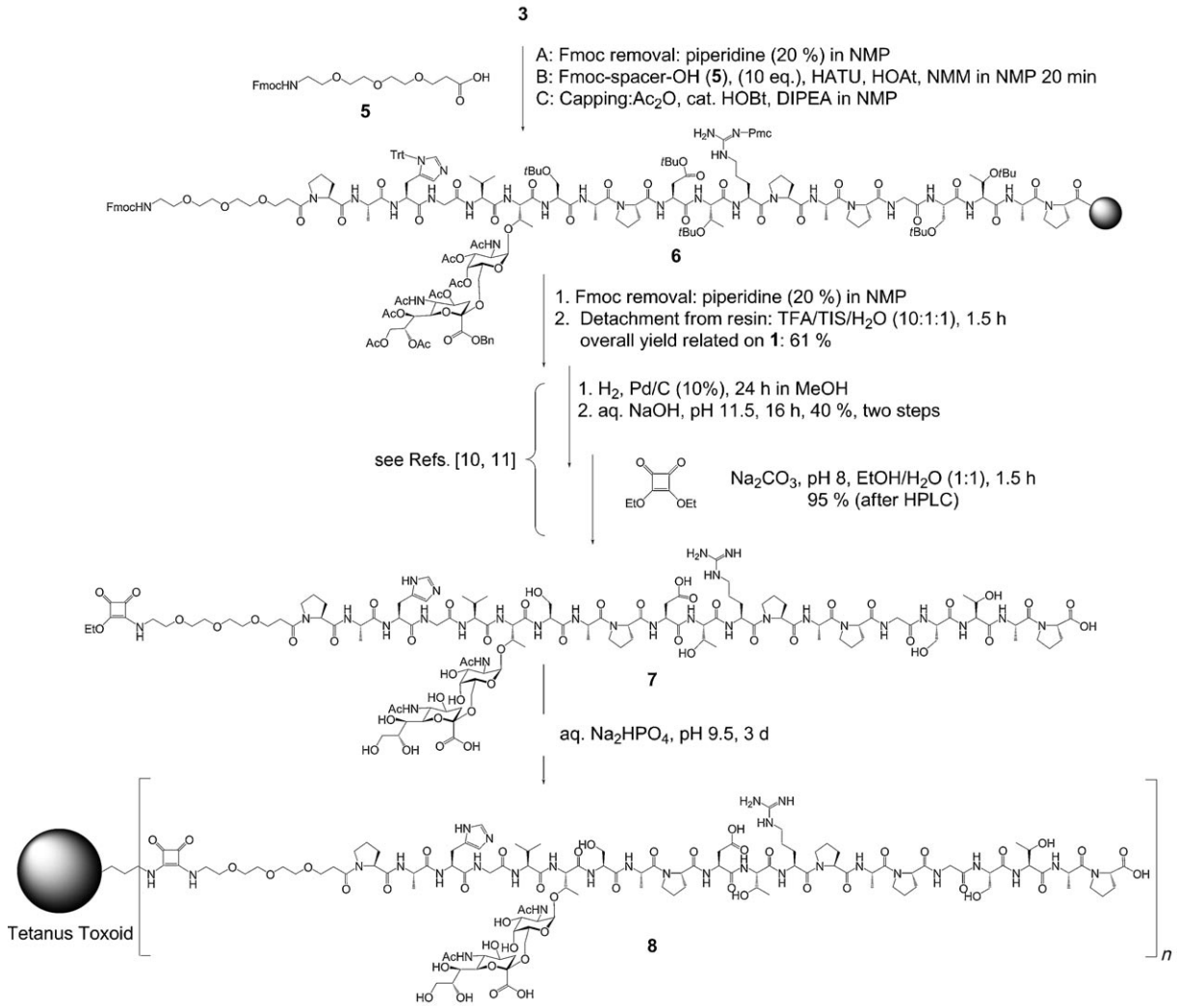
Scheme 1. Solid phase synthesis of the tumor-associated MUC1 tandem-repeat glycopeptide. Fmoc = 9-fluorenylmethoxycarbonyl, NMP = *N*-methylpyrrolidone, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIPEA = diisopropylethylamine, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = 7-aza-1-hydroxybenzotriazole, AA = amino acid.



Scheme 2. Synthesized MUC1 glycopeptide antigens according to Scheme 1.

after preparative HPLC methods, in a 61 % overall yield based on **1**. Hydrogenolysis of the benzyl ester and base-catalysed transesterification yielded the deprotected glycopeptide, which was converted into the monoamido ester **7**[22] with diethyl squarate[21] in ethanol/H_2O. The ester **7** was reacted with tetanus toxoid as the carrier protein in aquaeous sodium phophate buffer to give the synthetic sialyl-T_N-MUC1-TTox vaccine **8**. Low molecular weight compounds and unreacted ester **7** were removed through ultrafiltration (membrane 30 kDa). Washing with deionized water and lyophilization delivered vaccine **8** (7.5 mg). The increase in mass indicates the linking of about 40 molecules of ester **7** (molecular weight ca. 2700) per molecule of tetanus toxoid (molecular weight ca. 150000). In contrast to the MUC1-glycopeptide-BSA (bovine serum albumine) conjugates,[11] the loading of **8** could not be determined by Maldi-TOF mass spectrometry because of the high molecular weight.

Equal amounts (0.5 μg/well) each of the glycopeptide-TTox vaccine **8** and the analogous glycopeptide-BSA conjugate[11] **9** were subjected to comparative ELISA tests[10,11] using the serum of a mouse (Figure 1; see Figure 2a), immunized with the synthetic vaccine consisting of sialyl-T_N glycopeptide and a T-cell epitope from ovalbumine (described in reference [11]). The resuts showed the binding of almost equal amounts of the antibody. The binding of the antibody was photometrically determined using a biotinylated anti-mouse antibody, and subsequent treatment with horseradish peroxidase (HPO).[23] The glycopeptide-BSA conjugate **9** carries, on average, seven molecules of the MUC1 glycopeptide, and has about one third of the molecular weight of **8**. Thus, there must be at least 20 molecules of the synthetic glycopeptide bound per molecule **8**. This number is only an estimation because the relative number of glycopeptide antigens accessible to the antibody is smaller for the bulky TTox vaccine than for the



Scheme 3. Synthesis of sialyl-T_N-MUC1-tetanus-toxoid vaccine **8**.

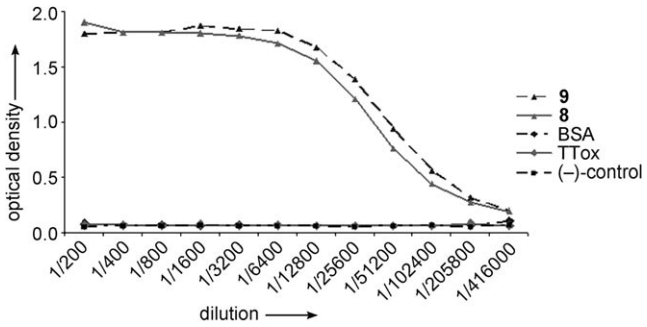


Figure 1. Comparative ELISA test to determine the loading of vaccine **8**.

BSA conjugate. In addition, the ELISA test (Figure 1) demonstrates that the antibody induced by **8** neither binds to BSA nor to the tetanus toxoid itself.

Ten eight-week old Balb/c-mice were immunized with 20 μg of vaccine **8** (in 40 μL PBS-buffer) together with complete Freund's adjuvant. After 21 days a booster immunization with 20 μg vaccine **8** and incomplete Freund's adjuvant was performed. Five days after the second immunization, blood was drawn from the mice and centrifuged. The serum was analyzed with regard to its binding to the sialyl-T_N-MUC1-glycopeptide-BSA conjugate **9**[11] in a dilution series (Figure 2b). The results show, that the sialyl-T_N-tetanus-toxoid vaccine **8** induces a strong immune response in all mice. Mice 8 and 6 show an antibody titer as high as 1/100 000–200 000. Therefore, the initial criterion that an anti-tumor vaccine must induce a strong, tolerance overriding immune response, has been fulfilled. In addition, the binding of the antigen to tetanus toxoid for the first time offers the possibility of transfering this vaccine immunization from mice to humans.

The selectivity of the immune response was investigated using serum from mouse 8 in neutralization experiments (Figure 2c). To this end, the sialyl-T_N-MUC1-glycopeptide-BSA conjugate **9** was coated onto a 96-well ELISA plate, washed, and blocked with a PBS buffer containing 1 % BSA. Three different dilutions of the serum were each incubated for 1 hour at 37 °C together with 100 μg of the glycopeptide antigens **4a**–**4h**, and then transferred to the ELISA plate. The

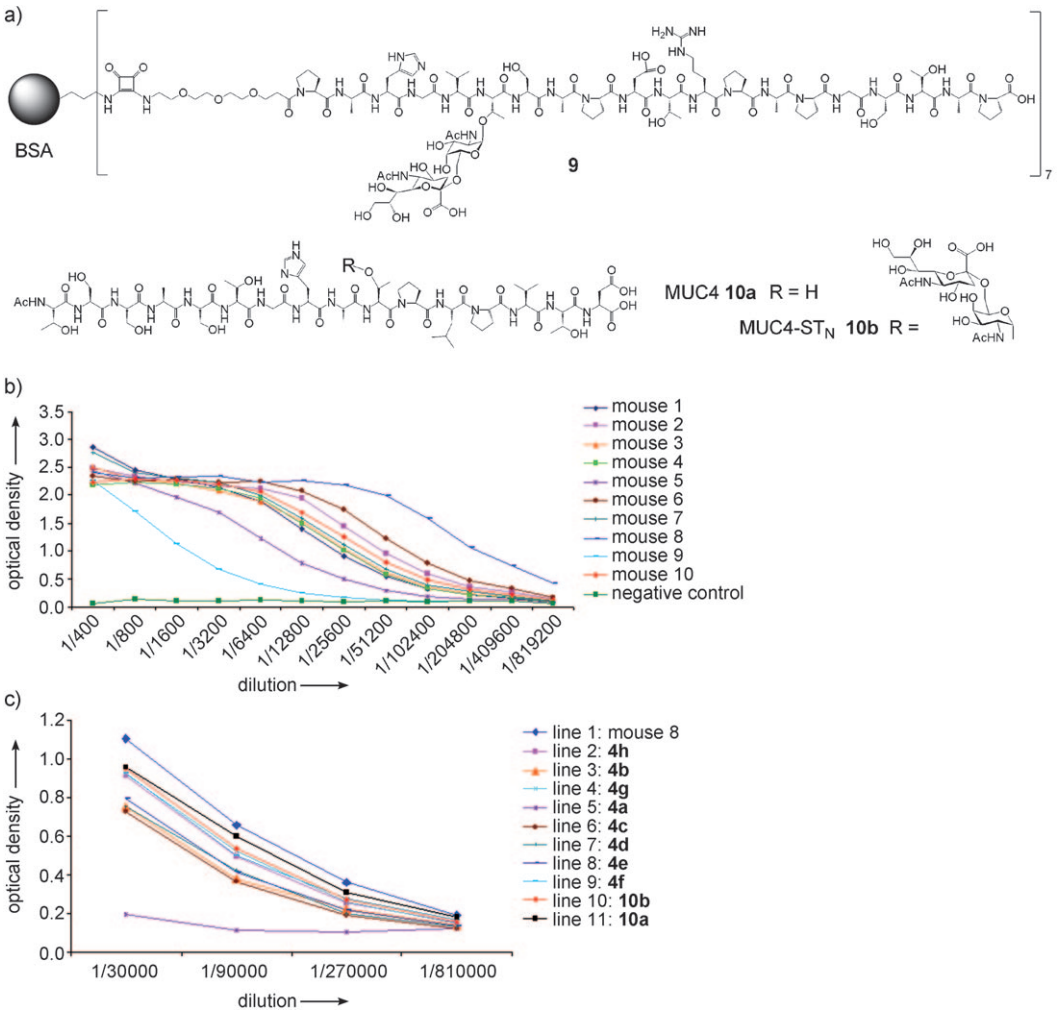


Figure 2. a) Chemical formulae of compounds **9** and **10**. b) ELISA of the serum from mice immunized with vaccine **8**, the microtiter plate was coated with the MUC1-BSA conjugate **9**. c) Neutralization of serum from mouse 8 immunized with vaccine **8**, using the (glyco)peptide antigens **4** and **10**.

plate was washed with a buffer solution, and then a biotinylated anti-mouse antibody and streptavidin–HPO conjugate[10,11] were sequentially added. The dye-forming peroxidase reaction[23] allowed measurement of the residual binding affinity of the serum to **9** at $\lambda = 410$ nm (Figure 2c).

The data indicate, that the binding of the untreated serum from mouse 8 (line 1) is completely neutralized when incubated with the sialyl-T_N-MUC1 antigen **4a** (line 5), the antigen contained in vaccine **8**. The MUC1-glycopeptide antigens **4b** (line 3), **4c** (line 6), **4d** (line 7), and **4e** (line 8) inhibit the antibodies induced with **8** to a considerable extent. All of them embody the same peptide sequence and fundamental parts of the sialyl-T_N disaccharide, and they too represent tumor-associated MUC1 structures. In contrast, for the unglycosylated MUC1-peptide of the same sequence (**4h**, line 2), the MUC1 glycopeptide incorporating the T antigen (**4g**, line 4), and the one with 2,3-sialyl-T side chain (**4f**, line 9) only a weak binding is observed, even though they include an identical peptide sequence. The MUC4 tandem-repeat sequence **10a** (line 11) and its glycopeptide with a sialyl-T_N-antigen side chain[12,24] (**10b**, line 10) do not neutralize the antibodies induced by **8**, emphasizing the high selectivity of the induced immune response towards the MUC1 peptide sequence.

By comparing the very weak neutralizing effect of **10b**, holding the sialyl-T_N saccharide bound to a different peptide sequence, and the nonglycosylated MUC1 tandem-repeat sequence **4h**, with the almost complete neutralization of the anitbodies by **4a**, the high selectivity of the immune response triggered by **8** becomes evident. Neither the saccharide, nor the peptide alone are recognized by the antibodies. However, the glycopeptide sturctures **4b–e**, which are closely related to **4a**, and are likewise tumor-associated MUC1 glycopeptide structures, are bound by the induced antibody to a reduced, but distinct degree. The scope of the recognition at the given high structural selectivity is an advantage for the practical application as an antitumor vaccine. It is of exceptional value, that the synthetic vaccine **8**, composed of sialyl-T_N-MUC1 glycopeptide antigen **4a** and tetanus toxoid, induces a very strong and highly selective immune reaction. In addition, based on the use of tetanus toxoid as carrier protein, an application of this immunization in humans appears possible.

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[22] **7**: $[\alpha]_{23}^{D} = -47.2$ ($c = 1.00$ g(100 mL)$^{-1}$, H_2O); MALDI-TOF-MS (dhb, positive): m/z: 2710.29 ($[M+H]^+$, calc.: 2708.23); ^{1}H NMR (400 MHz, D_2O, COSY, HSQC): $\delta = 8.50$ (d, 1H, H_ε, $J_{H\varepsilon,H\delta} = 1.44$ Hz), 7.21 (d, 1H, H_δ, $J_{H\delta,H\varepsilon} = 1.11$ Hz), 4.81 (d, 1H, H1, $J_{H1,H2} = 3.60$ Hz), 4.62–4.04 (m, 23H, D_α {4.60}, CH_2O squarate {4.59}, H_α {4.57}, R_α {4.52}, $A_{3\alpha}$ {4.47}, $A_{2\alpha}$ {4.44}, $S_{2\alpha}$ {4.38}, $A_{4\alpha}$ {4.34}, $S_{1\alpha}$ {4.31}, $P_{1,5\alpha}$ {4.28, 4.26, 4.23, 4.19, 4.17}, V_α {4.19}, $T_{STn\alpha}$ {4.17}, $T_{STn\beta}$ {4.14}, $A_{1\alpha}$ {4.12}, $T_{1\alpha}$ {4.10}, $T_{2\alpha}$ {4.08}, $T_{2\beta}$ {4.06}, $T_{1\beta}$ {4.05}), 3.97–3.59 (m, 23H, H2 {3.94}, $G_{1\alpha a}$ {3.88}, $G_{1\alpha b}$ {3.86}, H5 {3.77}, 4 × CH_2O spacer {3.76}, $S_{2\beta}$ {3.74}, $G_{2\alpha}$ {3.71}, H3 {3.69}, $S_{1\beta}$ {3.67}, 3-CH_2 spacer {3.66}, $H6_{a,b}$ {3.64}), 3.58–3.29 (m, 18H, H4 {3.57}, H6″ {3.56}, $H9_a$″ {3.56}, H7″ {3.55}, $P_{1,5\delta}$ {3.56, 3.53, 3.48, 3.44}, $H9_b$″{3.54}, H4″ {3.53}, H8″ {3.52}, H5″ {3.48}), 3.18 (d, 1H, $H_{\beta a}$, $J_{H\varepsilon,H\alpha} = 5.88$ Hz), 3.12–3.02 (m, 7H, CH_2-Spacer {3.08}, $H_{\beta b}$ {3.08}, $D_{\beta a}$ {3.07}, R_δ {3.04}), 2.81 (m, 1H, $D_{\beta b}$), 2.60 (t, 2H, 12-CH_2-Spacer, $J_{CH_2CH_2} = 6.32$ Hz), 2.57–2.48 (m, 3H, $H3_{eq}$″ {2.54}, CH_2 spacer {2.53}), 2.26–2.07 (m, 4H, $P_{1,2\beta a}$ {2.22, 2.19}, CH_2 spacer {2.14}), 2.01–1.67 (m, 26H, $P_{3,5\beta a}$ {1.97, 1.89, 1.84}, V_β {1.96}, $P_{1,5\gamma}$ {1.95–1.81}, $P_{1,5\beta b}$ {2.00, 1.96, 1.91, 1.87, 1.83}, AcNH {2 × s, 1.89, 1.89}, $R_{\beta a}$ {1.76}), 1.65–1.47 (m, 7H, $R_{\beta b}$ {1.60}, $H3_{ax}$″ {1.55}, R_γ {1.53}, CH_3CH_2 squarat {1.49}), 1.33–1.19 (m, 12H, $A_{2\beta}$, {1.25, d, $A_{3\beta}$ $J_{A\beta,A\alpha} = 7.0$ Hz}, {1.23 d, $A_{4\beta}$, $J_{A\beta,A\alpha} = 7.28$ Hz}, {1.20, d, $A_{1\beta}$, $J_{A\beta,A\alpha} = 7.28$ Hz}), 1.16 (d, 3H, $T_{STn\gamma}$, $J_{T\gamma,T\beta} = 6.16$ Hz), 1.08 (d, 3H, $T_{2\gamma}$, $J_{T\gamma,T\beta} = 6.50$ Hz), 1.05 (d, 3H, $T_{1\gamma}$, $J_{T\gamma,T\beta} = 6.44$ Hz), 0.84 (d, 3H, V_γ, $J_{V\gamma a,V\beta} = 6.52$ Hz), 0.82 ppm (d, 3H, V_γ, $J_{V\gamma b,V\beta} = 6.44$ Hz).

[23] HPO catalyzes the oxidation of the colorless 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) by H_2O_2 to form the metastable green radical cation having an absorption maximum at $\lambda = 414$ nm.

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