DOI: 10.1002/ejoc.201100304

Synthesis of Tn/T Antigen MUC1 Glycopeptide BSA Conjugates and Their Evaluation as Vaccines

Hui Cai,[a] Zhi-Hua Huang,[a] Lei Shi,[a] Peng Zou,[b] Yu-Fen Zhao,[a] Horst Kunz,*[c] and Yan-Mei Li*[a]

Keywords: Carbohydrates / Glycosylation / Glycopeptides / Antitumor agents / Solid-phase synthesis

The tumor-associated mucin MUC1 over-expressed in most epithelial tumor tissues is considered a promising target for immunotherapy. The extracellular part of MUC1 contains a domain of numerous tandem repeats of the amino acid sequence HGVTSAPDTRPAPGSTAPPA, including five potential *O*-glycosylation sites. In this study, T9 and S15 have been chosen as the positions of glycosylation. The glycopeptides *N*-terminally equipped with a triethylene glycol spacer were synthesized by microwave-assisted Fmoc solid-phase peptide synthesis. After detachment from the resin and deprotection, the MUC1 glycopeptides were conjugated to bovine serum albumin (BSA). To evaluate the immunological properties, balb/c mice were immunized with these BSA vaccines.

Introduction

MUC1 is a membrane glycoprotein expressed on almost all types of epithelial tissues. Its tumor-associated form has been found to be strongly over-expressed in breast, pancreatic, and ovarian cancers.[1] In its extracellular part it contains a large number of tandem repeats consisting of the sequence HGVTSAPDTRPAPGSTAPPA. Each tandem repeat includes five potential *O*-glycosylation sites (T4, S5, T9, S15, and T16). Glycosylation of MUC1 is essential for its biological functions.[2] On tumor cells, the glycosylation pattern of MUC1 is distinctly different to that of normal cells. Typically, *O*-glycosylation of the tumor cells is truncated, resulting in the Thomsen–Friedenreich antigen (T antigen), its precursor (Tn antigen), and their sialylated forms.[3] Due to aberrant truncated glycosylation, backbone peptide epitopes of tumor-associated MUC1 are demasked and exposed together with tumor-associated carbohydrate antigens.[4] These tumor-associated glycopeptide antigens are considered potential target structures for attack of the human immune system. Unfortunately, the immunogenicity of these glycopeptides as B-cell epitopes is low. In order to induce a sufficiently strong immune response, the synthetic glycopeptides need to be conjugated with an immunostimulating component.[5]

The type and site of glycosylation can distinctly influence the conformation and the immunogenicity of tumor-associated MUC1 glycopeptides.[6] For development of a vaccine, it is necessary to study the relationship between the glycosylation type and immunogenicity. The sequence PDTRP within the tandem repeat has been identified as an immunodominant motif.[7] It was also found that tumor cell recognizing monoclonal antibodies bind to this PDTRP motif when it contains the Tn antigen.[8] Therefore, the glycopeptides comprising the PDTRP sequence *O*-glycosylated with either Tn or T antigen were synthesized in this study as candidates for MUC1 antitumor vaccines.

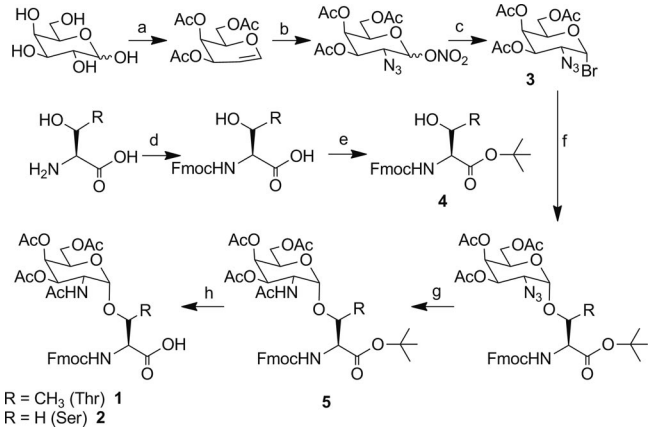
During the past years a number of potential antitumor vaccines consisting of tumor-associated MUC1 glycopeptides and different immunostimulants, such as BSA, tetanus toxin, or T cell epitope peptides from Ovalbumin (OVA), have been synthesized.[9] Most of the glycopeptides were glycosylated at the T4, T9, and T16 sites of the tandem repeat sequence. Taking into account this feature and the fact that the glycosylation of serine or threonine has different implications in the backbone conformation of the underlying amino acid,[10] we here focus on glycopeptides with glycosylation at sites S15 and T9 of the tandem repeat sequence. A series of glycopeptide antigens that contain the complete tandem repeat sequence and that bear the Tn and T antigen side chains was synthesized by solid-phase glycopeptides synthesis. These synthetic glycopeptides were conjugated to the carrier protein bovine serum albumin (BSA) through a triethylene glycol spacer.[5a]

[a] Department of Chemistry, Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology (Ministry of Education), Tsinghua University,
Beijing 100084, P. R. China
Fax: +0086-10-62781695
E-mail: liym@mail.tsinghua.edu.cn
[b] School of Life Sciences, Tsinghua University,
Beijing 100084, P. R. China
[c] Institute of Organic Chemistry, Johannes Gutenberg University of Mainz,
Duesbergweg 10-14, 55128 Mainz, Germany
Fax: +49-6131-392 4786
E-mail: hokunz@uni-mainz.de

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201100304.

Results and Discussion

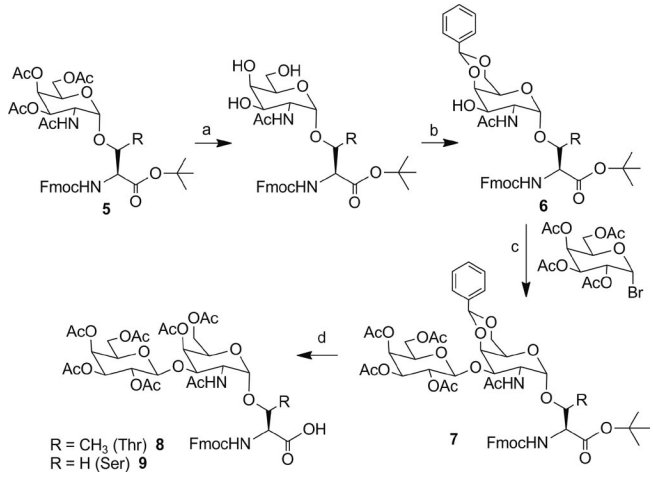
The Fmoc-protected Tn and T antigen threonine and serine building blocks bearing *O*-acetyl protection within the glycans were synthesized according to reported methods.[11] D-Galactose was transformed into 2-azidogalactosyl bromide **3** as a glycosyl donor in three steps (Scheme 1). Amino acid glycosyl acceptor **4** was obtained from serine or threonine after Fmoc protection of the amino group and introduction of the *tert*-butyl ester. After glycosylation according to modified Koenigs–Knorr methodology, the *O*-glycosyl amino acid was transformed into fully protected Tn antigen conjugate **5** through reduction of the azido group and acetylation. Removal of the *tert*-butyl ester by using TFA afforded Tn antigen building blocks **1** and **2**, which can be applied to solid-phase glycopeptide synthesis.



Scheme 1. Synthesis of Tn building block. R = H (Ser), CH_3 (Thr). Reagents and conditions: (a) 1. Ac_2O, pyridine; 2. 30% HBr/AcOH; 3. Zn, $CuSO_4$; 85% (3 steps); (b) CAN, NaN_3, 42%; (c) LiBr, 92%; (d) FmocOSu, $NaHCO_3$, quant.; (e) *t*BuOH, DCC, cat. CuCl, 67–70%; (f) $AgClO_4$, $AgCO_3$, 55–70%; (g) Zn, AcOH, Ac_2O, 69–77%; (h) TFA, anisole, 99%.

The Tn antigen was converted into the T antigen by β-galactosylation of the GalNAc 3-hydroxy group. After careful removal of the *O*-acetyl groups of **5**[11b] and protection of the 4- and 6-hydroxy groups as benzylidene acetal, the free 3-hydroxy group of glycosyl acceptor **6** was glycosylated with tetra-*O*-acetyl-galactosyl bromide under promotion by mercury(II) cyanide to give T antigen conjugate **7** in high yield and selectivity[12] (Scheme 2). Acidolytic removal, acetylation, and acidolysis of the *tert*-butyl ester afforded Fmoc T antigen building blocks **8** and **9**[11,13] applicable to solid-phase glycopeptide synthesis.

The microwave-assisted Fmoc solid-phase peptide synthesis of the MUC1 tandem repeat glycopeptides was performed according to a known procedure[14] starting from a 2-chlorotrityl resin preloaded with alanine (Scheme 3). The Fmoc amino acid (6 equiv.) was activated with HBTU/HOBt[15] in DMF in the presence of DIPEA. Fmoc protected Tn and T antigen building blocks **1**, **2** or **8**, **9** (2.0 equiv.), respectively, were activated by the more reactive reagent HATU/HOAt[16] in NMP by using *N*-methylmorpholine (NMM) as the base.[9f] After each coupling, the

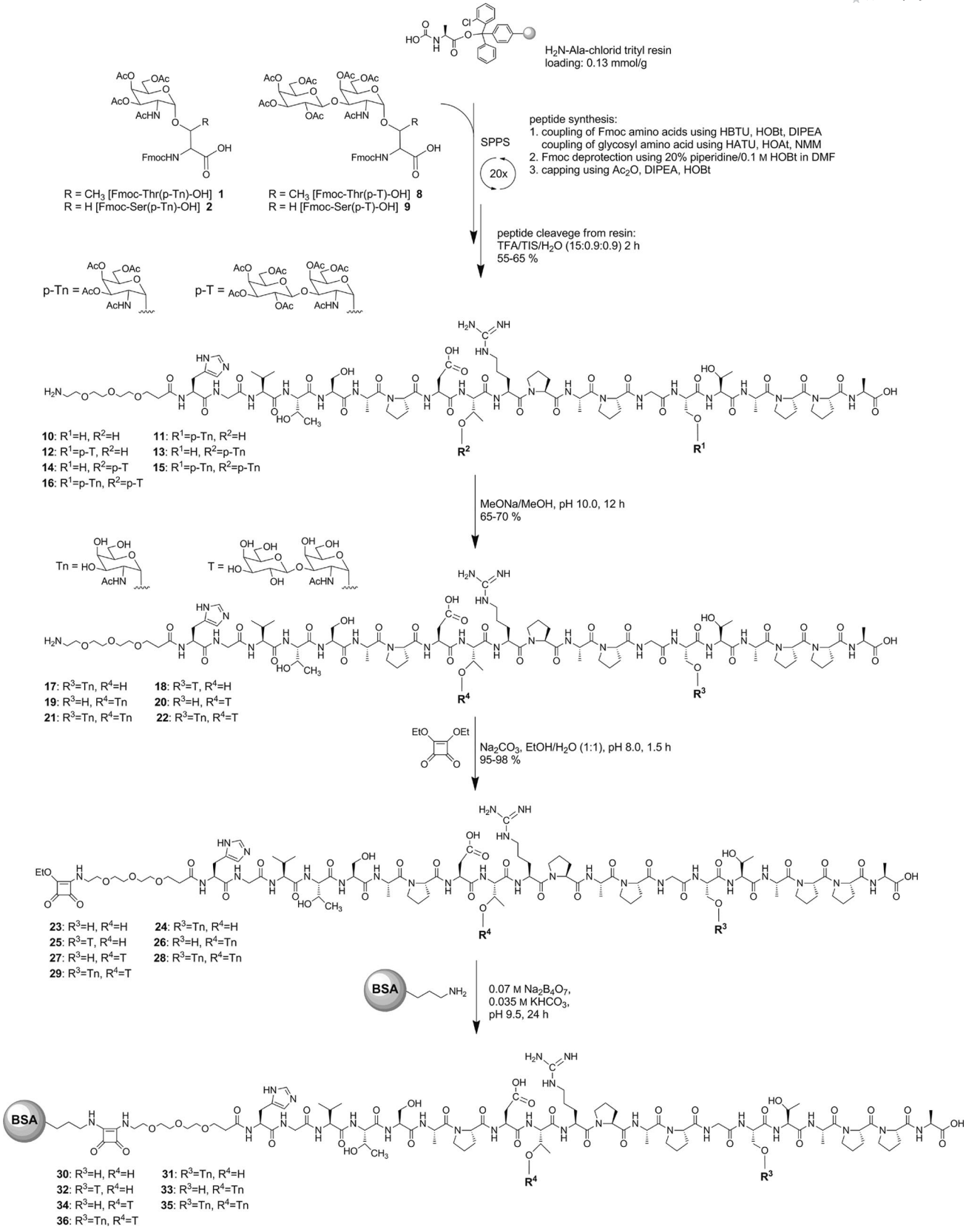


Scheme 2. Synthesis of T building blocks. Reagents and conditions: (a) NaOMe/MeOH, pH 9.0, 75%; (b) benzaldehyde–dimethylacetal, cat. *p*TsOH, 80%; (c) $Hg(CN)_2$, 65%; (d) 1. 80% AcOH/H_2O, 90%; 2. Ac_2O, pyridine, 95%; 3. TFA, anisole, 95%.

unreacted amino groups were capped using acetic anhydride/HOBt/DIPEA in DMF. Removal of the Fmoc group was performed with a solution of 20% piperidine/0.1 M HOBt in DMF. In order to later conjugate the glycopeptides with the carrier protein, each peptide was extended with a triethylene glycol spacer. After completion of the peptide synthesis, crude glycopeptides **10**–**16** were detached from the resin using trifluoroacetic acid (TFA)/(triisopropylsilane) TIS/water (15:0.9:0.9), while all acid-sensitive protecting groups of the amino acid side chains were removed concomitantly. However, the hydroxy groups of the carbohydrate portion remained *O*-acetylated. Obtained glycopeptides **10**–**16** were purified by preparative HPLC on a C-18 column and isolated in yields of 55–65%. The Fmoc group and the *O*-acetyl groups of the carbohydrate were removed by treatment with a MeONa/MeOH solution (pH 10.0) over 12 h. Free glycopeptides **17**–**22** were isolated after preparative HPLC in yields of 65–70%.

The terminal amino groups of **17**–**22** and **10** were treated with diethyl squarate in EtOH/H_2O (1:1) by adjusting the solution to pH 8 with a saturated aqueous Na_2CO_3 solution. Obtained squaric acid monoamides **23**–**29** of the glycopeptides were dissolved with BSA in a buffer solution at pH 9.5 to afford BSA-conjugated glycopeptides vaccines **30**–**36**. All the conjugated vaccines were purified by ultrafiltration against deionized water using a membrane of 12 kDa. The loading rates of the glycopeptide–BSA conjugates were determined by MALDI-TOF mass spectrometry (Figure 1, Table 1). On average, 8–12 molecules of the glycopeptides were found linked to one BSA protein molecule.

In order to evaluate the immunological properties of the glycopeptide–BSA conjugates, each synthetic vaccine containing 10 μg of the glycopeptide in combination with complete Freund's adjuvant (CFA) were injected into four balb/c mice. After an interval of two weeks, the second and third immunizations were performed with incomplete Freund's adjuvant (IFA). One week after the third immunization,



Scheme 3. Solid-phase synthesis of the Tn/T-antigen-MUC1 glycopeptides and their conjugation to the carrier protein BSA: SPPS = solid-phase peptide synthesis, Fmoc = fluorenyl-9-methoxycarbonyl, HBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetremethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole. DIPEA = diisopropylethylamine, HATU = *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = *N*-hydroxy-7-azabenzotriazole, NMM = *N*-methylmorpholine, TFA = trifluoroacetic acid, TIS = triisopropylsilane, BSA = bovine serum albumin.

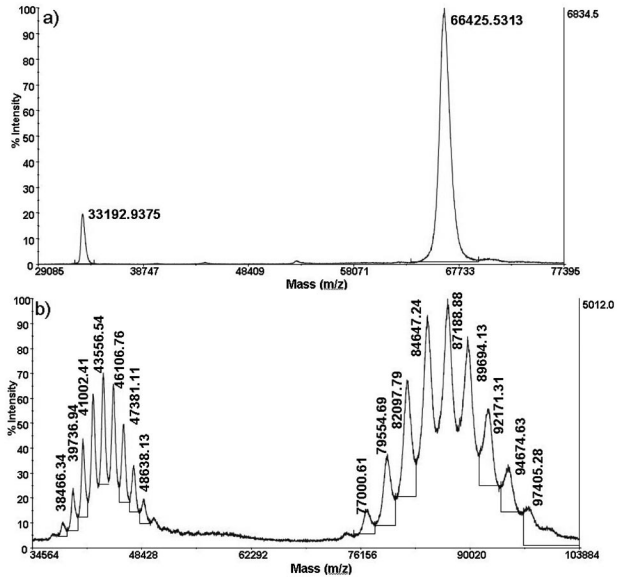


Figure 1. MALDI-TOF MS of (a) BSA and (b) vaccine **23**.

Table 1. MALDI-TOF mass analysis of the squaric acid monoamides of glycopeptides **23**–**29** and their BSA conjugates **30**–**36**.

Squaric acid monoamides glycopeptide	Detected molecular weight	BSA conjugates	Detected molecular weight	Loading of the glycopeptide
23	2214.07	**30**	85585.87	9
24	2417.85	**31**	90313.9	10
25	2579.18	**32**	91733.9	10
26	2417.11	**33**	87402.1	9
27	2579.13	**34**	96591.6	12
28	2620.22	**35**	87188.9	8
29	2782.32	**36**	88313.2	8

blood was drawn from the tail vein of each mouse, and the isolated sera were used for analysis of the induced antibodies by enzyme-linked immunoabsorbent assay (ELISA). In the ELISA tests, the microtiter plates were coated with unglycosylated peptide **10** and deprotected glycopeptides **17**–**22**. The sera were titrated at increasing dilution in a phosphate buffered saline (PBS) solution. The induced antibody concentration was detected photometrically by using a secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP, Figure 2). The immune response values corresponding to BSA conjugates of the glycopeptides can be classified into three groups. Unglycosylated peptide **30** and peptide **31**, glycosylated with Tn antigen at S15, elicited a lower immune response compared to the other glycopeptides vaccines. The second group showed a medium response and it comprises glycopeptides **32** and **36**, which carrying T or Tn antigens at S15. The higher immune responses were obtained with BSA conjugates **33**, **34**, and **35**, which present in common the feature that they are glycosylated with T or Tn at T9.

In conclusion and to rationalize these results, it is important to notice that in order to get a high immune response, it is necessary for the peptide sequence to be glycosylated,

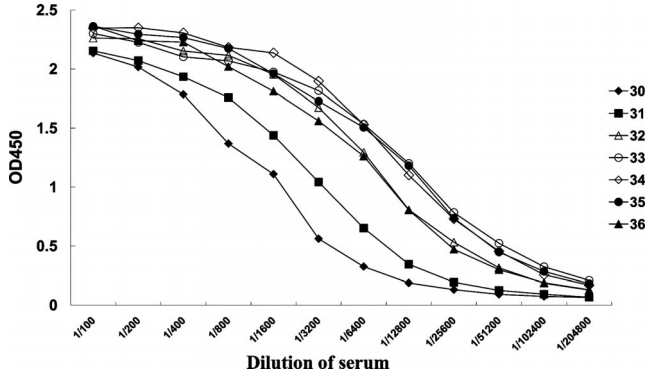


Figure 2. ELISA of sera isolated from mice immunized with **30**–**36** after third immunization, coating of the microtiter plate with **10** and **17**–**22**.

particularly at T9, which belong to the PDTRP immunodominant motif,[7,8] and in this case similar responses were obtained with both the T and Tn antigens. However, glycosylation at S15 is not a requisite to get a high immune response, as only good values were obtained when T9 is also glycosylated.

Conclusions

MUC1 glycopeptides containing a full-length tandem repeat sequence and bearing the Tn and/or T antigen at the glycosylation sites threonine 9 and serine 15 were synthesized by microwave-assisted solid-phase glycopeptides synthesis. These fully synthetic glycopeptides antigens were conjugated with BSA through a triethylene glycol spacer by using diethyl ester squarate as the coupling reagent. The obtained MUC1 glycopeptide vaccines were characterized by MALDI-TOF mass spectrometry. These BSA conjugates carried on average 8–12 glycopeptides per molecule protein. The MUC1-BSA vaccines were used for immunization of balb/c mice. The induced strong immune responses were evaluated by ELISA.

Experimental Section

General Methods: Reverse-phase HPLC separation was carried out with Waters 2487 (600E) equipment. Elution was performed with solution A (80% acetonitrile/water with 0.6% trifluoroacetic acid) or with solution B (100% water with 0.6% trifluoroacetic acid). Detection of the signal was achieved with a UV detector at a wavelength of 215 nm. Separation of the glycopeptides was performed by preparative HPLC using a C-18 column (10 × 250 mm) at a flow rate of 7 mL min^{-1}. Ultrafiltration purification of the BSA conjugates was performed by using a dialysis tube (12 kDa, Sigma). MALDI-TOF MS spectra were recorded by using α-cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB) with an Applied Biosystems 4700 Proteomics Analyzer 283.

General Procedure for the Synthesis of MUC1 Glycopeptides 10–16: Commercially available NH_2-Ala-2-chloro trityl resin was used for all the solid-phase syntheses of the glycopeptides. The loading of the resin in these synthesis was 0.13 mmol/g, and the scale used for each glycopeptide was 0.1 mmol. All cycles in the solid-phase

synthesis were executed in a microwave peptide synthesizer (Liberty, CEM Corporation). All Fmoc amino acid building blocks and the triethylene glycol linker were coupled by using HBTU/HOBt. Typically, coupling reactions of amino acids were conducted under microwave irradiation for 15 min with a power of 18 W at a temperature of 50 °C. The coupling was repeated two times for the coupling of Fmoc-Arg (Pbf)-OH. The coupling of Fmoc-His(Trt)-OH was performed at 40 °C for 25 min and also repeated two times. The amino acids (6 equiv.), HBTU (6 equiv.), and HOBt (6 equiv.) were dissolved in DMF, and DIPEA (12 equiv.) was dissolved in NMP. These solutions were added automatically. The protected Tn and T building blocks were coupled by using HATU/HOAt/NMM. The glycosyl amino acids building blocks (2.0 equiv.), HATU (2.5 equiv.), HOAt (2.5 equiv.), and NMM (5.0 equiv.) were dissolved in NMP, and these solutions were added manually. After the coupling, the unreacted free amino groups were capped by acetylation using 0.013 M HOBt in Ac_2O/DIPEA/DMF (4.75:2.25:93.0, v/v/v). The capping was performed under microwave irradiation at 50 °C for 180 s with a power of 20 W. A solution of 20% piperidine/0.1 M HOBt in DMF was used for Fmoc removal, which was conducted under microwave irradiation at 50 °C for 90 s and 300 s with a power of 24 W. After coupling all the building blocks, the resin was transferred from the peptide synthesizer into a reactor flask and treated with a mixture of TFA/TIS/H_2O (15:0.9:0.9, v/v/v) for 2 h in order to detach the glycopeptide from the resin.

Deacetylation of Glycopeptides 11–16: Glycopeptides **11**–**16** were dissolved in methanol. Then, 1% MeONa/MeOH solution was added carefully until pH 10.0 was reached. After 12 h, the solution was neutralized by acetic acid, and crude products **17**–**22** were purified by RP-HPLC.

General Procedure for the Synthesis of Glycopeptides Squaric Acid Monoamides 23–29: Deprotected glycopeptides **17**–**22** and **10** were dissolved in EtOH/H_2O (1:1). 3,4-Diethoxy-3-cyclobutene-1,2-dione (1.0 equiv.) was added. Then, a solution of sat. Na_2CO_3 (5 μL) was added in intervals of 5 min, until pH 8 was reached. After stirring for 1.5 h at room temperature (controlled by RP-HPLC), the reaction mixture was neutralized by adding HOAc. The solvent was removed in vacuo, and the water was removed by lyophilization. The products were purified by RP-HPLC.

General Procedure for the Conjugation of 23–29 with BSA: Glycopeptides squaric acid monoamides **23**–**29** and BSA were dissolved in 0.07 M $Na_2B_4O_7$ (600 μL) and 0.035 M $KHCO_3$ (600 μL) buffer solution. The glycopeptides and BSA mixture was kept 24 h at room temperature. The glycopeptide–BSA conjugate was dialyzed with deionized water for 48 h. Then the water was removed by lyophilization.

Enzyme-Linked Immunoabsorbent Assay (ELISA): 96-Well ELISA plates were coated with either unglycosylated peptide **10** or MUC1 glycopeptides **17**–**22** (5 μL/mL) dissolved in 0.1 M $NaHCO_3$ (pH 9.6, adjusted by NaOH) and incubated overnight at 4 °C. The plates were washed with 0.05% Tween PBS buffer and then blocked with PBS containing 0.25% gelatine for 2–3 h at room temperature. After washing with 0.05% Tween PBS buffer, the mouse sera diluted in 0.25% gelatine PBS were added to the plates at corresponding dilutions and incubated for 90 min at 37 °C. The plates were washed and incubated with horseradish peroxidase conjugated to rabbit anti-mouse IgG Ab for 2 h at 37 °C. Then the plates were washed and the substrate solution OPD and H_2O_2 was added. After 10–20 min, the absorbance was measured at 450 nm.

Supporting Information (see footnote on the first page of this article): Copies of mass spectra of the compounds.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (20825206 and 21028004) and by the Sino-German Center for Research Promotion (GZ561).

[1] J. Harris, D. E. Mason, J. Li, K. W. Burdick, B. J. Backes, T. Chen, A. Shipway, G. Van Heeke, L. Gough, A. Ghaemmaghami, F. Shakib, F. Debaene, N. Winssinger, *Chem. Biol.* **2004**, *11*, 1361–1372.
[2] S. Satoh, Y. Hinoda, T. Hayashi, M. D. Burdick, K. Imai, M. A. Hollingsworth, *Int. J. Cancer* **2000**, *88*, 507–518.
[3] G. F. Springer, *Science* **1984**, *224*, 1198–1206.
[4] K. O. Lloyd, J. Burchell, V. Kudryashov, B. W. T. Yin, J. Taylor Papadimitriou, *J. Biol. Chem.* **1996**, *271*, 33325–33334.
[5] a) F. G. Hanisch, *Biochem. Soc. Trans.* **2005**, *33*, 705–708; b) Z. H. Jiang, R. R. Koganty, *Curr. Med. Chem.* **2003**, *10*, 1423–1439; c) U. Westerlind, H. Kunz, *Carbohydrate Chemistry, Specialist Periodical Report*, RSC, Cambridge, UK, **2010**, vol. 36, p. 1.
[6] a) S. Dziadek, C. Griesinger, H. Kunz, U. M. Reinscheid, *Chem. Eur. J.* **2006**, *12*, 4981–4993; b) U. Westerlind, H. Schroder, A. Hobel, N. Gaidzik, A. Kaiser, C. M. Niemeyer, E. Schmitt, H. Waldmann, H. Kunz, *Angew. Chem.* **2009**, *121*, 8413; *Angew. Chem. Int. Ed.* **2009**, *48*, 8263–8267.
[7] a) O. J. Finn, K. R. Jerome, R. A. Henderson, G. Pecher, N. Domenech, J. Magarianblander, S. M. Barrattboyes, *Immunol. Rev.* **1995**, *145*, 61–89; b) P. Dokurno, P. A. Bates, H. A. Band, L. M. D. Stewart, J. M. Lally, J. M. Burchell, J. Taylor-Papadimitriou, D. Snary, M. J. E. Sternberg, P. S. Freemont, *J. Mol. Biol.* **1998**, *284*, 713–728.
[8] a) U. Karsten, C. Diotel, G. Klich, H. Paulsen, S. Goletz, S. Muller, F. G. Hanisch, *Cancer Res.* **1998**, *58*, 2541–2549; b) H. Moller, N. Serttas, H. Paulsen, J. M. Burchell, J. Taylor-Papadimitriou, B. Meyer, *Eur. J. Biochem.* **2002**, *269*, 1444–1455.
[9] a) S. Dziadek, A. Hobel, E. Schmitt, H. Kunz, *Angew. Chem.* **2005**, *117*, 7803; *Angew. Chem. Int. Ed.* **2005**, *44*, 7630–7635; b) S. Dziadek, D. Kowalczyk, H. Kunz, *Angew. Chem.* **2005**, *117*, 7798; *Angew. Chem. Int. Ed.* **2005**, *44*, 7624–7630; c) S. Wittrock, T. Becker, H. Kunz, *Angew. Chem.* **2007**, *119*, 5319; *Angew. Chem. Int. Ed.* **2007**, *46*, 5226–5230; d) U. Westerlind, A. Hobel, N. Gaidzik, E. Schmitt, H. Kunz, *Angew. Chem.* **2008**, *120*, 7662; *Angew. Chem. Int. Ed.* **2008**, *47*, 7551–7556; e) A. Kaiser, N. Gaidzik, U. Westerlind, D. Kowalczyk, A. Hobel, E. Schmitt, H. Kunz, *Angew. Chem.* **2009**, *121*, 7688; *Angew. Chem. Int. Ed.* **2009**, *48*, 7551–7555; f) A. Hoffmann-Roder, A. Kaiser, S. Wagner, N. Gaidzik, D. Kowalczyk, U. Westerlind, B. Gerlitzki, E. Schmitt, H. Kunz, *Angew. Chem. Int. Ed.* **2010**, *49*, 8498–8503.
[10] F. Corzana, J. H. Busto, G. Jimenez-Oses, M. G. de Luis, J. L. Asensio, J. Jimenez-Barbero, J. M. Peregrina, A. Avenoza, *J. Am. Chem. Soc.* **2007**, *129*, 9458–9467.
[11] a) T. Becker, S. Dziadek, S. Wittrock, H. Kunz, *Curr. Cancer Drug Targets* **2006**, *6*, 491–517; b) B. Liebe, H. Kunz, *Angew. Chem.* **1997**, *109*, 629; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 618–621.
[12] C. Brocke, H. Kunz, *Synthesis* **2004**, 525–542.
[13] A. Kuhn, H. Kunz, *Angew. Chem.* **2007**, *119*, 458; *Angew. Chem. Int. Ed.* **2007**, *46*, 454–458.
[14] T. Matsushita, H. Hinou, M. Kurogochi, H. Shimizu, S. I. Nishimura, *Org. Lett.* **2005**, *7*, 877–880.
[15] V. Dourtoglou, B. Gross, V. Lambropoulou, C. Ziodrou, *Synthesis* **1984**, 572–574.
[16] L. A. Carpino, *J. Am. Chem. Soc.* **1993**, *115*, 4397–4398.

Received: March 3, 2011
Published Online: May 2, 2011

