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Synthesis and Biological Evaluation of a Multiantigenic Tn/TF-Containing Glycopeptide Mimic of the Tumor-Related MUC1 Glycoprotein

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Glycoprotein glycans have been shown to play essential roles in many biological processes such as protein folding, cell–cell interactions, and tumor development. The isolation of native or recombinant glycoproteins yields heterogeneous carbohydrate moieties leading to different glycoforms, which may exhibit different properties. The chemical synthesis of *O*-glycoproteins presents the advantage of producing well-defined constructs.[1] However, such syntheses are truly challenging due to the multifunctionality of polypeptides and carbohydrates.[2] One of the principal synthetic strategies relies on the synthesis of peracetylated *O*-glycosyl amino acid building blocks, which are subsequently used in solid-phase glycopeptide syntheses.[3] For more complex or longer glycopeptides containing native *O*-glycosidic bonds, convergent strategies based on protected glycopeptides[4,5] or native chemical ligation[6–8] have been proposed.

O-Glycosylation is found on many soluble and membrane proteins. It is present in the variable tandem repeat part of mucins, which, like the membrane-associated mucin MUC1, are overexpressed as underglycosylated forms on the surface of tumor cells.[9] This altered expression of carbohydrate moieties that expose well-characterized tumor-associated epitopes such as Tn (α-GalNAc), TF (Galβ(1,3)- α-GalNAc), and their sialylated forms, as well as the APDTR peptide epitope from the repeat unit of MUC1, have been exploited in immunotherapy.[10,11] Elegant and demanding syntheses have afforded carbohydrate-based cancer vaccines[12] which are currently being tested in clinical trials.[13] However, carbohydrate antigens are mostly limited to IgM production. The switch to a carbohydrate-specific IgG response is possible if tumor-associated carbohydrate moieties are linked to a T-helper epitope.[5,14,15] However, the antibody response is not restricted to the carbohydrate moiety but may be directed against the aglycone backbone.[15–17] It is therefore preferable to find synthetic constructs composed of tumor-related MUC1 glycopeptides linked to a T-helper epitope. To evaluate these new immunogens at the biological and immunological levels, it is crucial to produce them in substantial amounts. Thus, a highly convergent strategy is needed. Moreover, to be useful for human immunotherapy the construct has to be immunogenic under conditions applied in the clinic, where it may be associated only with mild adjuvants. However, it must be capable of overcoming the immunological tolerance often observed with tumor-associated self antigens. Therefore, in this study, we focused on the synthesis of a glycopeptide construct containing the universal Pan DR epitope (PADRE)[18] and three MUC1 repeat units, two of them carrying the Tn and the TF carbohydrate epitopes, respectively, on the Thr residue of the immunodominant sequence PDTRP. Its immunogenic properties were evaluated in mice, and the antisera produced were characterized with synthetic and natural tumor antigens.

Our synthetic methodology is based on a modular multiligation scheme with the synthesis of a stem peptide bearing two aldehydes masked as 2-amino alcohol and acetal.[19] Successive unmaskings of the aldehyde groups with periodate oxidation[20] and trifluoroacetic acid (TFA) were followed by the oximation reaction with an aminooxyacetic acid (Aoa)-containing peptide. To adapt this convergent strategy to the synthesis of glycopeptides, two points need consideration: 1) the partner, aldehyde or aminooxy, on which the carbohydrate moieties will be introduced; 2) the deacetylation step of the hydroxy groups of the carbohydrate moieties by mild methanolysis. Herein, we demonstrate the potential of our approach by synthesizing a tri-branched glycoprotein construct using Aoa-containing glycopeptide building blocks carrying unprotected carbohydrate moieties.

Aoa-containing glycopeptides **1** and **2** were obtained by solid-phase peptide synthesis (SPPS, Scheme 1). The carbohydrate moieties were introduced at Thr 12 of the APDTR epitope as peracetylated N^{α}-Fmoc-Thr(Tn/TF)-OH building blocks.[3] The elongation was carried out with an automatic synthesizer using an Fmoc/*t*Bu strategy[21] starting from a *p*-benzyloxybenzyl alcohol resin (Wang resin)[22] using (1-*H*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)[23]/1-hydroxybenzotriazole (HOBt)[24]/*i*Pr$_2$NEt in *N*-methylpyrrolidine (NMP) as coupling reagents up to the *O*-glycosylated Thr. Peracetylated Fmoc-Thr(Tn)-OH and Fmoc-Thr(TF)-OH were introduced manually using only 1.1 equiv *O*-glycosyl amino acid/*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HATU)[25]/*i*Pr$_2$NEt in NMP. The remaining free amino groups were capped with acetic anhydride/HOBt/*i*Pr$_2$NEt. After the introduction of *O*-glycosylated Thr 12, the N-terminal amino acids were automatically coupled through activation with HBTU/HOBt/*i*Pr$_2$NEt (10 equiv). The Wang linker and the acid-labile side-chain protecting groups were then cleaved simultaneously with TFA/*i*Pr$_3$SiH/H$_2$O (95:2.5:2.5). Subsequent transesterification with NaOMe/methanol at pH 8.5 and 9 afforded the desired Aoa-containing glycopeptides **1** and **2**, respectively. It is notable that the unprotected Aoa group is compatible with the mild methanolysis treatment needed to remove the protecting groups from the carbohy-

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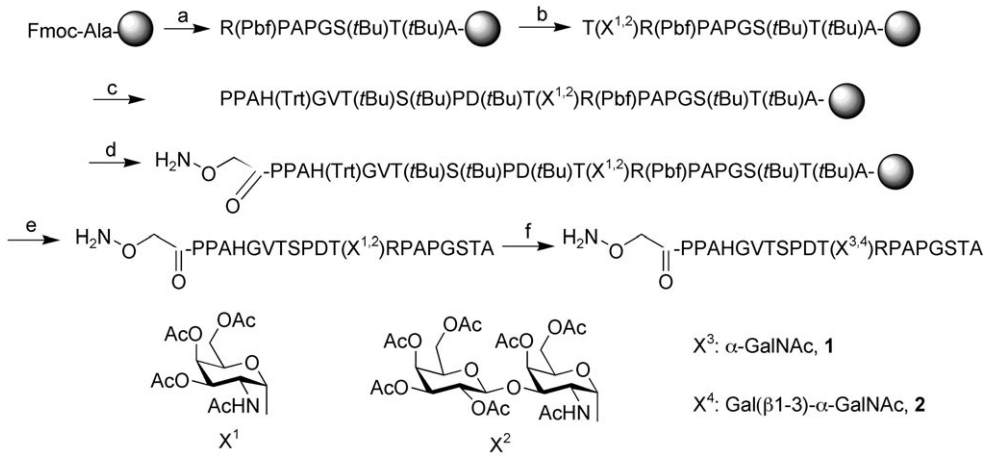
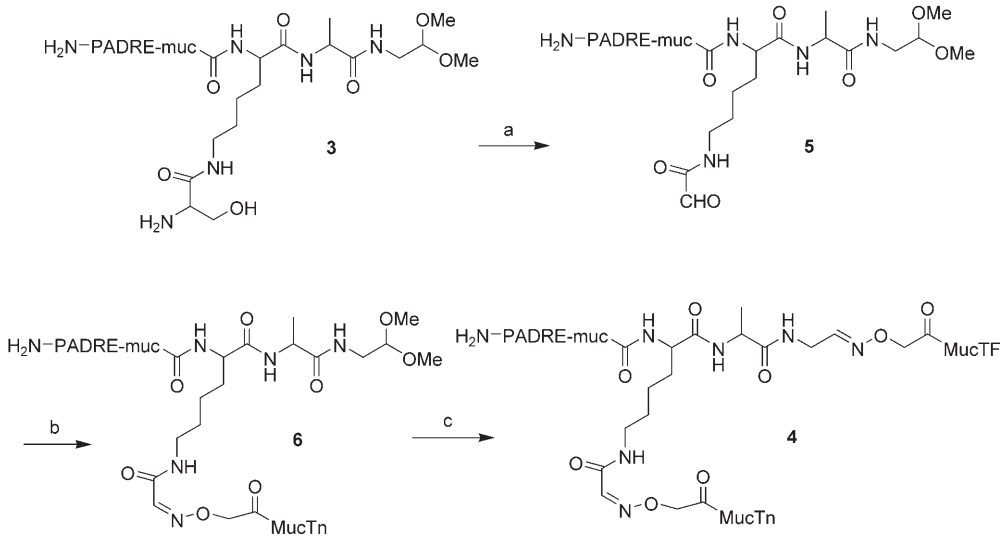


Scheme 1. Synthesis of Aoa MucTn **1** and Aoa MucTF **2**. a) SPPS (Fmoc/*t*Bu): 1 protected amino acid/HBTU/HOBt/iPr$_2$NEt, 10 equiv; 2 Ac$_2$O, iPr$_2$NEt; b) 1 Fmoc-Thr(Tn)-OH or Fmoc-Thr(TF)-OH/HATU/iPr$_2$NEt, 1.1 equiv; 2 Ac$_2$O, iPr$_2$NEt; c) 1 SPPS (Fmoc/*t*Bu): protected amino acid/HBTU/HOBt/iPr$_2$NEt, 10 equiv; 2 Ac$_2$O, iPr$_2$NEt; d) Boc-Aoa-OH/HATU/iPr$_2$NEt, 10 equiv; e) TFA/H$_2$O/iPr$_3$SiH (95:2.5:2.5); f) Aoa MucTn **1**: anhydrous MeOH adjusted to pH 8.5 with 1% NaOMe, 2 h; Aoa MucTF **2**: anhydrous MeOH adjusted to pH 9, 5 h. Fmoc = 9-fluorenylmethyloxycarbonyl.

drate moieties. The purity of the crude glycopeptides **1** and **2** was 64 and 70%, respectively. HPLC and mass spectrometry analyses revealed that the by-products were truncated acetylated peptides resulting from incomplete coupling of *O*-glycosylated Thr12 and the unreactive adducts of formaldehyde, acetaldehyde, and acetone to the Aoa-containing peptide.[26] Thus, the crude Aoa-containing glycopeptides **1** and **2** were directly engaged in oximation reactions (Scheme 2).

PADRE-muc(Ser)acetal **3**, the stem peptide bearing two masked aldehydes, is composed of the PADRE epitope colinearly synthesized at the N terminus of a MUC1 repeat unit with an additional Lys-Ala dipeptide at the C terminus: Lys for the introduction of Ser at the branch point and Ala as a unhindered amino acid for acetal installation at the C terminus by nucleophilic displacement.[27] PADRE-muc(Ser)acetal **3** was synthesized on a PEGA resin with the dipeptide WT introduced as the Fmoc-Trp(Boc)-Thr($\psi^{Me,Me}$ pro)-OH dimethyloxazolidine analogue[28] to improve the elongation yield of this difficult sequence.[29] $NaIO_4$ treatment of **3** and HPLC purification afforded keto-aldehyde **5** which was solubilized in 0.1 M NaOAc, pH 4.6 and ligated to Aoa MucTn **1** over 6 days. The reaction was not completely quantitative, and PADRE-muc(MucTn)acetal **6** was purified by HPLC. The acetal of **6** was then removed with TFA treatment. Those steps, including HPLC purification in the presence of 0.1% TFA and acetal removal proved to be particularly challenging. They have to be performed in the presence of



Scheme 2. Synthesis of the tri-branched glycopeptide conjugate **4**. a) $NaIO_4$, HPLC-purification; b) Aoa MucTn **1**, NaOAc (0.1 M, pH 4.6), HPLC purification; c) 10% TFA, Aoa MucTF **2**, HPLC purification. PADRE-muc(Ser)acetal **3**: aAKXVAAWTLKAaPPAHGVTSAPDTRPAPGSTAK(S)A-NH-CH$_2$-CH(OCH$_3$)$_2$ for which a = D-Ala and X = cyclohexylalanine.

AoaMucTF **2** to avoid side reactions resulting from the reaction of the free methylaldehyde in acidic medium with the preformed oxime bond.[19] Moreover, the amount of TFA needed to remove the acetal has to be carefully controlled to avoid the degradation of the *O*-glycosidic bond. After optimization, the acetal removal was carried out with 10% TFA at 0 °C over 10 min in the presence of AoaMucTF **2**. Under these conditions, the *O*-glycosidic bonds were stable. After TFA evaporation under vacuum, the reaction mixture was taken up with 0.1 M NaOAc, pH 4.6 and analyzed by HPLC. The calculated amount of AoaMucTF **2** was then added, leading to a quantitative reaction. The glycopeptide conjugate PADRE-muc-(MucTn)MucTF **4** was purified by HPLC. Electrospray mass spectrometry of purified glycopeptide **4** revealed the presence of a by-product with a Δm value of +162.1, attributed to the glycopeptide PADRE-muc(MucTF)MucTF. It can be explained by the fact that the first ligation on the keto-aldehyde **5** is not quantitative at pH 4.6 and that PADRE-muc(CHO)acetal **5**, having not reacted with AoaMuc **1**, is not separable from PADRE-muc(MucTn)acetal **6** by HPLC because of the very hydrophobic dominant character of the PADRE moiety. The keto-aldehyde could have been activated during the acidic treatment for acetal removal in the presence of AoaMucTF **2** leading to the glycopeptide PADRE-muc(MucTF)MucTF.

The immunogenicity of multiantigenic MUC1 glycopeptide conjugate **4**, composed of two diversely glycosylated MUC1 repeat units and one nonglycosylated unit, was then evaluated in mice. Mice were immunized four times with PADRE-muc-(MucTn)MucTF **4** adsorbed to alum, an adjuvant authorized in human immunotherapy. Sera collected 15 days after the last two boosts were tested by ELISA. Glycopeptide **4** elicited high titer antisera of mainly IgG type that recognized a recombinant MUC1 fragment of eight nonglycosylated repeat units (Table 1); only one mouse responded weakly to the antigen. These results demonstrate the high immunogenicity of the novel glycopeptide **4** associated with a mild adjuvant. To verify the specificity of the antibodies, the nonglycosylated MUC1 repeat unit and the unit glycosylated at Thr 12 with Tn or TF were separately coated on the ELISA plates (Figure 1). The mice produced antibodies that recognize the MucTn and MucTF glycopeptides. To test whether the antibodies are able to recognize MUC1 on tumor cells, we analyzed the binding of mouse sera to human MCF7 mammary adenocarcinoma cells, which have been extensively used to characterize tumor-associated MUC1[30] (Figure 2). The MCF7 cells are recognized remarkably well by antibodies elicited by glycopeptide **4** (sera diluted 1:1000), showing the potential of our approach to induce a highly specific humoral immune response directed against tumor-associated structures. These results provide the first example of the use of glycosylated MUC1 repeat units oxime-ligated to a universal T-helper epitope and associated with a mild adjuvant as a synthetic antitumor vaccine.

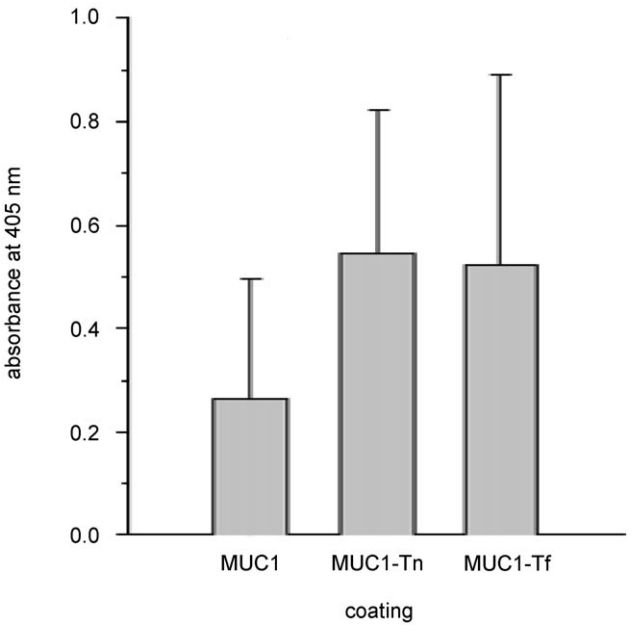


Figure 1. The PADRE-muc(MucTn)MucTF glycopeptide **4** induced specific antibodies. Five C57Bl/6 mice received four injections of 25 μg glycopeptide **4** adsorbed to alum. Sera were collected 15 days after the last boost, and antibodies were titrated in triplicate by ELISA on plates coated with one repeat unit MUC1, MUC1-Tn, or MUC1-TF. The histograms represent the mean absorbance of sera from the four responder mice diluted at 1:1000.

Table 1. Antibody titer of sera from mice immunized with glycopeptide **4**.[a]

Individual Mouse	Antibody Titer					
	After 3rd Immunization			After 4th Immunization		
	Ig	IgM	IgG	Ig	IgM	IgG
1	100	30	200	800	40	250
2	400	40	200	6400	80	1600
3	400	35	400	12000	50	5000
4	200	75	400	3200	70	1600
5	30	75	50	150	100	130
Average ±SD	220±160	50±20	250±120	4500±4300	70±20	1700±1750

[a] Five C57Bl/6 mice received four injections of 25 μg glycopeptide **4** adsorbed to alum. Sera were collected 15 days after the 3rd and 4th immunization, and antibodies were titrated in triplicate on plates coated with nonglycosylated recombinant MUC1 protein (eight repeat units).

In conclusion, we describe an efficient route for the synthesis of a multiantigenic glycopeptide conjugate, a mimic of the MUC1 glycoprotein, which in mice are able to elicit antibodies that recognize native tumor-associated MUC1 present on a human breast cancer cell line. Our synthetic approach opens the way towards the production of well-defined complex glycopeptides that may help to decipher the humoral and cellular immune responses induced by tumor-related glycopeptide antigens.

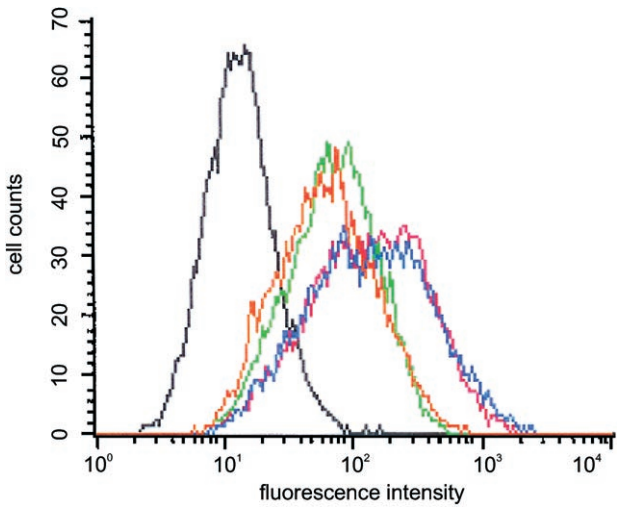


Figure 2. Recognition of the human breast cancer cell line MCF7 by sera diluted to 1:1000 from mice immunized with glycopeptide **4**. Colored lines: immune sera; black line: control serum.

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