ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 1487–1489

www.rsc.org/chemcomm

COMMUNICATION

Antibody recognition of fluorinated MUC1 glycopeptide antigens†‡

Thomas Oberbillig,[ab] Christian Mersch,[b] Sarah Wagner[b] and Anja Hoffmann-Röder*[b]

Received 18th August 2011, Accepted 28th September 2011
DOI: 10.1039/c1cc15139h

The syntheses of various fluorinated MUC1 glycopeptide antigens and their specific binding to serum antibodies from mice immunized with natural and fluorinated TF^6-MUC1-TTox conjugate vaccines are presented.

The identification of aberrant glycosylation patterns on cell-surface glycoproteins of malignant cells has spurred research efforts towards the development of cancer immunotherapy.[1] Unusual glycoforms of mucin glycoproteins which are expressed on most carcinoma represent attractive target structures for carbohydrate-based anti-cancer vaccines.[2] Although various tumor-associated carbohydrate antigens (TACA) have already been used for the design of anti-cancer vaccines,[3] their targeting is often hampered by a weak immunogenicity and a T-cell-independent immune response. Successful strategies to overcome these obstacles include the conjugation of MUC1-derived TACA to immunogenic peptides,[4] carrier proteins,[5] and lipopeptides.[6] Moreover, enhancement of the immunogenicity of the TACA by exploring structural variants such as non-natural glycosidic linkages and modified carbohydrate epitopes has been studied.[7] In this context, the strategic introduction of fluorine atoms into the glycan moiety seems to be a particularly promising strategy to improve the immunogenicity of carbohydrate-based antigens.[8] Furthermore, fluorosugars show enhanced chemical and metabolic stabilities which make fluorinated glycostructures attractive targets for medical applications including immunotherapy. However, owing to their challenging multi-step syntheses and lengthy chromatographic separations or separation procedures, only few examples of complex fluorinated TACA for immunotherapeutic purposes have been reported, so far.[8]

Herein, the syntheses and antibody binding properties of novel fluorinated MUC1 glycopeptide antigens are presented. All of these TACA feature at Thr6 of the 20 amino acid MUC1 tandem-repeat sequence a variably fluorinated Thomsen-Friedenreich antigen side-chain carrying up to three fluorine atoms at positions C2′, C6′ and C6. With the fluorine atoms in close proximity to the glycosidic linkages, these glycomimetics[9] should not only be more immunogenic than the natural compounds, but also more metabolically stable.

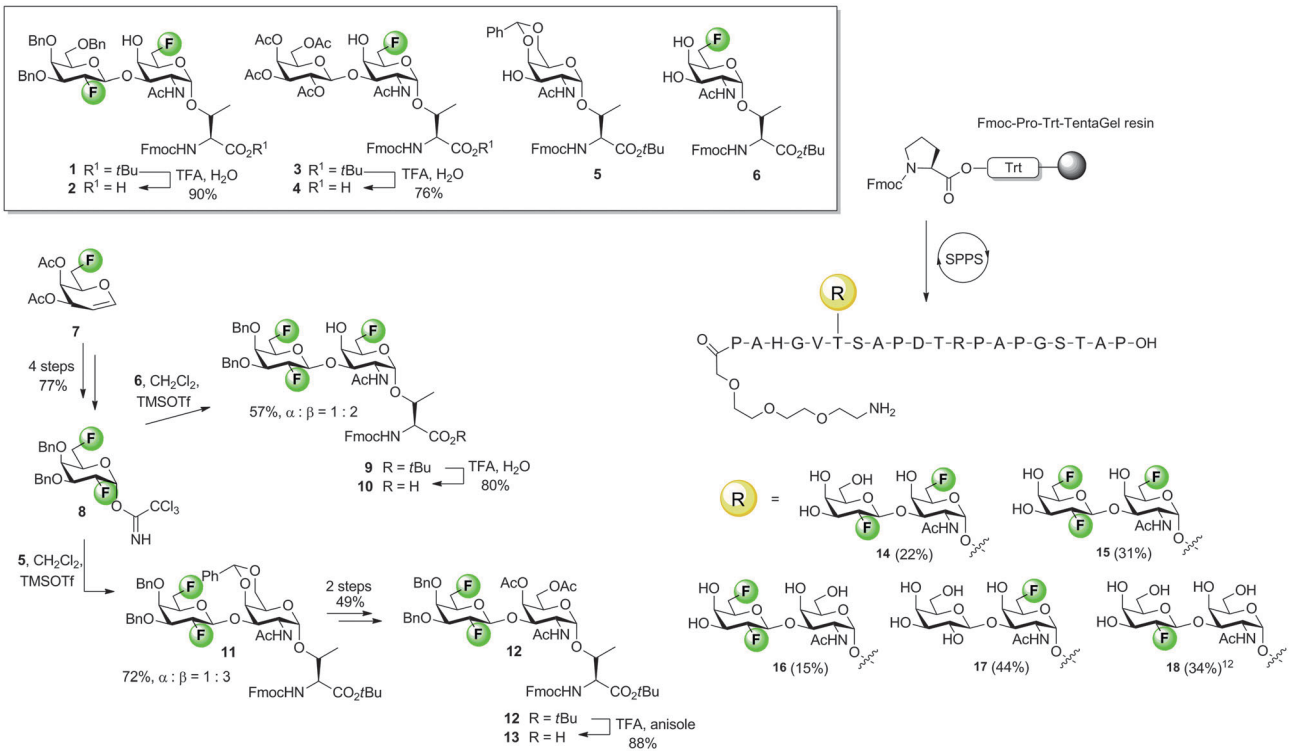
In addition to the previously synthesized TF antigen analog **1**,[10] the requisite fluorinated glycosyl amino ester building blocks **3**, **9** and **12** have been prepared from Fmoc-(αGalNAc)-Thr-O*t*Bu (**5**)[11] and its fluorinated analog **6**[12] (Scheme 1). Therefore, 6-fluorogalactal **7**[12] was converted to 3,4-di-*O*-benzyl-6-deoxy-6-fluoro-galactosyl trichloroacetimidate (**8**) through a reaction sequence of de-*O*-acetylation, benzylation, electrophilic Selectfluor®-mediated fluorination and base-catalyzed addition to trichloroacetonitrile (77%, 4 steps). The resulting fluorinated glycosyl donor **8** was coupled under Schmidt conditions (TMSOTf, CH_2Cl_2)[13] to T_N derivatives **5** and **6** to give the fluoro-TF antigen analogs **9** and **11** in good yields, albeit low stereoselectivities. Unfortunately, all attempts to increase the β-selectivities by varying the reaction conditions (solvent, promotor, temperature) failed, so far. Moreover, in some reactions of the 6-fluoro-T_N derivative **6**, small amounts of the corresponding 3,4-bisglycosylated product (<15%) were observed, which can be supressed by protection of the 4-OH prior to glycosylation. After purification of the fluoro-TF derivatives **9** and **11**, their benzylidene acetal protecting groups were exchanged for acetyl groups. Subsequent acidolysis of the *tert*-butyl esters yielded the desired glycosyl amino acids **10** and **13**, which were used together with their congeners **2** and **4** for the assembly of MUC1 tandem-repeat sequences.

The syntheses of the target MUC1 glycopeptides bearing *N*-terminal triethylene glycol units were achieved by Fmoc-based solid-phase peptide synthesis employing preloaded Fmoc-Pro-Trt-TentaGel resins (see Scheme 1 and ESI‡). In contrast to the standard procedure, the couplings of the fluorinated glycosyl amino acids always required longer reaction times (8 h) and usage of the more reactive reagent cocktail HATU/HOAt/NMM in NMP.[11] Moreover, to minimize the amount of byproducts, unreacted amino groups were capped with Ac_2O/HOBt/iPr_2NEt in NMP after each coupling cycle. The resulting glycopeptides were then cleaved from the resin with concomitant removal of the acid-labile side chain protecting groups using a mixture of TFA, triisopropylsilane (TIS) and water, followed by deprotection of the glycans *via* hydrogenolysis (H_2, $Pd(OAc)_2$)[12] or transesterification with NaOMe in MeOH.[14] Preparative RP-HPLC finally furnished the desired fluorinated TACA analogs **14–18**[12,15] in yields of 15–44% based on the original resin loadings.

[a] *Institut für Mikrotechnik Mainz GmbH, Carl-Zeiss-Strasse 18-20, 55129 Mainz, Germany*
[b] *Institut für Organische Chemie, Johannes Gutenberg-Universität Mainz, Duesbergweg 10-14, D-55128 Mainz, Germany. E-mail: hroeder@uni-mainz.de; Fax: +49 6231 3926066; Tel: +49 6131 3922417*

† This article is part of the *ChemComm* 'Emerging Investigators 2012' themed issue.

‡ Electronic supplementary information (ESI) available: Experimental procedures, characterization data and selected spectra of new compounds. See DOI: 10.1039/c1cc15139h



Scheme 1 Preparation of the requisite fluorinated glycosyl amino acid building blocks **2**, **4**, **10**, **13** and their use in solid-phase glycopeptide synthesis for the assembly of fluorinated mucin-type glycopeptide analogs **14–18**. For details of the syntheses, see ref. 8*c*, 10, 12, and ESI.‡

To investigate if the position and number of fluorine substituents at the glycan moieties affect the antibody binding properties, neutralization experiments of the glycopeptides **14–18** with anti-MUC1-serum antibodies were performed. For instance, four different concentrations of mouse antiserum obtained from immunization with a synthetic MUC1-TTox conjugate vaccine carrying the natural TF antigen glycan at Thr6[8c] were incubated with both the natural glycopeptide antigen and its fluorinated analogs **14–18** for 1 h at 37 °C (Fig. 1). The solutions were then transferred to an ELISA plate coated with the natural (non-fluorinated) MUC1 glycopeptide-BSA conjugate and a biotinylated secondary antibody was added. Subsequent treatment with streptavidin-horseradish peroxidase (HPO) allowed visualization of the residual antibodies' binding affinities.[5a,5d]

Fig. 1 shows that the binding of the untreated serum (positive control) was neutralized significantly by addition of the glycopeptide antigens. Similar results were obtained in the neutralization experiments performed with antisera from mice immunized with a 2′-fluoro-TF[6]-MUC1-TTox[16] (Fig. 2) and a 6,6′-difluoro-TF[6]-MUC1-TTox vaccine,[8c] respectively (Fig. 3).

Thus, for all fluorinated MUC1 antigens **14–18** strong binding of both antisera was observed, irrespective of the number and position of the fluorine atoms. However, a structural mismatch between the glycopeptide ligand and the cognate antigen can in some cases affect the binding affinity. For example, the antibodies elicited by the difluorinated

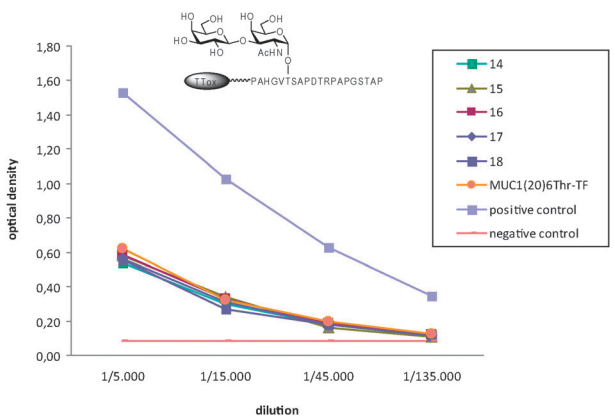


Fig. 1 Neutralization experiments using mouse antiserum raised by immunization with a TF-MUC1-TTox vaccine[8c] and mucin-type glycopeptide analogs **15–18**.

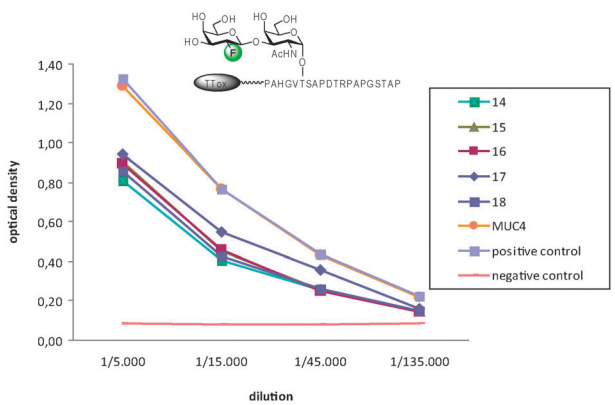


Fig. 2 Neutralization experiments using mouse antiserum raised by immunization with a 2′F-TF-MUC1-TTox vaccine[16] and mucin-type glycopeptide analogs **14–18** and a related MUC4 glycopeptide.[17]

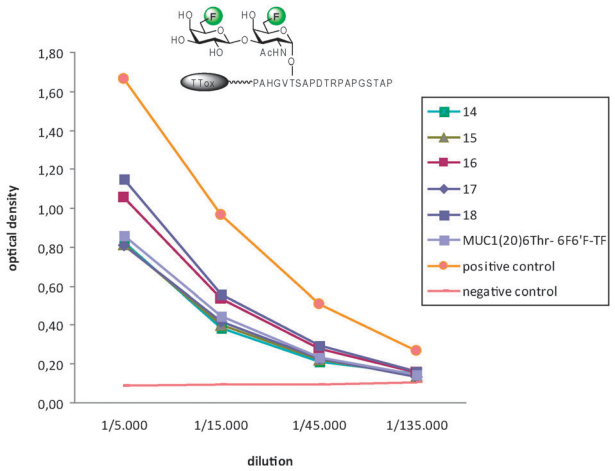


Fig. 3 Neutralization experiments using mouse antiserum raised by immunization with a 6F,6′F-TF-MUC1-TTox vaccine[8c] and mucin-type glycopeptide analogs **14–18**.

vaccine bound slightly weaker to glycopeptides **16** and **18**, whose glycan chains lack the fluorine atom in position 6 (Fig. 3). Likewise, the neutralization of the antiserum induced by the 2′-fluoro-TF[6]-MUC1-TTox vaccine was less effective with glycopeptide **17**, *i.e.*, in the absence of the 2′-fluoro substituent (Fig. 2). Nonetheless, the serum antibodies differentiate the modified glycan structures only slightly and their binding affinities seem mainly determined by the glycopeptide backbone. Thus, a related MUC4 glycopeptide[5a,17] with a deviating peptide sequence was not recognized at all (Fig. 3). This suggests that the majority of antibodies raised by both the natural and the fluorinated glycoconjugate vaccine were specific to the whole MUC1 glycopeptide antigen rather than to its partial structures such as the (fluorinated) carbohydrate part—a pre-requisite for the design of glycomimetic-based vaccines.

In summary, we have prepared various modified MUC1 glycopeptide antigens with fluorinated Thomsen-Friedenreich side chains and evaluated their specific binding to mouse antisera by means of ELISA experiments. The fact that the antisera derived from natural and fluorinated TF[6]-MUC1-TTox vaccines showed very little differences in binding to the fluorinated glycopeptide antigens **14–18** supports the idea of using strategically fluorinated TACA for the design of specific carbohydrate-based vaccines with enhanced immunogenicity and metabolic stability. Moreover, owing to the subtle differences in antibody affinity, glycopeptide antigens **14–18** are useful tools for analyzing humoral immune responses against MUC1 antigens with different glycan structures.

This work was supported by the Deutsche Forschungsgemeinschaft and the Institut für Mikrotechnik Mainz. We thank Prof. Dr H. Kunz and Prof. Dr E. Schmitt (University of Mainz) for fruitful discussions.

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