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COMMUNICATION

Synthesis of a MUC1-glycopeptide–BSA conjugate vaccine bearing the 3′-deoxy-3′-fluoro-Thomsen–Friedenreich antigen†‡

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A novel MUC1-glycopeptide–BSA conjugate vaccine with a specifically fluorinated Thomsen–Friedenreich antigen side chain at Thr6 was prepared. Preliminary immunological experiments reveal specific binding of the tumor-associated glycopeptide antigen analog by anti-MUC1-mouse antibodies.

Mucin glycoproteins are abundantly expressed on the surface of epithelial cells where they mediate crucial cellular interactions in the course of embryogenesis, organogenesis, carcinogenesis, and cancer metastasis.[1] Unusual glycoforms of mucins with specifically altered *O*-glycan chains expressed on carcinomas have been exploited as diagnostic biomarkers and are promising target structures in the quest for effective carbohydrate-based cancer vaccines and immunotherapeutics.[2] In this regard, the tumor-associated mucin MUC1[3] has attracted much attention, and synthetic glycopeptides of its tandem-repeat (TR) domain with cancer-related saccharides such as the T_N antigen, its sialylated congener (ST_N), and the Thomsen–Friedenreich (TF) antigen were shown to elicit highly specific immune responses in mice.[4]

An interesting concept for improving the immunological properties of tumor-associated antigens relies on the use of fluorine-substituted mimics. Thus, with a view to the absence of fluorine in most organisms and the high bond strength to carbon,[5] fluorination might enhance both the immunogenicity and the bioavailability of the antigen significantly. However, fluorine substitution has only recently been investigated as a tool in vaccine development,[4j,6] despite its frequent use in medicinal chemistry.[7] To date, many bioactive, fluorinated carbohydrates have been prepared ranging from simple monosaccharides to complex glycostructures, although the stereoselective assembly of the latter still remains a synthetic challenge.[8]

As part of a research programme devoted to the synthesis and immunological evaluation of selectively fluorinated MUC1-glycopeptide vaccines,[4j] we now report on the successful preparation of a first 3′-fluoro-TF antigen–MUC1–bovine serum albumin (BSA) conjugate. Because tumor-associated glycopeptides are T cell-independent self-antigens and as such tolerated by the immune system, their conjugation to suitable immunogenic carrier proteins, *e.g.* BSA, is needed to override this tolerance. Thereby, the carrier protein enhances the presentation of tumor-associated antigens to the immune system and provides peptide fragments that can activate T_H cells.

A key step in the synthesis of the 3′-fluoro-TF-antigen glycosyl amino acid building block **9** is the coupling of a 3-fluoro-galactosyl bromide **6**[8d] to the T_N-antigen–threonine conjugate **7**.[9] Analogously to the synthesis of Brimacombe *et al.*,[10] compound **6** was prepared in ten steps starting from glucose **1** (Scheme 1). Thus, **1** was converted into 1,2;5,6-di-*O*-isopropylidene-glucofuranose in acetone at 10 °C and oxidized at C3 with pyridinium dichromate (PDC)–acetic anhydride (Ac_2O) under reflux (46%, two steps).[11] To set up the correct stereochemistry, both stereocenters at C3 and C4 needed to be inverted. Therefore, ketone **2** was transformed into the corresponding hydrate and acetylated with Ac_2O–pyridine to furnish after β-elimination enol acetate precursor **3** (59%, two steps). The latter was then stereoselectively hydrogenated from the less hindered top side by Pd/C in MeOH[12] to provide 1,2;5,6-di-*O*-isopropylidene-3-*O*-acetyl-D-gulofuranose. Deprotection of the remaining acetate group finally furnished precursor **4** for the subsequent nucleophilic deoxy-fluorination reaction in a total yield of 19% over six steps. Thus, upon treatment of **4** with diethylaminosulfur trifluoride (DAST) and *N*,*N*-dimethylaminopyridine (DMAP),[13] smooth conversion to the desired 3-deoxy-3-fluoro-D-galactofuranose was observed. Acidolysis of the isopropylidene acetals then liberated 3-deoxy-3-fluoro-D-galactopyranose **5** which was peracetylated and converted to the anomeric bromide **6** in 69% yield over two steps.

The effectiveness of glycosyl bromides in synthesizing the TF antigen and its fluorinated derivatives has been demonstrated before,[14,8d,8h] and consequently, compound **6** was subjected to a β-selective Helferich-type glycosylation with T_N antigen acceptor **7**. Thus, the hitherto unknown 3′-deoxy-3′-fluoro-TF antigen analog **8** was stereoselectively formed in 67% yield by a $Hg(CN)_2$-promoted glycosylation of **7** at 80 °C under microwave irradiation. Subsequent protecting group manipulations, *i.e.*, cleavage of the benzylidene acetal followed by acetylation and acidolysis of the *tert*-butyl ester finally provided the target

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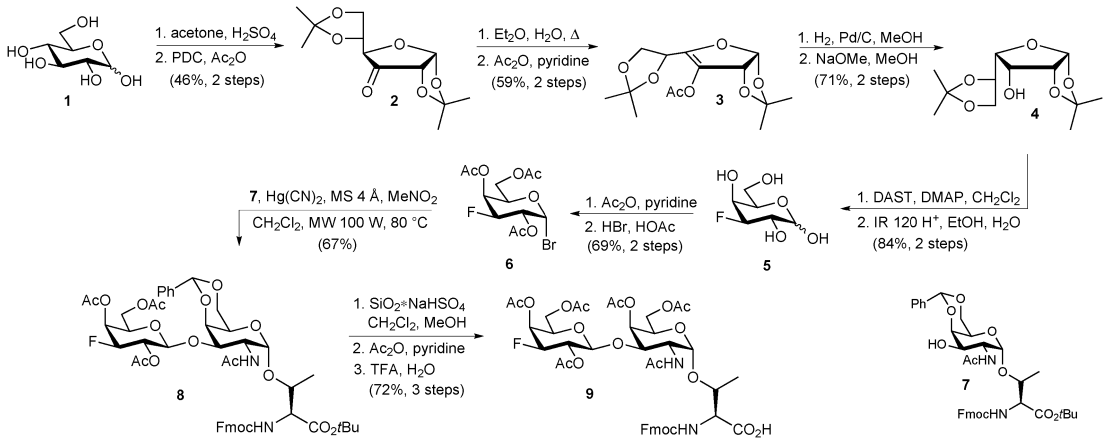
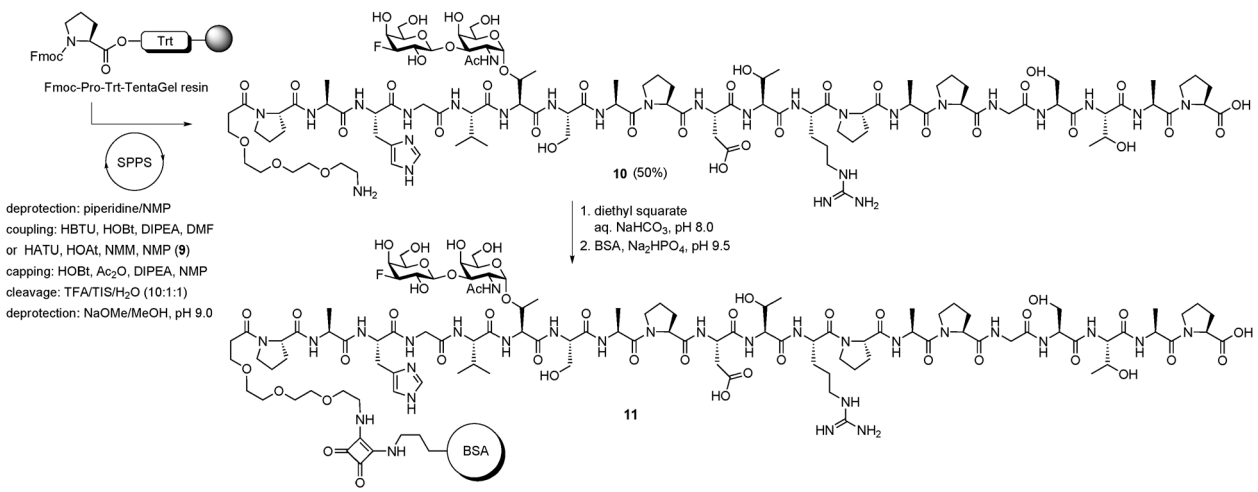


Scheme 1 Preparation of the 3′-deoxy-3′-fluoro-Thomsen–Friedenreich antigen building block for solid-phase glycopeptide synthesis. PDC = pyridinium dichromate, DAST = diethylaminosulfur trifluoride, DMAP = *N*,*N*-dimethylaminopyridine, TFA = trifluoroacetic acid.

3′-fluoro-TF antigen building block **9** in 72% yield over three steps.

Next, the corresponding MUC1-TR-glycopeptide antigen bearing the 3′-fluoro-TF analog at Thr6 was assembled in an automated synthesizer by Fmoc-SPPS employing trityl-TentaGel resin preloaded with Fmoc-Pro-OH, as previously described (Scheme 2 and ESI‡).[8h] Iterative couplings of the Fmoc-protected amino acids were performed with HBTU/HOBt and diisopropylethylamine (DIPEA) in DMF, while the coupling of the glycosyl amino acid **9** required the use of the more reactive HATU/HOAt cocktail and *N*-methylmorpholine (NMM) in *N*-methyl-pyrrolidone (NMP). At the end of the solid-phase synthesis, a triethylene glycol spacer[4a] was attached and the glycopeptide was released from the resin with simultaneous cleavage of the side chain protecting groups using an acidic cocktail of TFA–triisopropylsilane (TIS)–water (10 : 1 : 1). After deacetylation with NaOMe in MeOH at pH 9.0, glycopeptide **10**[15] was isolated in 50% yield by preparative RP-HPLC and subjected to conjugation to diethyl squarate in aqueous solution (pH 8).[16] Linking the resulting squarate monoamide to BSA was then achieved in aqueous phosphate buffer at pH 9.5, providing the desired vaccine conjugate **11** after dialysis (membrane 30 kDa). The antigen loading level of **11** was determined by MALDI-TOF mass spectrometry and indicated on average five molecules of glycopeptide per molecule of BSA (Fig. 1).

The binding of mouse anti-MUC1-serum antibodies, raised by immunization with the structurally related synthetic vaccines ^{6}TF–MUC1–TTox and 6′,6-difluoro-^{6}TF–MUC1–TTox,[4j] to the fluorinated glycopeptide antigen **10** was investigated. Therefore, four different dilutions of each serum were incubated for 1 h at 37 °C with 100 μL of the antigen **10**, and then transferred to ELISA plates coated with the parent antigen–BSA conjugates (Fig. 2 and 3). Upon addition of a biotinylated secondary anti-mouse antibody and treatment with streptavidin–horseradish peroxidase (HPO), the residual binding affinities of the serum antibodies to the antigen–BSA coatings were photometrically detected at λ = 410 nm.[4c,h] The data in Fig. 2 indicates that the binding of the untreated serum (positive control) was neutralized considerably when incubated with either the ^{6}TF–MUC1(20)-glycopeptide, the antigen contained in the vaccine, or its 3′-fluorinated derivative **10**. Similarly, the binding



Scheme 2 Solid-phase peptide synthesis (SPPS) of the 3′-fluoro-^{6}TF–MUC1 glycopeptide and its conjugation to BSA. NMP = *N*-methylpyrrolidone, HBTU = *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = *N*-hydroxybenzotriazole, DIPEA = diisopropylethylamine, HATU = *O*-(7-aza-benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = *N*-hydroxy-7-azabenzotriazole, NMM = *N*-methylmorpholine, TIS = triisopropylsilane, BSA = bovine serum albumin.

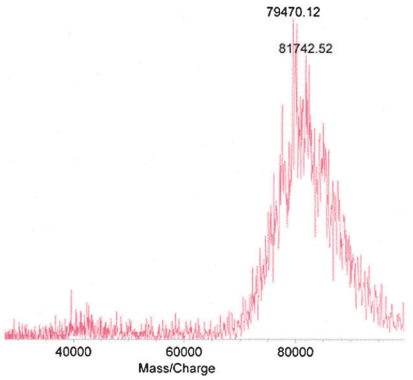


Fig. 1 MALDI-TOF spectrum of 3′-fluoro-^{6}TF–MUC1–BSA conjugate **11**.

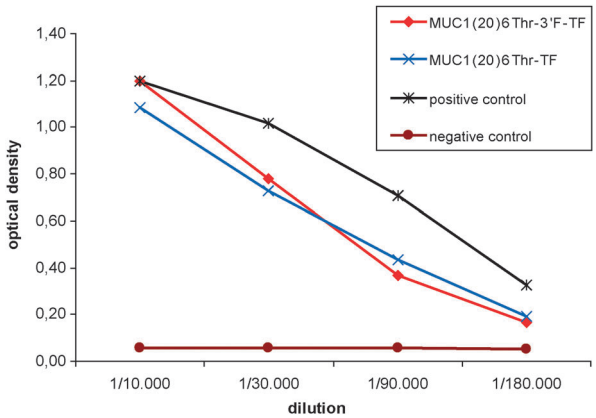


Fig. 2 Neutralization of serum antibodies from mouse immunized with a TF–MUC1–TTox vaccine using the natural ^{6}TF–MUC1(20)-glycopeptide antigen[4j] and its fluorinated derivative **10**.

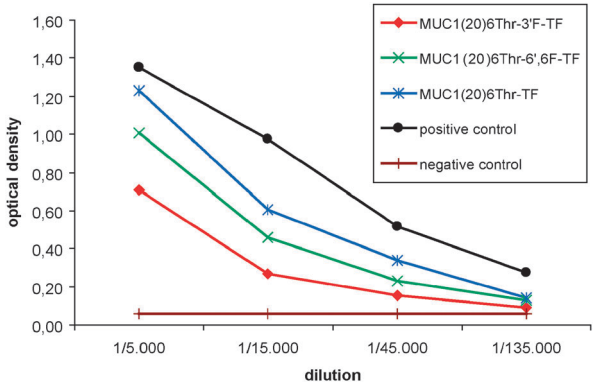


Fig. 3 Neutralization of serum antibodies from mouse immunized with a 6′,6-difluoro-^{6}TF–MUC1–TTox vaccine using the natural ^{6}TF–MUC1(20)-glycopeptide antigen,[4j] its related 6′,6-difluoro-^{6}TF-analog,[4j] and glycopeptide **10**.

of the serum antibodies raised by immunization with 6′,6-difluoro-^{6}TF–MUC1–TTox was neutralized in the presence of 3′-fluoro-^{6}TF–MUC1 glycopeptide **10** (Fig. 3). This cross-reactivity in epitope recognition supports the notion that the fluorinated glycopeptide conjugate **11** might be a promising vaccine candidate and further immunization studies in this direction are currently being pursued. Finally, glycoconjugate **11** is of particular interest for detailed analyses of the fine specificities of antibody responses.

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