Synthesis of MUC1–lipopeptide chimeras†

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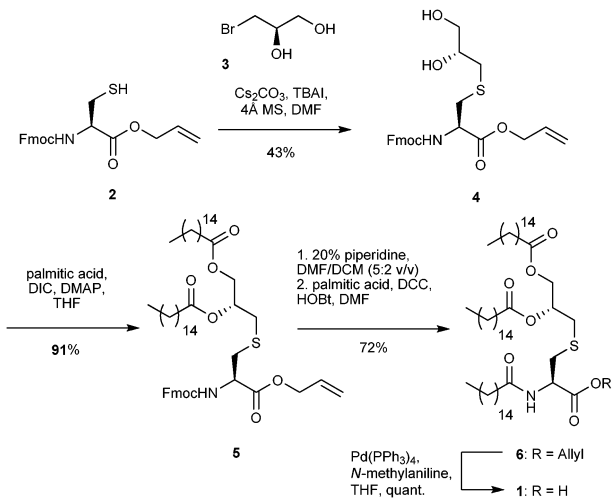
An efficient method for the convergent assembly of MUC1–lipopeptide vaccine candidates is described. Chimeras consisting of MUC1 glycopeptides (bearing multiple copies of the T_N and T tumour-associated carbohydrate antigens) tethered to the lipopeptide immunoadjuvant $Pam_3CysSer$ were synthesised in high yields using a fragment-based condensation strategy.

A classic feature of oncogenically transformed cells is the aberrant glycosylation of cell surface glycoproteins arising from the dysregulation of carbohydrate processing enzymes.[1] In the case of mucin glycoproteins located on the epithelial cell-surface, the over-expression of *O*-linked tumour-associated carbohydrate antigens (TACAs) such as the T_N, T and the corresponding sialylated derivatives has been directly correlated with tumour progression and growth.[2] These glycans exhibit poor immunogenicity, a direct consequence of over-riding self-tolerance by the immune system. As such, these TACAs cannot be employed as vaccines alone. Advances in our understanding of the relationship between innate and adaptive immunity have provided new opportunities in cancer vaccine development, thus stimulating significant interest in the rational design and preparation of synthetic MUC1-based glycopeptide vaccines.[3] This burgeoning knowledge base has culminated in the impressive work of Boons,[4] Danishefsky,[5] Kunz[6] and others.[7] In particular, it has been demonstrated that highly specific antibodies recognising tumour-associated epitopes can be achieved by utilising self-adjuvanting, multi-component MUC1 glycopeptide vaccines. These have been prepared *via* covalent attachment of segments of the cancer-associated MUC1 variable number tandem repeat domain (VNTR) to an immunostimulating adjuvant, with or without a helper T-cell epitope.[3,4] Recently, it was reported that hyperglycosylation of the MUC1 VNTR sequence (GVTSAPDTRPAPGSTAPPAH), whereby all five potential *O*-glycosylation sites are occupied, is an important feature for the production of a strong antibody response against the glycopeptides.[2,5a] Hyperglycosylated MUC1 peptides conjugated to an immunostimulating adjuvant represent an underexplored class of vaccine candidates. Herein, we report the design and synthesis of a new class of self-adjuvanting MUC1 vaccine candidates possessing a full copy of the 20 amino acid MUC1 peptide VNTR domain and the immunostimulating Toll-like receptor 2 (TLR-2) ligand, $Pam_3CysSer$, first described by Jung and Bessler.[8] These constructs represent the first fully-synthetic MUC1-based multi-component vaccine candidates in which all five potential *O*-glycosylation sites are derivatised with the cancer associated T_N and T antigens.

Our synthetic strategy can be divided into three independent stages: solution phase synthesis of the TLR-2 ligand Pam_3Cys, solid-phase synthesis of MUC1 peptides, glycopeptides and a $Pam_3CysSer$ fragment (bearing a *C*-terminal triethylene glycolic acid linker) using Fmoc-based techniques, and finally a pentafluorophenyl ester-mediated fragment condensation of the triethylene glycolic acid-derivatised $Pam_3CysSer$ lipopeptide to completely deprotected MUC1 (glyco)peptides in order to furnish the corresponding target MUC1–lipopeptide chimeras.

The synthesis of Pam_3Cys **1** was achieved over five steps starting from Fmoc-Cys-OAll **2**[9] (Scheme 1). Treatment of **2** with the optically pure (*R*)-bromo-1,2-propane diol **3**[10] in the presence of Cs_2CO_3 and TBAI[11] furnished diol **4** in moderate yield (43%).[12] Carbodiimide-promoted esterification of **4** using palmitic acid resulted in smooth conversion to the dipalmitoylated cysteine derivative **5** in excellent yield.[13] One-pot deprotection of the Fmoc-carbamate and palmitoylation gave allyl protected Pam_3Cys precursor **6** in 72% yield. Finally, Tsuji–Trost deallylation using tetrakis(triphenylphosphine)palladium(0) and *N*-methylaniline as a scavenger provided Pam_3Cys **1** in quantitative yield.

With optically pure Pam_3Cys in hand, our attention focused on the preparation of the lipopeptide component of the proposed chimeras bearing a short triethylene glycolic acid spacer[14] *via* solid-phase peptide synthesis (SPPS) (Scheme 2). Ethylene glycol units are often incorporated to serve as flexible, polar and immunosilent linkers between the various recognition elements of a vaccine construct.[6] The flexible spacer is thought to minimise any potential conformational distortion caused by the lipopeptide fragment, thus ensuring all elements of the vaccine bind with maximum affinity to their cognate biological



Scheme 1 Synthesis of Pam_3Cys **1**.

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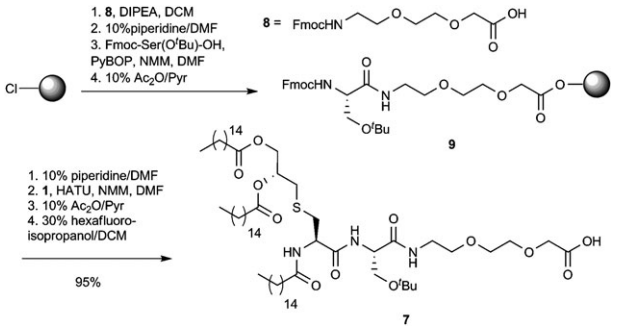
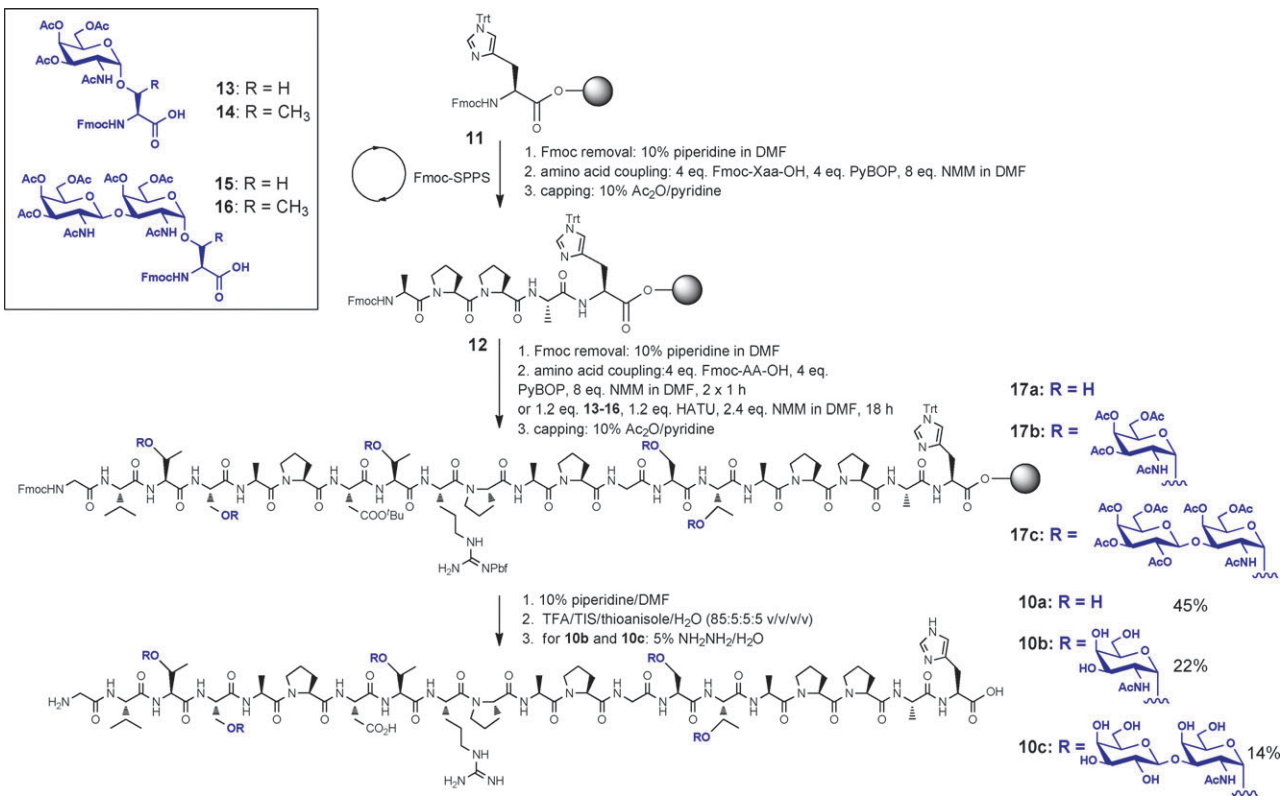


Scheme 2 SPPS of lipopeptide component **7**.

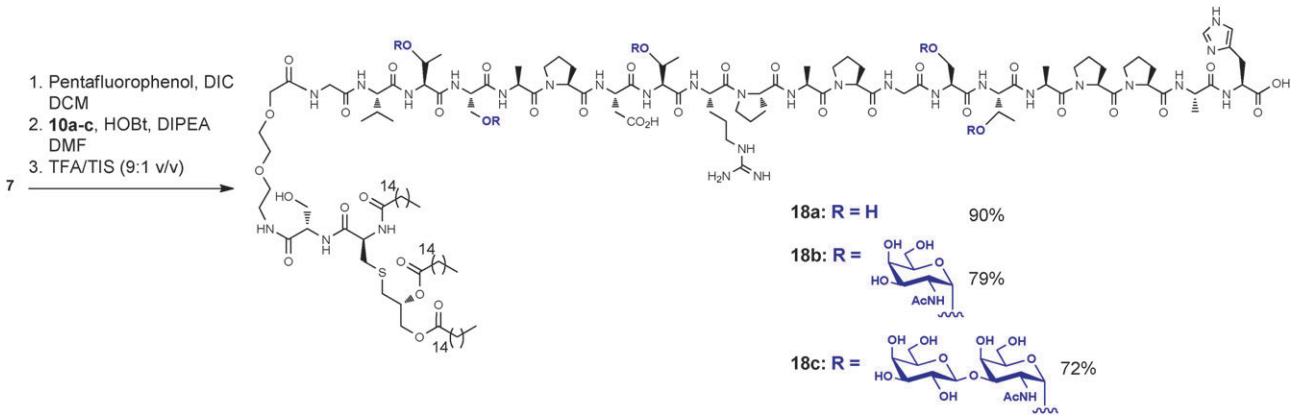
targets.[6] The desired lipopeptide **7** was assembled on 2-chlorotrityl chloride resin which was first loaded with Fmoc-protected triethylene glycolic acid **8**.[15] The solid-supported linker was subsequently deprotected with 10% piperidine in DMF, and coupled to Fmoc-Ser(O^{t}Bu)-OH using benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and *N*-methylmorpholine (NMM) to afford resin bound **9**. Following Fmoc deprotection, Pam_3Cys was installed by treatment with 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) and NMM in DMF for 20 h. Following cleavage and purification by flash column chromatography, the desired lipopeptide **7** was isolated in 95% yield.

Having successfully prepared lipopeptide fragment **7**, we embarked on the synthesis of peptide **10a** and glycopeptides **10b** and **10c** bearing multiple copies of the T_N and T TACAs, respectively. The synthesis of these targets was conducted from 2-chlorotrityl chloride resin preloaded with Fmoc-His(Trt)-OH **11** (Scheme 3). Resin bound pentapeptide **12** was assembled *via* Fmoc-strategy SPPS. At this stage, the synthesis diverged at the first *O*-glycosylation site. Synthesis of the solid supported unglycosylated peptide **10a** was achieved by iterative Fmoc-strategy SPPS using PyBOP and NMM. In the case of the resin bound glycopeptides **10b** and **10c**, coupling of the glycosylamino acids **13–16**[16,17] was achieved using only 1.2 equivalents of the precious building blocks and HATU as the coupling reagent. These conditions were employed so as to prevent excess waste of precious glycosyl amino acid building blocks whilst maintaining high coupling yields. Upon complete assembly of peptides **17a–c**, the resins were treated with 10% piperidine in DMF to remove the *N*-terminal Fmoc-carbamate moiety, followed by treatment with TFA/TIS/thioanisole/H_2O (85 : 5 : 5 : 5 v/v/v/v) to facilitate side chain deprotection and cleavage from the resin. At this stage peptide **10a** was purified by HPLC and isolated in 45% yield (based on the original resin loading). De-*O*-acetylation of glycopeptides was achieved using aqueous hydrazine and afforded target glycopeptides **10b** and **10c**, in 22% and 14% yields respectively after HPLC purification.

Having successfully synthesised the desired peptide and glycopeptide fragments by SPPS, we next explored possible convergent conjugation methods to lipopeptide **7**. Recently, Kunz and co-workers reported the HATU-promoted fragment condensation of a protected lipopeptide with deprotected MUC1 glycopeptides in solution which provided conjugates in 20–25% yields.[6c] In addition, Boons and co-workers have demonstrated that glycopeptides can be conjugated to lipopeptide thioesters using native chemical ligation.[4] However, owing to the hydrophobicity of the lipopeptide fragment, it was necessary to conduct these reactions in liposomes.



Scheme 3 SPPS of peptide **10a** and glycopeptides **10b** and **10c**.



Scheme 4 Synthesis of MUC1–lipopeptide chimeras **18a–c** *via* pentafluorophenyl ester-mediated fragment condensation.

We were interested in investigating an alternative fragment condensation approach for the high yielding preparation of MUC1–lipopeptide chimeras to serve as cancer vaccine candidates. To this end, we decided to explore the utility of pentafluorophenyl esters as *N*-acylation donors to prepare these constructs.[18] In a preliminary reaction, the free carboxylate group was pre-activated by treatment of lipopeptide **7** with *N*,*N*′-diisopropylcarbodiimide and pentafluorophenol.[19] Formation of the desired active ester could be monitored by TLC analysis and proceeded to completion within 1 h. The pentafluorophenyl ester was subsequently condensed with a slight excess (1.2 equivalents) of the unglycosylated MUC1 eicosopeptide in the presence of 1-hydroxybenzotriazole (HOBt) and DIPEA and the reaction monitored by LC-MS (Scheme 4). After complete consumption of lipopeptide **7** the side chain *tert*-butyl protecting group was removed *in situ* using an acidic cocktail. Purification by preparative HPLC furnished the target MUC1–lipopeptide chimera **18a** in excellent yield (90%).

Having established a simple and efficient method for the fragment-based condensation reaction of the unglycosylated MUC1 peptide **10a**, we shifted our attention towards the preparation of chimeras bearing multiple copies of the TACAs. To this end, homogeneous MUC1 glycopeptides bearing five copies of the T_N **10b** and T antigen **10c**, respectively, were subjected to the above conditions. Gratifyingly, MUC1 glycopeptide–lipopeptide constructs **18b** and **18c** were furnished in excellent yields (79% and 72%, respectively) after HPLC purification.

In summary, we have successfully exploited a convergent fragment condensation approach for the high yielding synthesis of chimeras for use as cancer vaccine candidates. Specifically, a pentafluorophenyl ester-mediated condensation allowed for the rapid construction of a number of MUC1 lipopeptide chimeras incorporating the full length MUC1 tandem repeat sequence and the TLR-2 ligand Pam_3Cys. These constructs represent the first fully synthetic glycopeptide MUC1-based vaccine candidates carrying the full length tandem repeat domain where all five potential *O*-glycosylation sites are occupied with either the T_N or T TACAs.

Such constructs should serve as useful leads for immunological studies which will be reported in due course. In addition, it is anticipated that the synthetic methodology described will be broadly applicable to the assembly of larger peptides and proteins in the future.

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