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Efficient Sequential Segment Coupling Using *N*-Alkylcysteine-Assisted Thioesterification for Glycopeptide Dendrimer Synthesis

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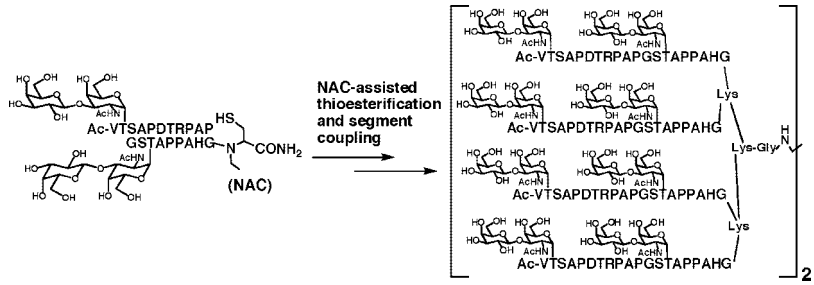
ABSTRACT



A highly pure MUC1-derived glycopeptide dendrimer of 22 kDa was prepared by a sequential segment coupling, achieved by an *N*-alkylcysteine (NAC)-assisted thioesterification. The glycopeptide having C-terminal NAC was prepared by the Fmoc method and converted to the thioester by 3-mercaptopropionic acid treatment. The thioester was condensed with a lysine trimer carrying NAC to afford tetramer, which was then converted to the thioester. Two tetramers were condensed with ethylenediamine to give the octameric glycopeptide dendrimer.

Mucins are large glycoproteins that cover the surface of epithelial cells. It is well-known that the carbohydrate structure of mucins alters during the course of malignancy.[1] Typical tumor markers, such as T (Gal-GalNAc), Tn (GalNAc), and sialyl-T antigens, are highly expressed on mucins derived from adenocarcinomas due to the incomplete elongation of the carbohydrate chains. Thus, mucins could be potential clinical targets for cancers by inducing effective antibodies against these tumor markers. On the basis of this idea, conjugates composed of a cancer-related glycopeptide and a carrier protein have been prepared as vaccine candidates.[2] In several cases, clinical trials are underway. However, the conjugates have limitations, such as the irrelevant immune response against the carrier proteins and the ambiguous composition of the materials. These points might be disadvantages if the conjugates are used as human vaccines. Various fully synthetic glycopeptide conjugates have also been examined as vaccine candidates.[2a,b,d,f,3] The glycopeptide dendrimers, one of the glycopeptide conjugates, are

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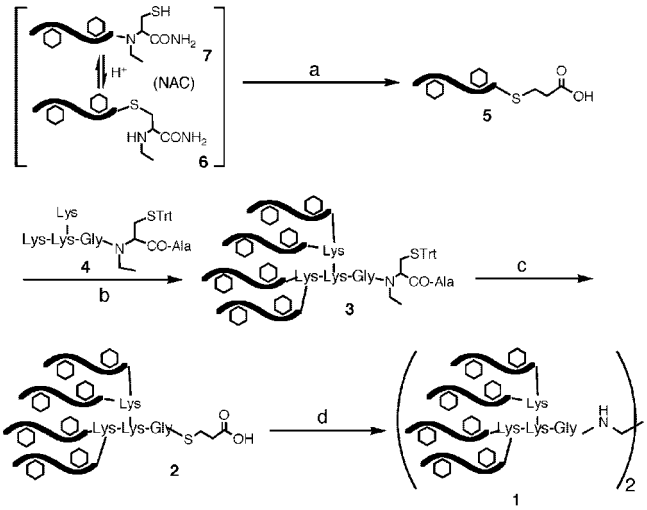
particularly advantageous as they are self-immunogenic due to their large size.[4] Dendrimeric compounds can be prepared by the convergent approach, in which the glycopeptide and the branching unit are prepared separately and condensed. However, when the number of glycopeptide chain increases, the condensation reaction becomes difficult to proceed completely. We recently applied a similar strategy for the synthesis of a glycopeptide dendrimer composed of eight glycopeptide chains.[5] As with other cases, the reaction gave a mixture of the desired product and imperfect dendrimers, and we successfully isolated the desired glycopeptide dendrimer in high purity using preparative gel-electrophoresis. However, considering the general applicability of the method, an establishment of a more general synthetic route for glycopeptide dendrimer is desirable.

In this paper, we designed a new method for synthesizing a highly pure glycopeptide dendrimer using the potential of the post-solid-phase peptide synthesis (SPPS) thioesterification, in which *N*-alkyl cysteine (NAC) at the C-terminus of the peptide was used as an *N*- to *S*- acyl migratory device.[6] The efficiency of the method was demonstrated by the synthesis of the glycopeptide dendrimer **1**, which consisted of eight peptide chains of the tandem repeat region of MUC1[1] carrying two T-antigens (Scheme 1). First, the glycopeptide thioester **5** was

Scheme 1. Novel Route for Glycopeptide Dendrimer Synthesis[a]



[a]Conditions: (a) (1) 5% aq MPA, 2 d, (2) RPHPLC, 20% from the loading of C-terminal Gly on resin; (b) AgCl, HOOBt, DIEA, DMSO, overnight; (c) (1) 10% 1,2-ethanedithiol−TFA, 1 h, (2) 5% aq MPA containing 6 M urea, 4 d, (3) GFC, (4) RPHPLC, 17% (total of b and c); (d) (1) ethylenediamine, AgCl, HOOBt, DIEA, DMSO, overnight, (2) GFC, 76%.

synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) method combined with the NAC method as shown in Figure 1. To prepare the peptide resin **8**, Fmoc-*N*-ethyl-*S*-trityl cysteine (Fmoc-(Et)Cys(Trt)) was introduced into CLEAR amide resin. Then, a glycine, the C-terminal amino acid in the MUC1 sequence, was introduced using Fmoc-Gly activated by *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIEA).

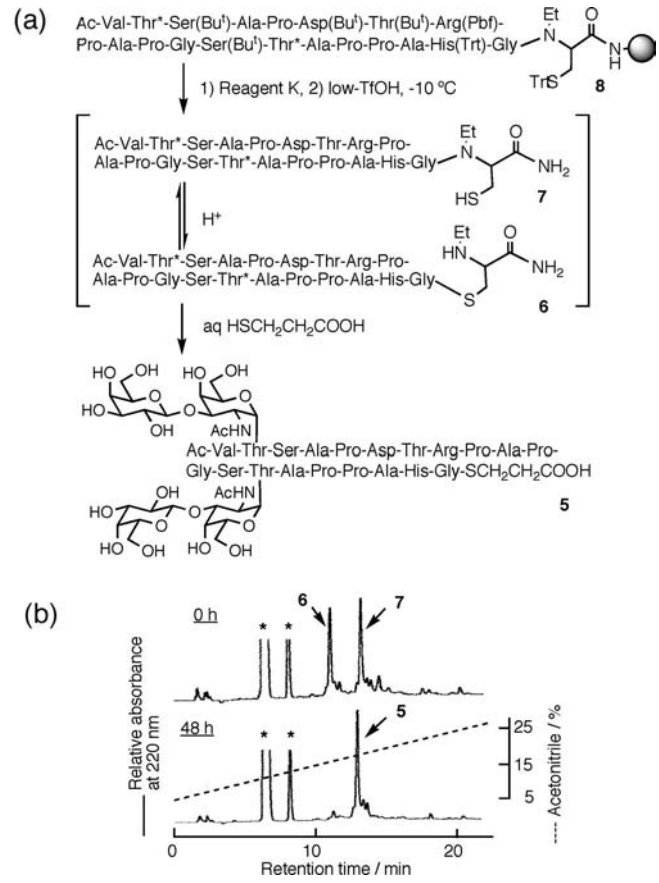


Figure 1. Synthesis of glycopeptide thioester **5**: (a) synthetic route, Thr* denotes threonine residue carrying benzyl-protected Gal-GalNAc moiety. (b) RPHPLC profile of crude glycopeptides **6** and **7** and thioester **5**. Elution conditions: column, Mightysil RP-18 GP (4.6 × 150 mm, Kanto, Japan) at a flow rate of 1 mL /min; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA. The asterisked peaks were derived from nonpeptidic components.

The glycine residue was quantitatively introduced after double coupling at 50 °C, which was monitored by the amino acid

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analysis of the acid-hydrolyzed resin sample. The remaining amino acids were introduced by a peptide synthesizer (Applied Biosystems, 433A) using the FastMoc protocol, which uses piperidine in 1-methyl-2-pyrrolidinone (NMP) for the Fmoc removal and *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) for the activation of amino acids, except that Fmoc-Thr carrying the benzyl-protected Gal-GalNAc moiety (T-antigen)[7] activated by HBTU was introduced manually. The obtained resin **8** was treated with Reagent K (aq TFA, phenol, thioanisole, 1,2-ethanedithiol)[8] to achieve deprotection of the peptide part. After precipitation by diethyl ether, the crude peptide was further treated with low-acidity TfOH[9] to remove benzyl groups of the carbohydrate portion. As expected, the peptide was in an equilibrium between the amide **7** and thioester **6** forms as shown in Figure 1. The peptide was then dissolved in a 5% 3-mercaptopropionic acid (MPA) solution to obtain the glycopeptide thioester **5**. After 2 d, the NAC-containing peptides were almost converted to the thioester **5** without significant decomposition of the carbohydrate and the thioester portions as shown in Figure 1b. The yield of the purified glycosylated peptide thioester **5** was 20% based on the initial loading amount of Gly on the resin. This yield was significantly higher than those of glycosylated peptide thioesters prepared by modified Fmoc strategy using a resin, which immobilizes a peptide via a thioester linkage.[9b,c] This result demonstrated the practical applicability of the NAC method for glycopeptide thioester synthesis. The lysine trimer carrying NAC **4**, in which the *N*−*S* acyl transfer activity is blocked by the Trt group, was synthesized by the Fmoc SPPS method using 2-chlorotrityl alcohol resin (see the Supporting Information).

Next, the glycopeptide dendrimer **1** was synthesized by the thioester method[10] according to Scheme 1. The glycopeptide thioester **5** and dendrimer core **4** were dissolved in DMSO containing 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOOBt) and DIEA. AgCl was then added to initiate the coupling reaction. The reversed-phase (RP)HPLC analysis of the solution indicated that the thioester **5** was almost consumed within 6 h and the new peak corresponding to the desired tetramer **3** appeared. The tetramer **3** was precipitated by ether and used in the next reaction without further purification.

The Trt group of the C-terminal NAC residue was removed by TFA containing 5% 1,2-ethanedithiol, and thioesterification was carried out by 5% aq MPA treatment. The reaction proceeded without significant side reactions within 4 d. Then the desired tetrabranched thioester **2** was isolated by RPHPLC in high purity. Finally, the thioester **2** was condensed with ethylenediamine by the thioester method to obtain the octamer. After isolation by gel filtration chromatography (GFC), the isolated product was analyzed by SDS-PAGE, MALDI-TOF mass (Figure 2), and amino acid composition analyses, which demonstrated that the product **1** was obtained in high purity.

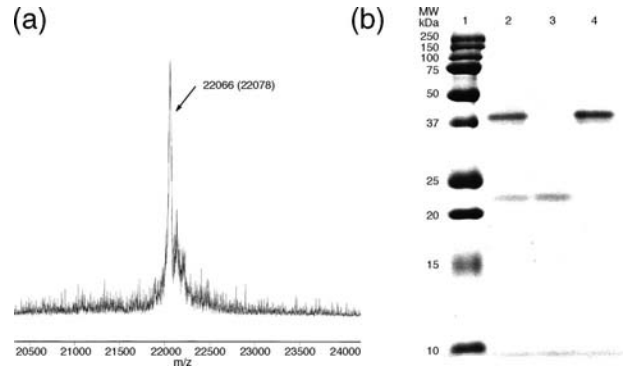

Figure 2. (a) MALDI-TOF mass spectrum of **1** and (b) SDS-PAGE of the glycopeptide dendrimer. Key: lane 1, m.w. standard; lane 2, coupling mixture for the preparation of the product **1**; lane 3, purified tetramer **2**; lane 4, purified octamer **1**.

The NAC-assisted thioesterification reaction proved to be an efficient method to obtain highly pure glycopeptide thioester in good yield. The consecutive application of the NAC method to the peptide and tetrameric dendrimer successfully gave a highly pure octabranched glycopeptide dendrimer with molecular weight larger than 20 kDa, which will be a candidate for unambiguous cancer vaccines. In this sequential coupling method, the final stage is a condensation of the two tetrabranched dendrimers. Thus, the purification was easily achieved by the GFC, due to the large difference in molecular weight between the product and the starting material. This strategy can realize the stepwise introduction of tetramer to dendrimer core, which would achieve the preparation of dendrimers having different components, such as B and T cell epitopes, and glycopeptides and fluorescent tag. These molecules are useful for the induction of a more efficient immune response and for the trace of the cellular uptake of the dendrimers. Along this line, the synthesis of these dendrimers is now in progress.

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Supporting Information Available: Synthetic procedures for glycopeptide thioester **5**, dendrimer core **4**, and glycopeptide dendrimers **2** and **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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