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Synthesis of a Mimic for the Heterogeneous Surface of Core 2 Sialoglycan-Linked Glycoprotein

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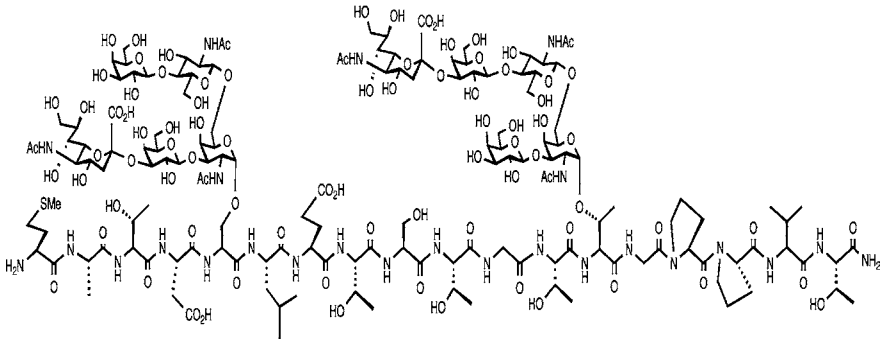
ABSTRACT



An O-glycosylated peptide carrying two core 2 sialopentasaccharides of different glycoform was synthesized as a model of the heterogeneous surface of mucin glycoprotein. Solid-phase synthesis and the subsequent enzymatic sialylation gave two segments, which were coupled by selective thioester activation to produce the title compound.

Mucins and mucin-like glycoproteins carry a serine/threonine-rich peptide backbone that serves as a scaffold for O-glycosylation to form a dense hydrophilic glycan coat. Mucin *O*-glycans usually display an extreme heterogeneity in the structure by varying saccharide compositions, lengths, and anomeric linkages. The structures of the *O*-glycans are classified into eight subtypes referred to as cores 1−8 depending upon the saccharyl substitution on the common GalNAc−Ser/Thr motif.[1] Elongation of *O*-glycans often involves an addition of repeating *N*-acetyllactosamine that is terminated by sialylation as well as fucosylation. Such densely clustered glycans are thought to function in protection of mucosal surfaces from desiccation, pathogenic microorganisms, and mechanical injury.[1] On the other hand, alteration of mucin expression in amount and changes in the *O*-glycan structure by malignant transformation are the particularly important topics in glycobiology. A number of efforts have, therefore, been made on characterization of the tumor-associated epitopes on mucin, since more insight into the alteration mechanisms will lead to immune-based therapies and vaccine preparation. Synthetic homogeneous glycopeptides have also been used to identify the immunogenic structures.[2] Recently, we developed a facile synthetic procedure for a disialylated core 2 glycan-linked glycopeptide by combination of solid-phase assembly of the building blocks and enzymatic sialylations.[3] Expansion of the repertoire of synthetic methodology would help toward the construction of diverse glycopeptide molecules useful as the tools in biological and physicochemical investigations. We present here a novel chemoenzymatic approach to the

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synthetic glycopeptide carrying a couple of distinctively sialylated core 2 *O*-glycans that might suitably mimic a heterogeneous mucosal surface. Notably, only a few glycopeptides possessing two different *O*-glycans in a molecule have been synthesized so far.[4d] The majority of the synthetic methods of the glycopeptides bearing plural *O*-glycans have employed a homogeneous glycan.[4,5]

The synthetic target chosen in this study is the fragment of glycoprotein CD43 (leukosialin $Met^{170}-Thr^{187}$) **1**, which corresponds to a repeating sequence in the highly O-glycosylated domain. The expression of core 2 *O*-glycans in CD43 is of biological significance as evidenced in the IL 2-activated T-lymphocyte,[6] the resting T-lymphocyte derived from the patients of immunodeficiency syndromes,[7] and the human colon carcinoma-derived Caco-2 cells through enterocytic differentiation.[8] We designed a synthetic route to glycopeptide **1** based on a strategy of coupling two shorter segments **2** ($Met^{170}-Gly^{180}$) and **3** ($Thr^{181}-Thr^{187}$) by the peptide−thioester method.[9] The key coupling reaction was anticipated to be feasible even in the presence of the unmasked sialic acid residues, since selective activation of peptide thioester has been established under specific conditions. Consequently, enzymatic sialylation of the glycopeptide substituted as a labile C-terminal thioester became the major challenging target in this study.

The C-terminal segment was readily synthesized on Rink amide MBHA resin in the Fmoc procedure by manual operation with a vortex mixer. Fmoc amino acids were condensed by activation with DCC−HOBt in NMP at ambient temperature for 1 h, while tetrasaccharide building block **4b**[3] (2 equiv) was activated with HATU in NMP to be incorporated into the peptide at 50 °C for 4 h. N-Deprotection was performed with 20% piperidine/NMP. Washing steps with NMP and CH_2Cl_2 were involved during each operation. The synthesized glycopeptide was released from the resin with reagent K (aq TFA, phenol, thioanisole, 1,2-ethanedithiol), and the crude product precipitated from ether, a mixture of partly debenzylated glycopeptides, was collected and treated with a "low-acidity TfOH" mixture (TfOH, Me_2S, *m*-cresol, 1,2-ethanedithiol, TFA) at −15 °C for 2 h as reported previously.[3] The major product was collected by reversed-phase HPLC to afford **6** in 70% overall yield. The yield of the synthetic glycopeptide was estimated by the amino acid analysis. The structure was confirmed by MALDI TOF MS ($M + Na^+$: *m*/*z* 1423.74).[2]

In contrast, the thioester for the N-terminal segment was more carefully synthesized on CLEAR amide resin. In view of the base-labile thioester linkage, a modified procedure for solid-phase synthesis of Fmoc peptide thioester was employed.[10] Attachment of Fmoc-Gly-$SCH_2CH_2CO_2H$ to the resin was followed by deprotection of the *N*-Fmoc group with a mild base mixture of 1-methylpyrrolidine, hexamethyleneimine, and HOBt in NMP−DMSO.[11] The resultant Gly thioester-loaded resin was reacted with the second amino acid building block, Tsoc-Thr(Bn)-OPfp (*N*-triisopropylsilyloxycarbonyl O^3-benzyl threonine pentafluorophenyl ester). Then N-deprotection initiated by a catalytic fluoride ion (Bu_4NF) led to the concomitant condensation of a third amino acid with Fmoc-Ser(Bu^t)-F. Thus, formation of diketopiperazine, a crucial side reaction in the early stage of solid-phase synthesis, was diminished.[12] The peptide elongation and introduction of building block **4a**[3] were performed in a manner similar to that described for **6** by combination with the mild base N-deprotection. The last four amino acids were attached each by a double coupling method with prolonged reaction time. The synthesized glycopeptide was cleaved from the resin, and the benzyl protecting groups in the oligosaccharide moiety were removed by an analogous procedure used for **6**. The resulting *N*-Fmoc glycopeptide thioester **5** was isolated by HPLC in 24% overall yield ($M + Na^+$: *m*/*z* 2173.96).

Both synthetic glycopeptides **5** and **6** were then subjected to enzymatic monosialylation to prepare segments **2** and **3**, respectively. Sialylation of **5** was achieved with commercially available recombinant rat β-Gal-β1,3-GalNAc-α2,3-sialyltransferase and CMP-sialic acid (5 equiv). By incubation in a cacodylate buffer (pH 6.0) at 37 °C with an additive of BSA, transformation of **5** was successful within 2 h with little loss of the thioester functionality as shown by HPLC in Figure 1a. The purified glycopeptide **2** was obtained in 70% yield by HPLC separation ($M + Na^+$: *m*/*z* 2464.67). Similarly, segment **3** was prepared by reaction of **6** and the donor nucleotide with recombinant rat β-Gal-β1,3/4-GlcNAc-α2,3-sialyltransferase at 37 °C in 0.5 M MOPS buffer (pH 7.4). Figure 1b shows the chromatogram of the product after incubation for 2 h. Isolation by reversed-phase HPLC afforded **3** in 63% yield ($M + Na^+$: *m*/*z* 1714.74). Analysis

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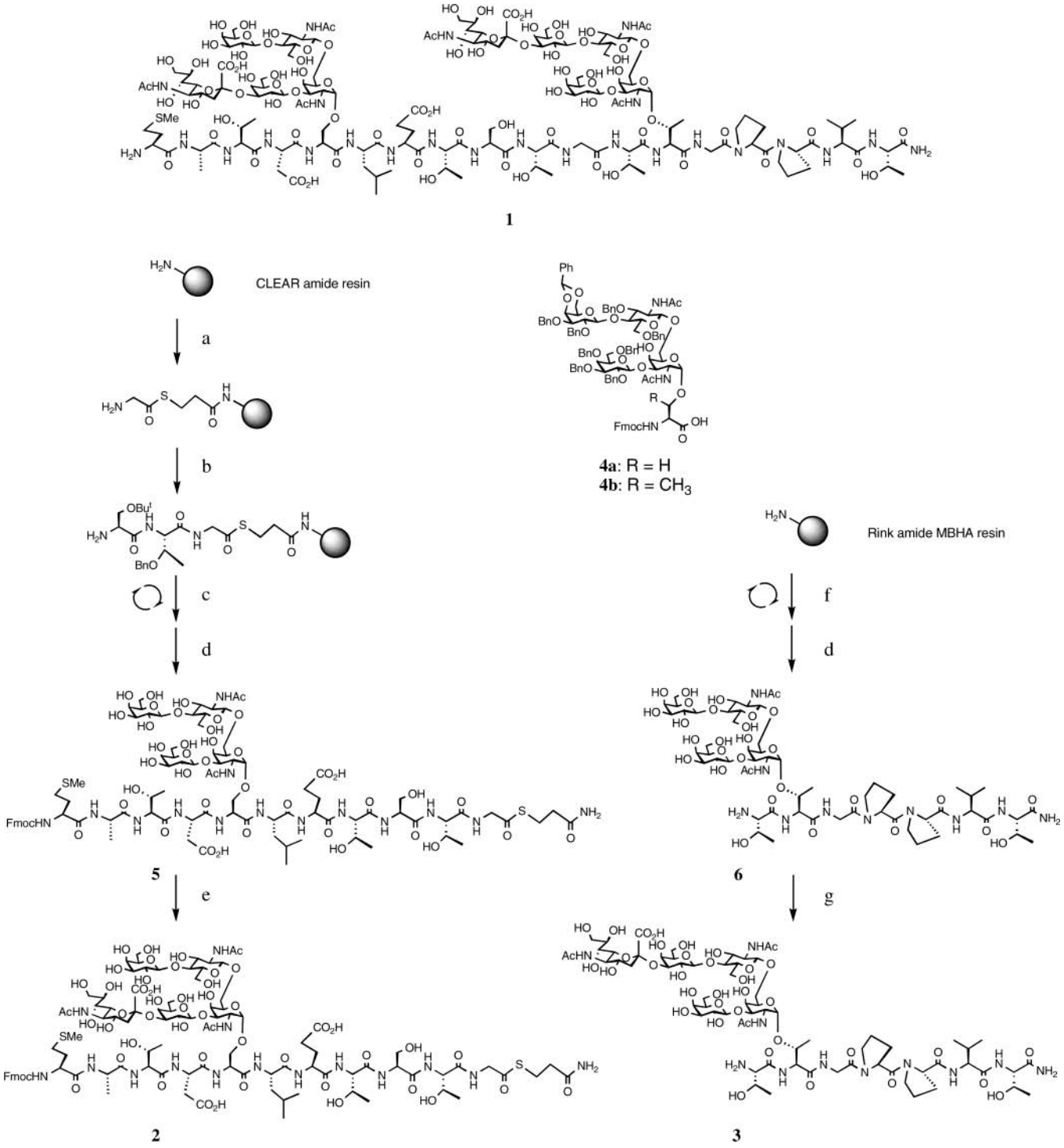
Scheme 1. Structure of the Target Glycopeptide **1** and Syntheses of the Segments **2** and **3**[a]



[a] Conditions: (a) (1) Fmoc-Gly-$SCH_2CH_2CO_2H$, DCC, HOBt; (2) 1-methylpyrrolidine, hexamethyleneimine, HOBt. (b) (1) Tsoc-Thr(Bn)-OPfp; (2) Fmoc-Ser(Bu^t)-F, Bu_4NF. (c) (1) 1-Methylpyrrolidine, hexamethyleneimine, HOBt; (2) Fmoc-AA, DCC, HOBt, (or **4a**, HATU DIEA). (d) (1) Reagent K; (2) DMS, *m*-cresol, EDT, TFA, then TfOH. (e) β-Gal-β1,3-GalNAc-α2,3-Sialyltransferase, CMP-sialic acid, BSA, cacodylate buffer (pH 6.0). (f) (1) Fmoc-AA, DCC, HOBt, (or **4b**, HATU, DIEA); (2) 20% piperidine. (g) β-Gal-β1,3/4-GlcNAc-α2,3-sialyltransferase, CMP-sialic acid, BSA, MOPS buffer (pH 7.4).

of the isolated samples by HPLC revealed that **2** and **3** were contaminated by a small amount (5−6%) of asialo derivatives **5** and **6**, respectively. The contaminants were most likely produced from **2** and **3** by desialylation during lyophilization of the eluate containing 0.1% TFA. Taking into account the difficulties in full suppression of the desialylation in the isolation steps of **2** and **3**, the samples were used for the next reaction without further purification.

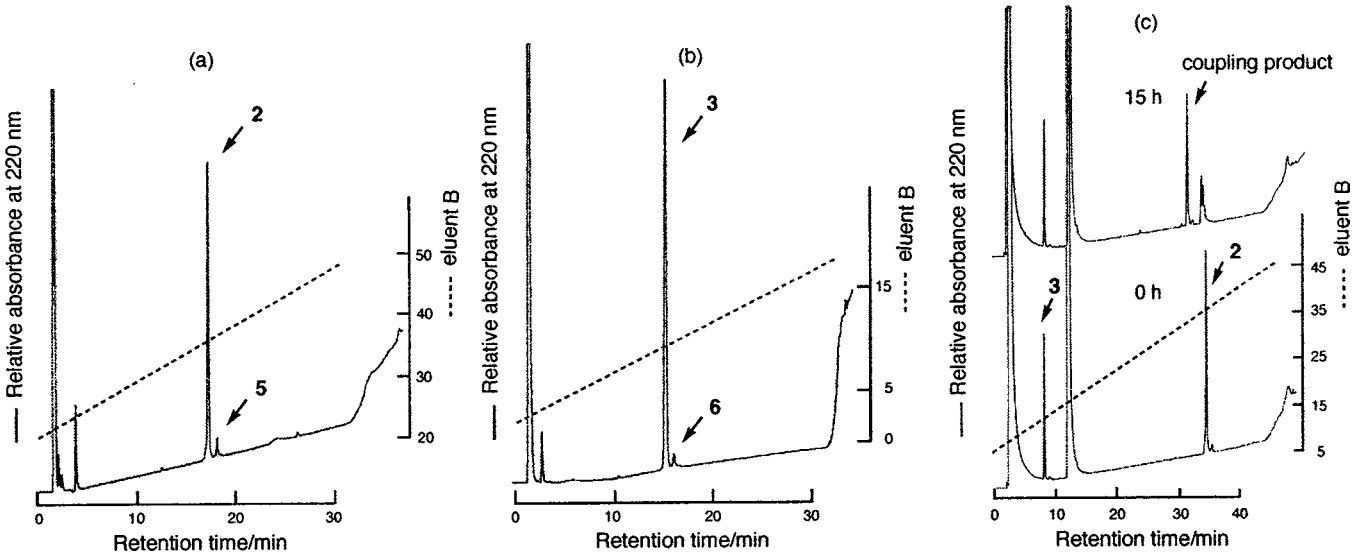


Figure 1. HPLC of the reaction product. Column: Mightysil, RP-18 (150 × 4.6 mm), Flow rate: 1 mL/min. (a) Sialylation of **5**. Eluent A: distilled water containing 0.1% TFA. Eluent B: acetonitrile containing 0.1% TFA. (b) Sialylation of **6**. Eluent A: 10 mM ammonium acetate (pH 5.8). Eluent B: acetonitrile containing 10% eluent A. (c) Coupling of **2** and **3** (at 0 and 15 h, respectively). Eluent A: distilled water containing 0.1% TFA. Eluent B: acetonitrile containing 0.1% TFA.

With segments **2** and **3**, the crucial coupling was performed. Both segments were carefully dried over P_2O_5, and then a mixture of **2** and **3** (1.4 equiv) was treated in the dark with AgCl and HOOBt in DMSO at ambient temperature according to the established procedure.[9] The reaction was monitored by HPLC. Figure 1c demonstrates the result of coupling reaction after 15 h. Thioester **2** was completely consumed, and a new peak appeared in a more mobile fraction as the major product. Its molecular ion mass supported the coupling reaction as being successful. The product was N-deprotected by adding piperidine to the mixture. The crude product was purified by duplicate HPLC, first with aqueous CH_3CN containing TFA and next with ammonium acetate buffer. Thus, pure glycopeptide **1** was obtained in 16% overall yield ($M - H^-$: *m*/*z* 3805.98). The monosialo byproducts were separated in the minor fraction of the HPLC (see Supporting Information).

In conclusion, the glycopeptide thioester corresponding to the segment of human leukosialin (Met^{170}−Gly^{180}) was prepared by the Fmoc solid-phase method and specifically sialylated using a commercial enzyme. Chemoselective activation of the thioester led to smooth condensation with the sialoglycopeptide counterpart (Thr^{181}−Thr^{187}) synthesized by using another commercial sialyltransferase. Thus, an octadecapeptidyl repeating sequence carrying two core 2 sialopentasaccharides in different glycoforms was synthesized. In this study we have demonstrated a feasible approach to the glycodiversification in the mucin-type sialoglycoproteins. The synthetic glycopeptides, including the intermediates, were obtainable in a milligram (μmol) scale. Although the yields shown here were obtained by an indirect means on the basis of amino acid analysis of the acid-hydrolyzed samples, high efficiency was established in this series of conversions as evidenced by the HPLC data. It is remarkable that application of the enzymatic procedure could avoid the problems present in the long-step chemical synthesis of the sialylated glycoamino acid building blocks.[13]

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Supporting Information Available: Experimental procedures for **1**−**3**, **5**, and **6** and HPLC data of **1**, **5**, and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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