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Convenient synthesis of a sialylglycopeptide-thioester having an intact and homogeneous complex-type disialyl-oligosaccharide

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Abstract—Access to glycopeptides with C-terminal thioester functionality is essential for the synthesis of large glycopeptides and glycoproteins through the use of native chemical ligation. Toward that end, we have developed a concise method for the synthesis of a glycopeptide thioester having an intact complex-type dibranched disialyl-oligosaccharide. The synthesis employed a coupling reaction between benzylthiol and a free carboxylic acid at the C-terminus of a glycopeptide in which the peptide side chains are protected. After construction of glycopeptide on the HMPB-PEGA resin through the Fmoc-strategy, the protected glycopeptide was released upon treatment with acetic acid/trifluoroethanol and then the C-terminal carboxylic acid was coupled with benzylthiol at −20 °C in DMF. For this coupling, PyBOP/DIPEA was found to be the best for the formation of the thioester, while avoiding racemization. Finally, the protecting groups were removed in good yield with 95% TFA, thus affording a glycopeptide-thioester having an intact and homogeneous complex-type disialyl-oligosaccharide.

Keywords: Glycopeptide; Complex-type oligosaccharide; Thioester; Solid-phase peptide synthesis; Sialyl-oligosaccharyl-peptide

1. Introduction

Oligosaccharide chains on proteins play important roles in several biological events.[1–4] The function of protein glycosylation has been studied and it now appreciated that the oligosaccharide chains of glycoproteins are essential for proper activity. However, oligosaccharide chains on glycoproteins are heterogeneous, giving rise to a number of glycoforms and this has hindered investigation of the exact oligosaccharide structure implicated in a given biological phenomenon. Therefore, the synthesis of glycoproteins and glycopeptides having homogeneous oligosaccharide chains is essential. To address this issue, we developed a convenient preparation of asparagine-linked complex-type oligosaccharides through the synthesis of a Fmoc-protected disialyl-oligosaccharyl-asparagine derivative and used it to synthesize sialylglycopeptides.[5–7]

For the synthesis of proteins, native chemical ligation is a powerful method for coupling two peptide fragments.[8] The ligation occurs between a C-terminal thioester in one peptide and a cysteine at the N-terminus of another peptide leading to amide bond formation. A convenient method for the synthesis of glycopeptide-thioesters is, therefore, essential for the synthesis of glycoproteins by native chemical ligation. Several synthetic methods have been reported to perform thioesterification.[9–20] Of these, the safety-catch linker[21,22] is the most utilized. However, before release of glycopeptide-thioester from the safety-catch linker, the linker should be activated by alkylation of the amide nitrogen by cyanomethyl iodide or an excess of diazomethane.[13–15,23] When we have examined this

Abbreviations: DEPBT, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one; DIPCI, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HMPA, 4-hydroxymethylphenoxyacetic acid; HMPB, 4-hydroxymethyl-3-methoxy-phenoxyacetic acid; HOBt, *N*-hydroxybenzotriazole; MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole; PEGA, poly(ethyleneglycol)/poly(dimethylacrylamide) co-polymer; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid.

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method for glycopeptides having complex-type oligosaccharides in which all sugar hydroxyl groups are free, the sugar hydroxyl groups were easily alkylated by these reagents. To circumvent this problem, Unverzagt has reported a method in which the sugar hydroxyl groups are protected and thus alkylation is avoided.[17] The advantage of using an oligosaccharide having free hydroxyl groups is that the deprotection steps can be shortened after construction of the glycopeptide on the solid phase. Therefore, we investigated the conditions that are suitable for the synthesis of glycopeptide-thioesters having oligosaccharides with free hydroxyl groups. In this paper, we report a concise thioesterification of a glycopeptide containing a homogeneous complex-type disialyl-oligosaccharide.

2. Results and discussion

Our strategy for the synthesis of glycopeptide-thioesters was based on approaches by Futaki,[9] Beyermann,[10] and Imperiali,[11] in which a protected peptide was synthesized on trityl resin, which was then released from the resin by hydrolysis with acetic acid. The peptide obtained after cleavage had a C-terminal carboxylic acid, which was then converted into a thioester by use of a conventional condensation reaction before all side chain protecting groups were removed. However, these conditions frequently gave rise to racemization of the amino acid at the C-terminus of the peptide. To prevent this racemization, Futaki[9] substituted the C-terminus of the peptide with glycine, and other research groups have reported optimized conditions that avoid racemization (PyBOP/dichloromethane[10] or HBTU/THF[11]). However, application of these latter approaches to glycopeptides containing oligosaccharides with free hydroxyl groups is problematic as these compounds are hydrophilic and thus their dissolution in THF and dichloromethane is often difficult. Therefore, to achieve thioesterification of C-terminal carboxylic acids in such glycopeptides, a solvent that would both dissolve the substrate and which would also be compatible with the condensation reaction was essential. We considered DMF and *N*-methyl-2-pyrrolidone as the most suitable solvents for our hydrophilic glycopeptides.

We therefore investigated the conditions that are suitable for avoiding racemization in DMF. We selected a model tetrapeptide, Ac-NH-HAAF-OH, and coupled it with benzylthiol using several condensation reagents (DIPCI/HOBt, HBTU/DIPEA, DEPBT[24]/DIPEA, and PyBOP/DIPEA). As shown in Table 1, DIPCI afforded the desired thioester, but the reaction required 9 h to reach completion. Two of the other reagents, HBTU and DEPBT, did not afford the thioester in good yield in this solvent. Under these conditions, only PyBOP/DIPEA afforded the desired thioester in good yield within 2 h.

Table 1. Thioesterification with several coupling reagents[a]

Reagents	Time (h)	Yield (%)	Racemization
HOBt (5 equiv), DIPCI (5 equiv)	32	75	97:3
HOBt (10 equiv), DIPCI (10 equiv)	9	98	95:5
DEPBT (5 equiv), DIPEA (5 equiv)	27	10	—[b]
HBTU (5 equiv), DIPEA (5 equiv)	5	<10	—[b]
PyBOP (5 equiv), DIPEA (5 equiv)	2	98	94:6

[a] The Ac-NH-HAAF-OH tetrapeptide was used for the esterification with benzylthiol.
[b] Not determined.

The amount of racemization was determined by HPLC and MS/MS analysis. As shown in Figure 1a, a by-product was produced, which appeared as an HPLC peak beside that for the desired product. After purification of these peaks, both products were analyzed by MS/MS to determine not only the amino acid sequence and molecular weight but also whether both peaks were isomeric to each other due to racemization. The analysis clearly indicated that PyBOP/DIPEA afforded less than 6% racemization (Table 1). To investigate when racemization occurred, the activation time of the C-terminal carboxylic acid by PyBOP was varied. As shown in Figure 1b, the racemization product increased as a function of activation time. In a separate experiment, when the pure peptide-thioester was treated under basic conditions (10 equiv of DIPEA) no racemization was observed (Fig. 2). These data indicate that immediate coupling with excess alkylthiol is essential for decreasing C-terminal racemization. Carrying out the coupling reaction with a large excess of alkylthiol at low temperature (−20 °C) was also examined. As shown in Figure 3, under these conditions, C-terminal racemization was suppressed to less than 2%.

Because the amino acid histidine is known to be racemized easily, we also examined the thioesterification of a peptide with a C-terminal histidine, Ac-NH-CCEH-OH. Although the product was obtained in good yield, HPLC analysis could not determine the percentage of racemization. Therefore, we prepared authentic products racemized by coupling at 30 °C and then compared this mixture with the products formed when the reaction was done at −20 °C. After purification of products by HPLC, each product was analyzed by ^{1}H NMR spectroscopy. As shown in Figure 4, this analysis suggests that less than 1% of the C-terminal histidine residue in this peptide was racemized under the optimized conditions.

It is known that the first amide bond formation between the C-terminal amino acid and the sulfonamide on the safety-catch linker should be performed at low temperature for a few hours to avoid racemization. Because our coupling velocity is obviously faster than that of the formation of the first amide coupling to the safety-catch linker, our conditions appeared to be suit-

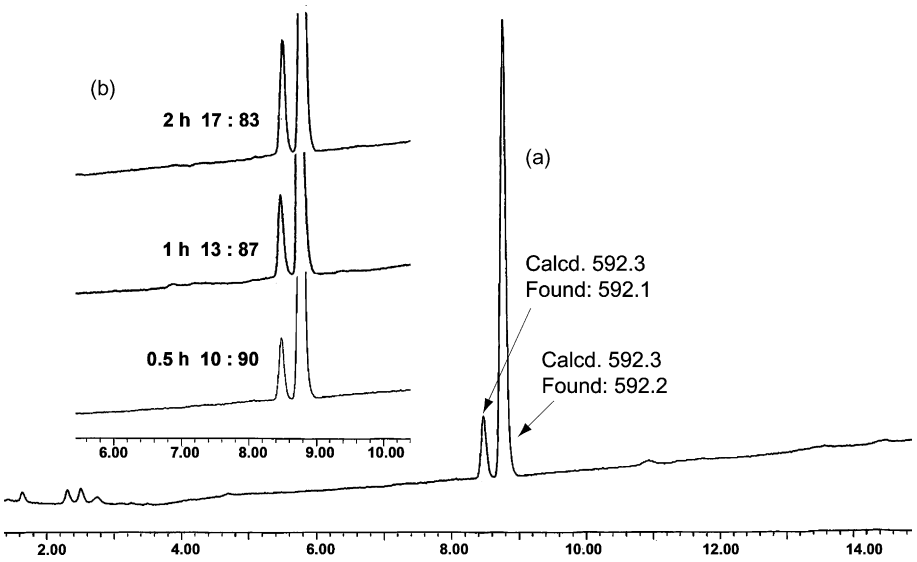


Figure 1. Monitoring of racemization at the C-terminal amino acid during thioester formation. (a) The C-terminus was 6% racemized upon immediate coupling with benzylthiol at 0 °C. (b) Effect of increasing activation time with PyBOP from 0.5 to 2.0 h.

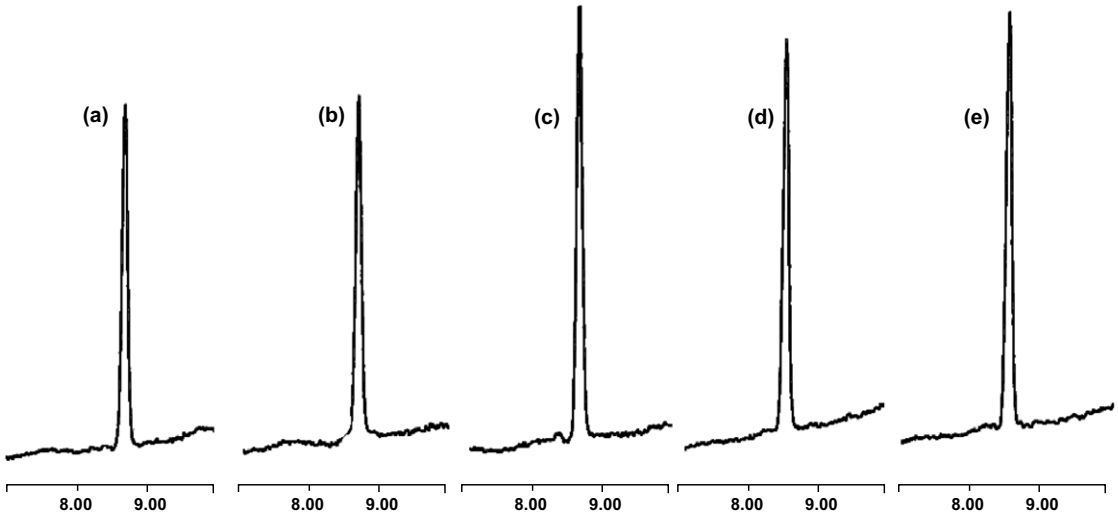


Figure 2. Monitoring the racemization of Ac-NH-HAAF-COSBn in the presence of DIPEA. (a) 0 °C for 30 min; (b) 0 °C for 1 h; (c) 0 °C for 7 h; (d) rt for 2 h; (e) rt for 4 h. There is no racemization within 2 h.

able for avoiding considerable racemization and we employed these conditions in the solid-phase synthesis of the glycopeptide-thioester.

We have already investigated the conditions that are suitable for the coupling of our Fmoc-protected disialyl-oligosaccharyl-asparagine derivative (**1**, Fig. 5) into the resin-bound peptide.[5] This highly hydrophilic glycosyl amino acid building block was compatible with hydrophilic PEGA resin,[25] which has a high swelling capacity. In the cases we have studied, the coupling of this building block did not work well with conventional (e.g., polystyrene) resins. Furthermore, commercially available trityl resin also did not work well. We believe this is due to the hydrophobicity of the resin, which appears to hinder access of **1** to the resin. Although we have used HMPA-PEGA resin for glycopeptide synthesis, cleavage from the resin requires 95% TFA, which also cleaves the protecting groups on the peptide side chains. The preparation of glycopeptide-thioesters, requires protected glycopeptides with a C-terminal carboxylic acid and thus HMPA-PEGA resin is not suitable. For all of the reasons stated above, we selected HMPB-PEGA resin for the synthesis of the target glycopeptide-thioester. The HMPB linker has a *m*-methoxy

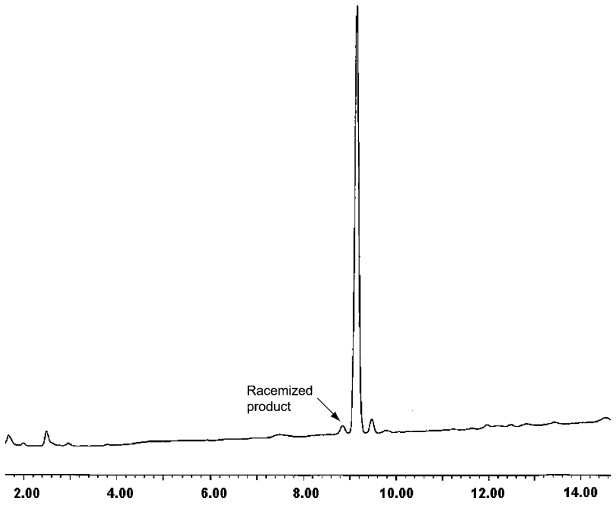


Figure 3. HPLC profile of the thioesterification product of Ac-NH-HAAF-OH at −20 °C.

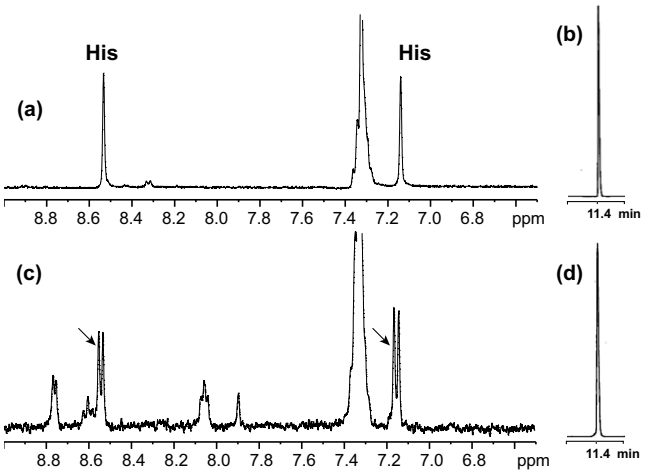


Figure 4. HPLC profiles and ^{1}H NMR spectra of thioesterification of Ac-NH-CCEH-OH while monitoring for C-terminal racemization. (a) ^{1}H NMR spectrum of the product obtained from the reaction carried out at −20 °C; (b) HPLC profile of the product obtained from the reaction carried out at −20 °C; (c) ^{1}H NMR spectrum of the product obtained from the reaction carried out at 30 °C; (d) HPLC profile of the product obtained from the reaction carried out at 30 °C. The signal of the racemized product is shown by arrows.

substituent on the phenyl group and, compared to the HMPA linker, it can be cleaved under milder conditions (1% TFA/CH_2Cl_2). These conditions do not lead to deprotection of the peptide side chains.

To test the limitations of our methodology for the synthesis of glycopeptide-thioesters, we selected a decapeptide segment from HIV GP120 (372T–381S) as a model. After attaching the first amino acid to the HMPB-PEGA resin by MSNT, the peptide was elongated as shown in Scheme 1. For the coupling of **1**, DEPBT and DIPEA were used to avoid undesired aspartimide formation and this coupling proceeded in good yield.[5] The coupling yield was estimated by the Kaiser test and HPLC analysis after cleavage of glycopeptide from the resin. After the sugar was added to the growing peptide on the HMPB-PEGA resin, further amino acids were added via coupling with DIPCI/HOBt. In our hands, these are the best conditions to avoid undesired esterification of the sugar hydroxyl groups during peptide elongation.

After construction of glycopeptide on the resin, we examined its release. Treatment with 95% TFA produced a glycopeptide that was completely deprotected except for the benzyl esters of the sialic acid residues. As shown in Figure 6a, the desired glycopeptide was obtained in good yield. We next examined the release of the protected glycopeptide **2** from the resin with 1% TFA/CH_2Cl_2. However, these conditions did not release **2** in good yield but fortunately, the use of a 1:1 mixture of acetic acid and trifluoroethanol was found to be suitable for release of **2** (Fig. 6b). Because the glycopeptide has a number of sugar hydroxyl groups, a hydrophilic reagent such as trifluoroethanol appears to be necessary for successful cleavage as the use of such solvents would be expected to facilitate access of the acid to the resin.

With an effective method for the preparation of **2** in hand, thioesterification of the C-terminal carboxylic acid was examined by use of the optimized conditions, which provided glycopeptide **3** (Fig. 6c). All the protecting groups in **3** were then removed by treatment with a

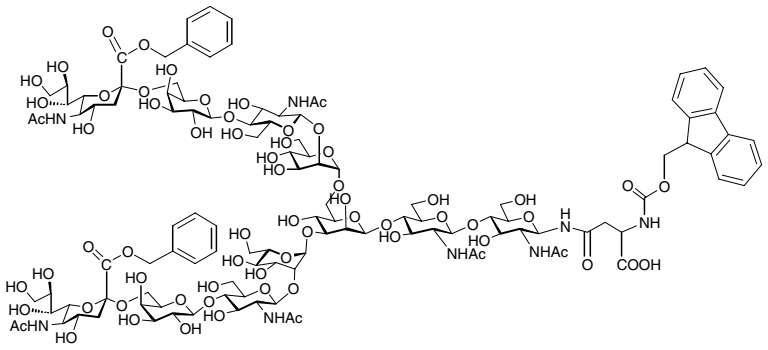
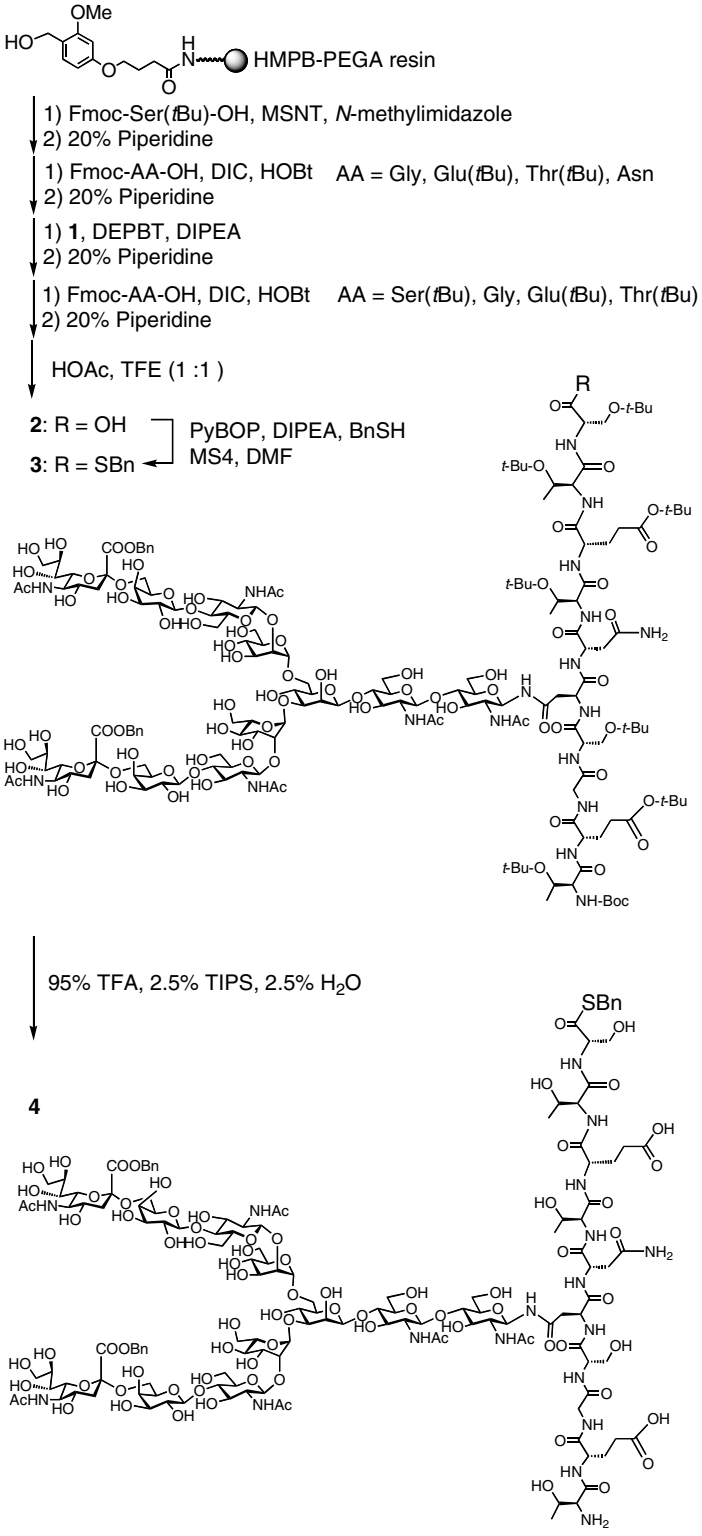


Figure 5. Structure of the Fmoc-protected disialyl-oligosaccharyl-asparagine derivative **1**.



Scheme 1.

95% TFA solution containing 2.5% H_2O and 2.5% triisopropylsilane (Fig. 6d). Purification of the crude glycopeptide-thioester afforded the desired glycopeptide-thioester **4** (Fig. 6e) in good yield. Analysis of the product by ^1H NMR spectroscopy (Fig. 7) indicated that there is no shoulder signals or minor-signals corresponding to a glycopeptide-thioester that has been racemized at the C-terminus. Although we cannot assert that

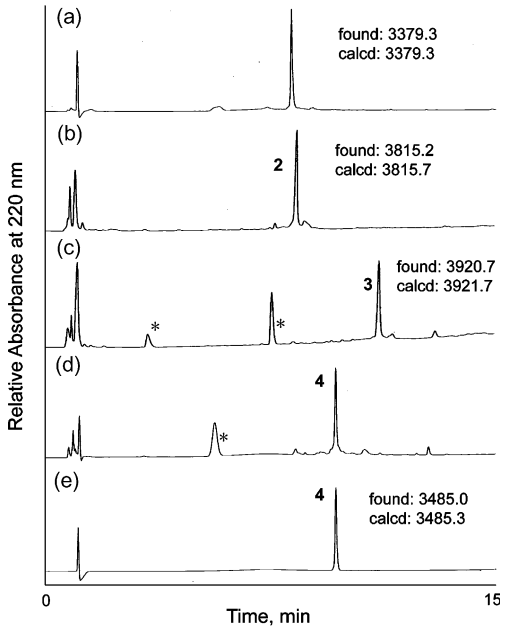


Figure 6. HPLC profiles of the synthesis of glycopeptide-thioesters. (a) Crude material after 95% TFA treatment of the resin following construction of the glycopeptide; (b) crude **2** after treatment of the resin with acetic acid and trifluoroethanol; (c) reaction mixture obtained after thioesterification of **2**; (d) crude **4** after 95% TFA treatment of **3**; (e) purified sialylglycopeptide-thioester **4**. HPLC elution conditions: Cadenza CD-18 column (4.6 × 75 mm); flow rate 1.0 mL min^{-1} at rt. For (a) and (c), a linear gradient of 36–90% CH_3CN containing 0.09% TFA over 15 min was used. For (b), (d), and (e), a linear gradient of 4.5–67.5% CH_3CN containing 0.09% TFA over 15 min was used. Impurities are indicated with an asterisk.

glycopeptide-thioester **4** does not contain racemized product, the purity of **4** is over 95% based on the HPLC profile and the ^1H NMR spectrum.

3. Conclusion

To synthesize large glycopeptides and glycoproteins, native chemical ligation is a powerful method for the coupling of smaller glycopeptides with each other. The use of the method requires an efficient method for thioesterification of any glycopeptide. Our optimized method reported here is straightforward for the synthesis of such thioesters. Of particular note is that the sugar hydroxyl groups in our complex-type oligosaccharide are free and the only protecting groups employed on the glycan are benzyl esters of the sialic acid residues. Therefore, several deprotection steps after the construction of large glycopeptides can be omitted. This is highly advantageous because as the number of sugar residues in the oligosaccharide increases, problems in the deprotection steps are often encountered. The conditions developed for the introduction of the thioester into the glycopeptide proceed with minimal C-terminal racemization and thus afford the product in good yield. Glycopeptides synthesized by this method will be useful in the preparation of both large glycopeptide and glycoproteins. Research is in progress to synthesize several glycoproteins.

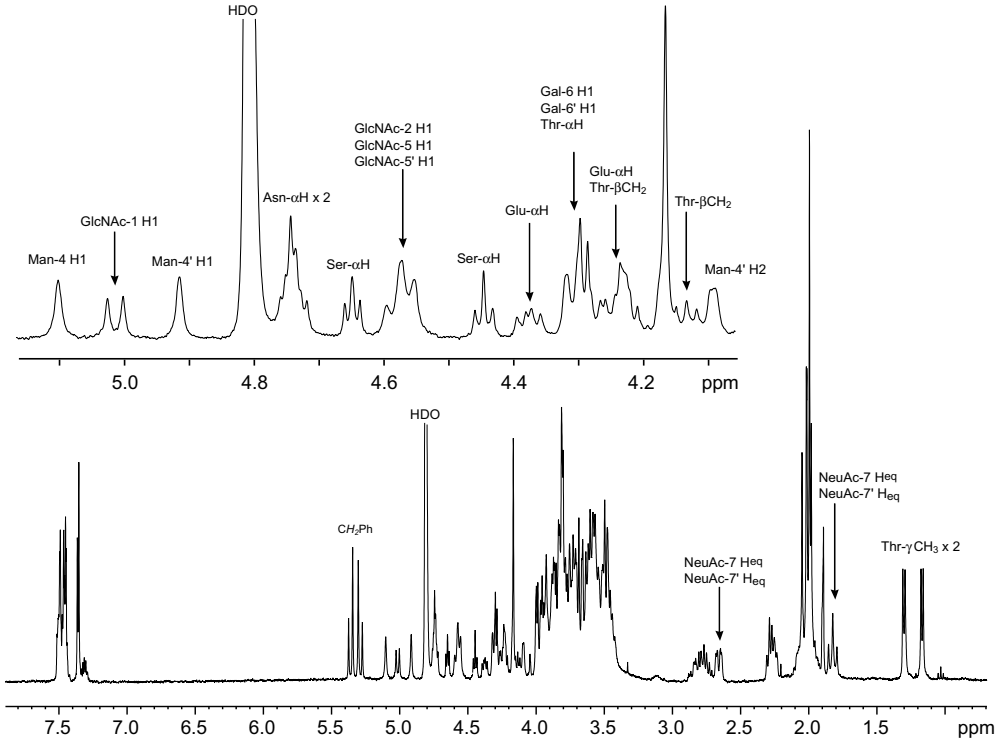


Figure 7. ^1H NMR spectrum of glycopeptide thioester **4**.

4. Experimental

4.1. General methods

NMR spectra were measured on a Bruker Avance 400 spectrometer (23 °C, internal standard HDO, δ 4.81 ppm). The glycosylated Fmoc-Asn-OH derivative, **1**, was prepared in our laboratory as reported earlier.[7] Fmoc-protected amino acid derivatives and coupling reagents were commercially available. Reverse Phase HPLC (RP-HPLC) analyses were carried out on a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using a Cadenza column (Imtakt Corporation, 3 μm, 75 × 4.6 mm) at the pump rate of 1.0 mL/min. ESI mass measurements were carried out on a Bruker Daltonics/Esquire 3000 plus. DEPBT was purchased from Sigma. HBTU, HOBt, and PyBOP were purchased from Novabiochem.

4.2. Thioesterification through the use of several coupling reagents in DMF

A model peptide (Ac-NH-HAAF-OH), in which side chains were unprotected was synthesized manually using solid-phase synthesis and then released from the resin with 95% TFA. The crude peptide was purified by HPLC and then its esterification with benzylthiol was examined. A solution of DMF (0.1 mL) containing benzylthiol (30 equiv), molecular sieves 4 Å (10 mg) and the peptide (1 μmol) was stirred for 1 h at 0 °C and then reagents (one of DEPBT, HBTU, and PyBOP) and DIPEA were added. Coupling with HOBt and DIPCI was also investigated. Thus, a solution of DMF (0.1 mL) containing the peptide (1 μmol), HOBt (5 or 10 equiv), molecular sieves 4 Å (10 mg) was stirred for 1 h at 0 °C and then benzylthiol (30 equiv) and DIPCI were added to this mixture. The solution was stirred for the appropriate time and then analyzed by HPLC. The conditions and results are summarized in Table 1.

4.3. Monitoring racemization of the C-terminal amino acid in DMF

To a solution of a peptide (1 μmol) in DMF (0.1 mL), was added the coupling reagent and the solution was stirred for the corresponding time (0.5, 1, and 2 h) at 0 °C and then excess benzylthiol (30 equiv) was added. After the coupling was finished, the ratio of racemization was estimated by HPLC. To confirm whether the corresponding peak in the HPLC profile was a racemized product, the HPLC peaks were isolated and then analyzed by both ^{1}H NMR and the Biotool® method of MS/MS analysis. The results are shown in Figure 1.

4.4. Synthesis of Ac-CCEH-COSBn

A solution containing the peptide (Ac-C(Acm)C(Trityl)E(*t*-Bu)H(Trityl)-OH, 12 μmol), benzylthiol (30 equiv), and molecular sieves 4 Å (10 mg) in DMF (0.5 mL) was stirred at −20 °C for 1 h and then PyBOP (60 μmol) and DIPEA (60 μmol) were added to this mixture. To obtain the racemized product, a solution of DMF (0.5 mL) containing peptide (Ac-C(Acm)C(Trityl)E(*t*-Bu)H(Trityl)-OH, 12 μmol), PyBOP (60 μmol), DIPEA (60 μmol), and molecular sieves 4 Å (10 mg) was stirred at 30 °C for 2 h and then benzylthiol (30 equiv) was added to this mixture. The mixture was stirred for 2 h at 30 °C. After 2 h, Et_2O (3 mL) was added to this mixture to precipitate the peptide. To this precipitate was added a solution containing 92.5% TFA, 2.5% H_2O, 2.5% triisopropylsilane, and 2.5% ethanedithiol and this mixture was stirred for 3 h for deprotection. The results are shown in Figure 4a–d.

4.5. Solid-phase synthesis of sialylglycopeptide-thioesters 2–4

The synthesis of the sialylglycopeptide having protecting groups on the amino acid side chains was performed manually using the Fmoc procedure on a polypropylene column (Tokyo Rika, No. 183470) and HMPB-PEGA resin (1 μmol scale). The first amino acid (3 equiv) was coupled to the resin with MSNT (3.0 equiv) and *N*-methylimidazole (2.75 equiv) in CH_2Cl_2 (250 mM). Peptide elongation was performed with DIPCI (5.0 equiv), HOBt (5.0 equiv) in DMF (0.4 M) for 1.0 h until attaching **1** to the peptide-resin. The glycosylated Fmoc-Asn-OH (**1**, 2.0 equiv) was coupled employing DEPBT (3.0 equiv) and DIPEA (2.0 equiv) in DMF (30 mM). After introduction of **1**, the peptide was elongated by DIPCI and HOBt; the concentration of Fmoc-amino acid was chosen to be 0.04 M in DMF to avoid unexpected esterification of the hydroxyl groups on the oligosaccharide. Deprotection of Fmoc groups was performed with 20% piperidine in DMF for 20 min.

After all coupling steps were completed, a glycopeptide (**2**) in which the peptide side chains were protected was released from the resin by use of HOAc/trifluoroethanol (1:1, 2.0 mL); this treatment was repeated twice. The trifluoroethanol solution was concentrated in vacuo and the residue containing the crude **2** was dissolved in DMF and then concentrated by co-evaporation three times. To a solution of crude glycopeptide **2** in DMF (0.1 mL, 4 mM) was added molecular sieves 4 Å (10 mg) and benzylthiol (30 equiv) and then this mixture was stirred at −20 °C. After 1 h, PyBOP (5 equiv) and DIPEA (5 equiv) were added to this mixture and the mixture was stirred at −20 °C. After 4 h, the solution was filtered and Et_2O (3.0 mL) was added to this filtrate to give a precipitate of glycopeptide-thioester **3**. The

precipitate was collected by centrifugation. Toward this precipitate was added a solution containing 95% TFA, 2.5% TIPS (triisopropylsilane), and 2.5% H_2O to remove protecting groups for 2 h and then the solution was concentrated in vacuo. Purification of the residue by RP-HPLC afforded the desired sialylglycopeptide-thioester **4** (0.3 mg, 22%).

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