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An Approach for a Synthesis of Asparagine-Linked Sialylglycopeptides Having Intact and Homogeneous Complex-Type Undecadisialyloligosaccharides

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Abstract: This paper describes synthesis of asparagine-linked sialylglycopeptides. The typical feature of our strategy for the synthesis of a sialylglycopeptide is to employ undecadisialyloligosaccharyl Fmoc-asparagine (Fmoc-Asn(CHO)-OH) **1** without protecting groups on its hydroxyl groups except for the benzyl ester of the NeuAc residues. Our synthetic methodology solved the problem of esterification toward sugar hydroxyl groups by activated amino acids during the elongation of a peptide chain. When employing high concentrations of the Fmoc-amino acid, esterification markedly occurred, but the esterification scarcely occurred when employing low concentrations of reactants. Taking advantage of these findings, we examined the synthesis of a high molecular sialylglycopeptide, CTLA-4 fragment (113–150) **13** having two complex-type sialyloligosaccharides by use of native chemical ligation (NCL). As a result, we succeeded in the synthesis of a sialylglycopeptide having a cysteine residue at the N-terminus (CTLA-4: 129–150 fragment) **11** and a sialylglycopeptide-thioester (CTLA-4: 113–128 fragment) **12**. Finally, the sialylglycopeptides synthesized were applied to NCL reactions. The reaction successfully afforded the desired product, CTLA-4 (113–150) **13** containing mature and pure complex-type sialyloligosaccharides in excellent purity.

Keywords: glycopeptides · ligation · oligosaccharides · sialic acid · solid-phase synthesis

Introduction

Glycoproteins and glycopeptides contain oligosaccharides on their backbone and play central roles in several biological events. Much attention is also paid the function of the oligosaccharides.[1] Protein glycosylation is a predominant modification of proteins. In the post-genome era, it is essential to determine how oligosaccharides behave to express its latent ability as well as to support protein functions.

Oligosaccharides on proteins are divided into mainly two groups, O-linked and N-linked oligosaccharides. The O-linked oligosaccharides are attached to the hydroxyl group of threonine or serine, while N-linked oligosaccharides are attached to the nitrogen of the amide group of the asparagine side-chain. Furthermore, the structures of N-linked oligosaccharides are divided into three categories: high-mannose-, complex-, and hybrid-type. All N-linked oligosaccharides have a common pentasaccharide core structure, $(Man)_3$-$(GlcNAc)_2$-Asn, but vary in the structure and nature of the nonreducing terminal where is the outer and remote position to Asn residues.

In order to investigate the role of such oligosaccharides on proteins, glycoproteins and glycopeptides with structurally well-defined oligosaccharides would be of great value. However, due to the microheterogeneity of oligosaccharides on the proteins, the so-called glycoform, it is difficult to obtain a glycoprotein with a homogeneous oligosaccharide form using a mammalian expressing system. Therefore, chemical synthesis would be a powerful tool to obtain pure glycoproteins and glycopeptides.

In the chemical synthesis of N-linked glycoproteins and glycopeptides, the most problematic issue is the limited quantity of oligosaccharides such as N-linked oligosaccharide containing over 10 sugar residues, which are difficult to

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Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author: Preparation of **3**, **4**, **9**, **10**, **14**, and **15**; ^{1}H NMR spectra of **3** and **4**; analysis by RP-HPLC and ESI mass measurement of NCL reaction.

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synthesize on a large-scale (gram scale) due to the complexity of their structure.[2] Therefore, it has been thought that chemical synthesis of a pure glycoprotein containing a homogeneous N-linked oligosaccharide would be difficult.

Recently, to overcome the problem, Meinjohanns has reported the preparation of a di- and triantennary complex-type oligosaccharide from natural sources by use of the hydrazinolysis procedure and the synthesis of N-linked glycopeptides using the oligosaccharides thus obtained in the building-block approach.[3] Moreover, several groups have reported the strenuous chemical or chemoenzymatic synthesis of high-mannose-, hybrid-, and complex-type oligosaccharides and then demonstrated synthesis of N-linked glycopeptides.[2a,b,4] Thus, the solid-phase synthesis of N-linked glycopeptides has been continuously attempted and the synthesis of homogeneous glycoproteins and glycopeptides has been examined in various approaches.[5] However, synthesis of sialylglycopeptides was difficult because of the nature of the sialyl linkage, which is prone to be hydrolyzed by acid treatment during the final cleavage step of solid-phase glycopeptide synthesis.[6] The sialic acid residue, which usually locates at the nonreducing end of both O-linked and N-linked oligosaccharide, has been shown to change the property of the glycoprotein and glycopeptide and to play essential roles in many biological events such as cell–cell interaction and virus invasion.[1] To investigate the roles of sialyloligosaccharides it is essential to synthesize a sialylglycopeptide efficiently.

To overcome these problems, we have established a preparation method of a diantennary complex-type sialyloligosaccharide from SGP (sialylglycopeptide)[7] obtained from egg yolk on a gram scale and examined the solid-phase synthesis of sialylglycopeptides by use of complex-type disialyloligosaccharyl Fmoc-asparagine (Fmoc-Asn(CHO)-OH) **1**.[8] However, the synthesis of glycoproteins over 10 kDa and glycopeptides having several sialyloligosaccharides on their backbone like an intact glycoprotein remains a difficult task. To address this issue, an efficient synthetic method for higher molecular sialylglycopeptides is essential. Over the past few years, it has been shown that full-length proteins can be synthesized using the native chemical ligation (NCL) strategy.[9] In this approach, two peptides are separately prepared, one bearing a C-terminal thioester, the other one having an N-terminal cysteine. Then, the two unprotected fragments are ligated using chemoselective reaction in aqueous solution. In order to use this approach in the synthesis of high molecular sialylglycopeptides, it is essential to prepare two sialylglycopeptides, sialylglycopeptide-thioester and sialylglycopeptide having a cysteine at the N-terminus. However, there is no report on synthesis of an N-linked sialylglycopeptide-thioester up to now.[10] Moreover, even for a sialylglycopeptide consisted of over 20 amino acid residues there are little efficient synthetic methods.

Therefore, we attempted to solve several problems faced during a solid-phase sialylglycopeptide synthesis by use of Fmoc-Asn(CHO)-OH **1**, and then examined a synthesis of a high molecular sialylglycopeptide using an NCL reaction.

As a synthetic model sialylglycopeptide, we chose the peptide sequence of cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) fragment (113–150), which consists of 38 amino acid residues and includes a biologically important binding motif. CTLA-4 is a glycoprotein related to the immune system.[11] To activate a T-cell, two signals are simultaneously essential. One signal is provided by a foreign peptide bound to the major histocompatibilty complex (MHC) protein on the surface of an antigen-presenting cell (APC). The peptide–MHC complex signal is transmitted into a T-cell through a T-cell receptor and its associated proteins. The essential second signal is provided by costimulatory proteins. One well-known costimulatory protein on the surface of the T-cell is CD28. On the other hand, the activation of the T-cell is controlled by negative feedback. During the activation process, the T-cell starts to express another cell-surface protein, called CTLA-4, which acts to inhibit intracellular signaling by binding to B7 proteins on the surface of an APC.[12] The binding motif of CTLA-4 with B7 protein[13] is proline-rich motif (PRM); in addition there are two N-linked oligosaccharides on both sides of the binding motif. PRM are important for protein–protein interaction;[14] therefore, if the glycopeptide including a PRM can be prepared, such compounds would be utilized not only in the study of the function of oligosaccharides but also in the development of new antiinflammatory drugs. For making glycopeptide drugs, sialylglycopeptide can be expected to lengthen the lifetime of sialylglycopeptides in blood such as erythropoietin.[15] Therefore, we examined a synthesis of the CTLA-4 fragment having two sialyloligosaccharides.[16]

Herein, we describe our examination to overcome several problems in the solid-phase sialylglycopeptide synthesis in detail and the results enabled us to obtain sialylglycopeptide consisted of over 20 amino acid residues. In addition, we also describe the synthesis of sialylglycopeptide-thioester for the first time and successful synthesis of a drug candidate, that is the CTLA-4 (113–150) fragment, by use of the NCL reaction.

Results and Discussion

Efficiently coupling of Fmoc-Asn(CHO)-OH 1: In order to synthesize glycopeptides, coupling of the precious oligosaccharyl Fmoc-Asn onto a peptide-resin should be performed in good yields. However, previously, we have reported unexpected aspartimide formation between the amide nitrogen at the reducing end and α-carboxyl acid of oligosaccharyl Fmoc-asparagine **1** during activation of α-carboxyl acid (Figure 1). This side reaction resulted in low coupling yields of oligosaccharyl Fmoc-asparagine **1** with peptide-resin.[17]

In order to investigate the conditions avoiding the aspartimide derivative **2** in detail, the reaction rate under several coupling conditions was extensively determined. In addition to 2-(1*H*-9-azobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphonate (HATU),[18] benzotriazole-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphonate

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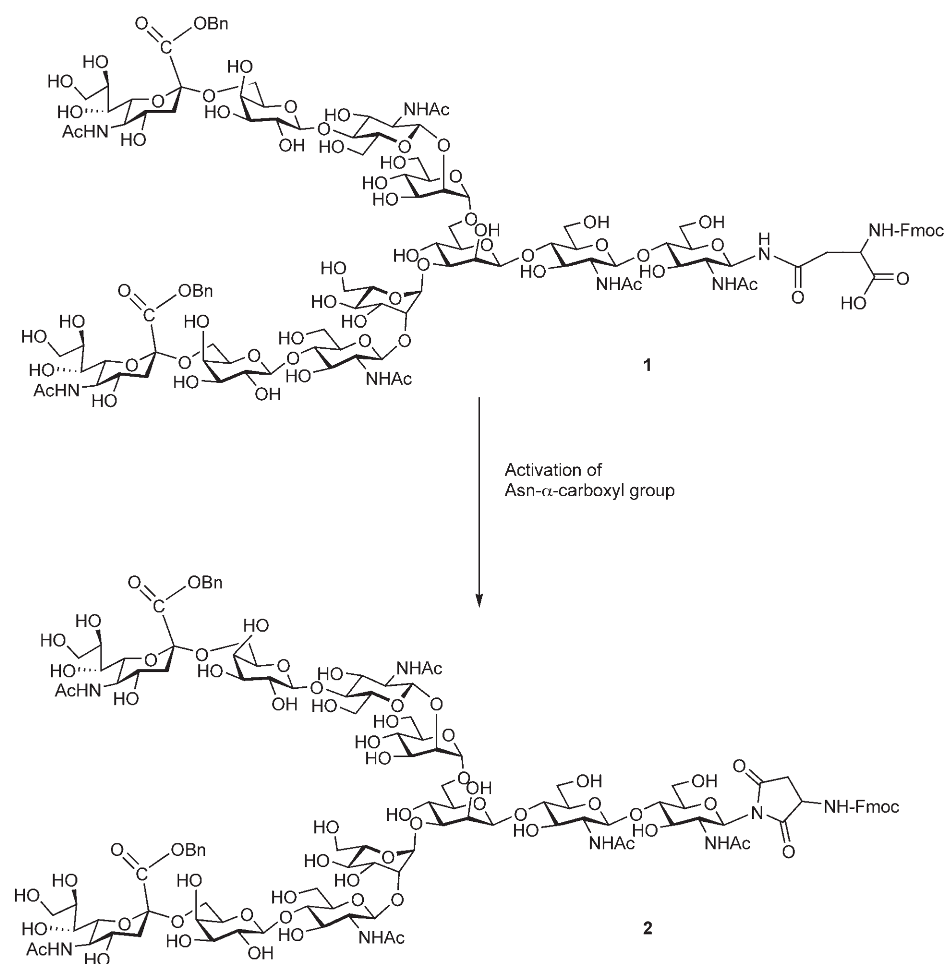

Figure 1. Unexpected formation of aspartimide during activation of Asn-α-COOH.

(PyBOP)[19] and 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT),[20] which were used previously,[17] 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU),[21] 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphonate (HBTU)[22] and *N*,*N'*-diisopropyl carbodiimide (DIC)/1-hydroxy benzotriazole (HOBt) were chosen as additional coupling reagents. These coupling reagents are commonly used in solid-phase peptide synthesis.

Fmoc-Asn(CHO)-OH **1** was activated by 1.5 equivalents each of the above-mentioned coupling reagents in the presence of *N*,*N'*-diisopropylethylamine (DIPEA) (1.0 equiv or 3.0 equiv), except for DIC/HOBt, and each reaction mixture was analyzed by RP-HPLC after 5 and 30 min, respectively. The results are summarized in Figure 2.

As shown in Figure 2, it was found that the aspartimide derivative **2** was generated under all coupling conditions examined and that the reaction rate was faster under the conditions using 3.0 equivalents DIPEA compared with 1.0 equivalent DIPEA. Furthermore, nearly half of Fmoc-Asn-(CHO)-OH **1** was already converted to aspartimide derivative **2** after 5 min under the conditions of PyBOP/DIPEA (1.5 equiv/3.0 equiv) and TBTU/DIPEA (1.5 equiv/3.0 equiv). On the other hand, for 1.0 equivalent DIPEA, the generation of aspartimide derivative **2** was mildly suppressed; in particular, DEPBT and HATU afforded minor amounts of **2**. In addition, for DIC/HOBt, which does not require any base, small amounts of **2** were obtained within 30 min. In this case, poor nucleophilicity of the amide nitrogen attached to the GlcNAc of **1** under the nonbasic condition may be the reason that an aspartimide formation was suppressed. However, Hojo reported on a coupling reaction of Fmoc oligosaccharyl asparagine with a peptide on the solid phase which did not proceed by using of the 1,3-dicyclohexylcarbodiimide (DCC)/HOBt method, but a very similar procedure to the DIC/HOBt method.[10a] In addition, it is known that a partial amino group of peptide-resin reacts with HATU during peptide elongation.[23] Therefore, we selected DEPBT as a coupling reagent, which was the most suitable reagent to avoid aspartimide formation, and we determined whether DEPBT could afford several glycopeptides in good yields. To achieve this, we chose two kinds of sialylglycopeptides, NDTNTN(sialyloligosaccharide)SSS (107–114) **3** and TDLKN(sialyloligosaccharide)DTNT (102–110) **4**, which are contained in gp120[24]—envelope proteins of human immunodeficiency virus (HIV) (Figure S1 in the Supporting Information). The solid-phase synthesis of sialylglycopeptides **3** and **4** was performed on the poly(ethylene glycol)-poly(dimethylacrylamide) copolymer (PEGA)[25] resin having an acid-labile linker, hydroxymethylphenoxyacetic acid (HMPA). Considering that the aspartimide **2** is inevitably generated during a coupling reaction of Fmoc-Asn-(CHO)-OH **1**, the coupling conditions for Fmoc-Asn-(CHO)-OH **1** with the peptide-resin employed 2.0 equivalents of Fmoc-Asn(CHO)-OH **1** and DIPEA, 3.0 equivalents of DEPBT to the resin (1.0 μmol scale). In addition, as far as we could determine, aspartimide **2** did not react with the peptide-resin to afford the isoasparaginyl sialylglycopeptide. Other peptide elongations were performed by the DIC/HOBt method and each coupling reaction progressed quantitatively monitored by the Kaiser test.[26] In both syntheses

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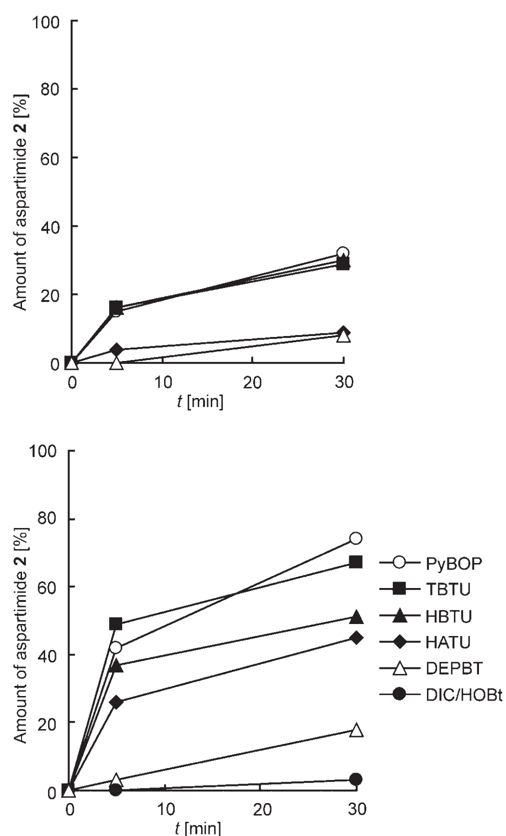

Figure 2. Tendency toward the formation of aspartimide derivative **2** under several coupling conditions; coupling reagents (1.5 equiv), and DIPEA (1.0 equiv: top, 3.0 equiv: bottom) in DMF except for DIC/HOBt. DIC/HOBt (1.0 equiv/1.0 equiv) was used without DIPEA and its result is shown in only the bottom panel.

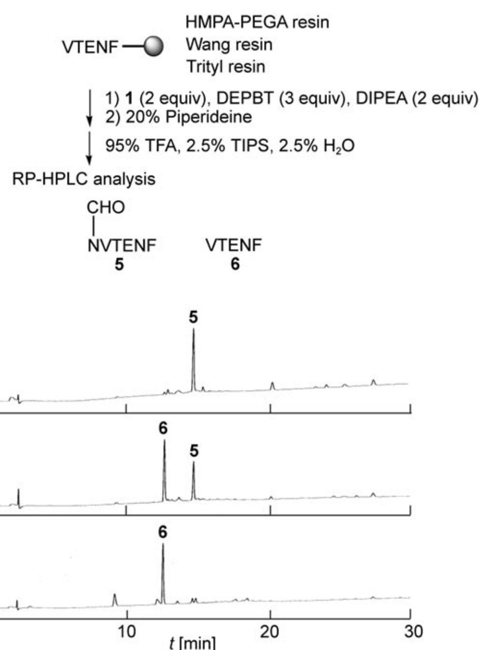

Figure 3. Experimental procedure for comparison of coupling efficiency and resin type. HPLC profiles of crude sample after cleavage from a) HMPA-PEGA resin, b) Wang resin, c) Trt resin. HPLC elution condition of a)–c): column, Inertsil ODS-2 (5 μm, 4.6×150 mm) at a flow rate of 1.0 mL min^{-1}, linear gradient of 4.5→67.5% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 30 min.

of **3** and **4**, the coupling reaction of Fmoc-Asn(CHO)-OH **1** afforded quantitative yields, which resulted successfully in the effective synthesis of sialylglycopeptides **3** and **4** (Figures S2 and S3, Supporting Information). The sialylglycopeptides **3** and **4** were characterized by NMR spectroscopy and mass spectrometry. As the result, it was found that DEPBT was a suitable reagent for the coupling of Fmoc-Asn(CHO)-OH **1** to the peptide-resin.

Suitable resin for a solid-phase glycopeptide synthesis: Several different types of resins were used in the solid-phase glycopeptide synthesis. To investigate which type of resin would be suitable for a solid-phase sialylglycopeptide synthesis, we compared the coupling efficiency of Fmoc-Asn-(CHO)-OH **1** of the following three resins, the Wang resin,[27] Trityl (Trt) resin,[28] and HMPA-PEGA resin. The Wang and Trt resin are derived from poly(chloromethylstyrene-styrene-divinylbenzene) and are standard resins for the Fmoc solid-phase peptide synthesis. HMPA-PEGA resin is derived from poly(ethylene glycol)-poly(dimethylacrylamide) copolymer and is known to have excellent swelling properties.[29] As a model compound, we selected the sequence N(sialyloligosaccharide)VTENF, which is part of gp120. As shown in Figure 3, using the DEPBT/DIPEA method, 2.0 equivalents Fmoc-Asn(CHO)-OH **1** (based on the resin) was added to the VTENF-resin (Wang, Trt, or HMPA-PEGA), which was prepared manually by Fmoc solid-phase peptide synthesis. After 21 h, each resin was washed with DMF and then treated with 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), and 2.5% water, followed by 20% piperidine. The residues obtained were analyzed by RP-HPLC, respectively (Figure 3).

The desired product **5** and peptide **6**, which is a result of incomplete introduction of Fmoc-Asn(CHO)-OH **1**, were characterized by mass spectrometry (**5**: MALDI: *m*/*z* calcd for $[M+Na]^+$: 3130.18, found 3129.78; **6**: ESI: *m*/*z* calcd for $[M+H]^+$: 609.28, found 609.15). As shown in Figure 3, in the case of HMPA-PEGA resin Fmoc-Asn(CHO)-OH **1** coupled to the peptide-resin quantitatively (Figure 3a); however, when using Trt resin, the coupling reaction of Fmoc-Asn(CHO)-OH **1** to the peptide-resin afforded minimal amount of the desired glycopeptide **5** (Figure 3c). It was found that the coupling efficiency of Fmoc-Asn(CHO)-OH **1** increased in the order of HMPA-PEGA resin > Wang resin > Trt resin. As has been reported by several groups,[3,4f,8b] the swelling property of the resin seems to influence the coupling yield of oligosaccharyl Fmoc-asparagine. These results suggest that the PEGA resin with its hydrophilic properties is suitable for the synthesis of a sialylglycopeptide using a large hydrophilic sialyloligosaccharyl Fmoc-asparagine derivative such as Fmoc-Asn(CHO)-OH **1**.

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Determination of the esterification of sugar hydroxyl groups by an activated amino acid residue: The typical feature of our synthetic strategy in the solid-phase glycopeptide synthesis is the use of a sialyloligosaccharyl Fmoc-asparagine without protecting groups on the oligosaccharide hydroxyl groups except for benzyl ester on the NeuAc residues. This strategy would dramatically shorten the processes for removal of the protecting groups and accordingly this strategy is thought to be advantageous if this protocol can avoid esterification toward sugar hydroxyl groups by activated amino acid derivatives during a solid-phase glycopeptide synthesis. Therefore, it is essential to confirm our strategy. In order to investigate how many amino acids can be assembled to form the desired glycopeptide sequence without undesired esterification of the oligosaccharyl asparagine containing over 40 free-hydroxyl groups, we examined glycopeptide synthesis using a model compound.

As the model compound, we chose the sialylglycopeptide, GN(sialyloligosaccharide)GTQIY, which was contained in CTLA-4. This sequence is suitable for this examination because the amino acid after introduction of oligosaccharyl asparagine is a glycine, which is thought to be the most reactive amino acid and has minimum steric hindrance among twenty kinds of amino acids.

The N(CHO)GTQIY-HMPA-PEGA resin was prepared manually by Fmoc solid-phase peptide synthesis using DEPBT for the coupling of Fmoc-Asn(CHO)-OH **1**. The aim of this esterification experiment was to evaluate how many sugar hydroxyl groups were esterified by Fmoc-Gly-OH during amide bond formation between Fmoc-Gly-OH and the N(CHO)GTQIY-resin. However, if multiple esterification reactions occurred toward sugar hydroxyl groups, the resulting HPLC peaks would make it difficult to identify the reaction terminus. Therefore, the coupling reaction of Fmoc-Asn(CHO)-OH **1** was arranged to be approximately 60% yield in order to use the peptide derived from the incomplete introduction of Fmoc-Asn(CHO)-OH **1** as an internal reference. If the nonglycosylated peptide (GTQIY) was converted into GGTQIY by the next addition of excess Fmoc-Gly-OH, we could confirm completion of each experiment. Toward this resin, Fmoc-Gly-OH was treated under different conditions as shown in Table 1.

Table 1. Fmoc-Gly-OH coupling conditions.

Entry	Fmoc-Gly-OH (equiv)	DIC (equiv)	HOBt (equiv)	DMF [mM][a]
1	5	5	5	40
2	10	10	10	100
3	25	25	25	400

[a] Concentration in the parenthesis indicates concentrations of Fmoc-Gly-OH in DMF.

To obtain clear results, we largely varied the conditions with regard to both the concentrations and amounts of Fmoc-Gly-OH. After Fmoc-Gly-OH was treated with DIC/HOBt, the glycopeptide on the resin was cleaved by TFA treatment following removal of the Fmoc group. The obtained mixtures (filtrate) were evaporated in vacuo and then the residues were analyzed by RP-HPLC. The results are shown in Figure 4a–c. It was found that the desired product

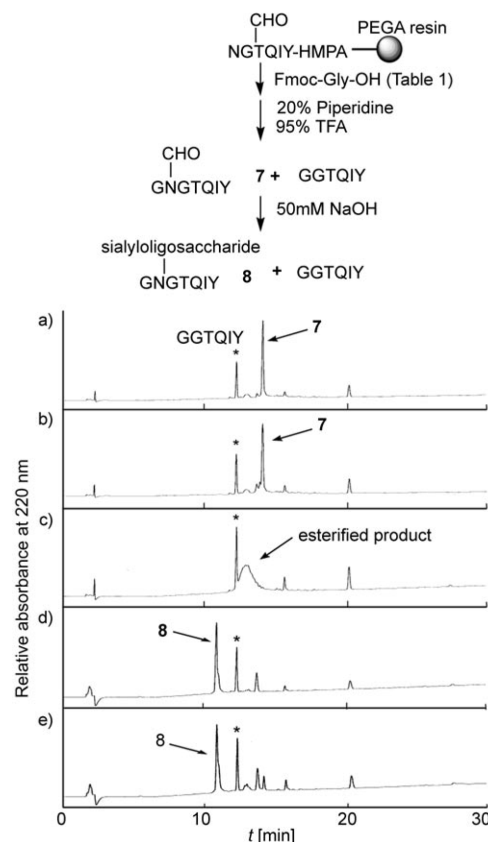

Figure 4. Esterification toward hydroxyl groups of oligosaccharides with Fmoc-Gly-OH on the resin. HPLC profiles of crude sample after 95% TFA treatment; a), b), and c) correspond with results of entry, 1, 2, and 3, respectively. HPLC profiles d) and e) correspond with crude samples of a) and c) after 50 mM NaOH treatment, respectively. Asterisk (*) indicates an internal standard peptide derived from incomplete introduction of Fmoc-Asn(CHO)-OH **1**. HPLC elution condition of (a)-(e): column, Inertsil ODS-2 (5 μm, 4.6×150 mm) at a flow rate of 1.0 mL min^{-1}, linear gradient of 4.5→67.5% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 30 min.

7 (MALDI: m/z calcd for $[M+Na]^+$ 3159.2, found 3159.0) was obtained using the condition of entry 1. This result indicates that Fmoc-Gly-OH could quantitatively coupled with the amino group of the glycosylated and the nonglycosylated peptides on the solid phase even at lower concentrations (40 mM). On the other hand, higher concentrations and excess of Fmoc-Gly-OH (entry 3) did not afford the desired product (Figure 4c). As shown in Figure 4c, we observed a broadened peak at 12 min. To confirm whether this broadened peak corresponded to multi-esterified products by a glycine, we treated this crude sample with 50 mM NaOH. As a result, the broadened peak disappeared and then a new peak **8** at 10.6 min was generated (Figure 4e). In order to confirm whether this peak is identical to that of the product

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obtained using the conditions of entry 1, we also examined the same saponification toward a crude product prepared using the conditions of entry 1. As shown in Figure 4d and e, both experiments afforded the same HPLC profiles and their mass data were in good agreement with that of the desired sialylglycopeptide **8** (ESI: m/z calcd for $[M+H]^+$ 2957.1, found 2956.9). The results indicate not only that the esterification toward sugar hydroxyl groups by activated amino acid occurs remarkably well when employing high concentrations of Fmoc-amino acid and coupling reagent, but also the esterification would scarcely occur when employing low concentrations of the reactants.

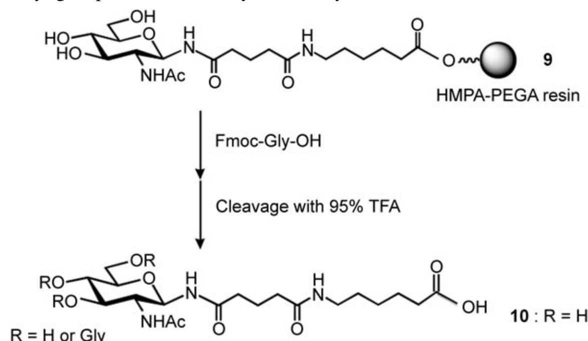
In these experiments, we employed only DIC/HOBt as a coupling reagent for the peptide elongation. We also examined whether esterification toward sugar hydroxyl groups occurred when several other conventional coupling reagents were employed under the low concentration conditions. As shown in Table 2, a structurally simple sugar, *N*-acetyl glucosamine (GlcNAc) derivative linked HMPA-PEGA resin **9** was prepared. Onto this resin Fmoc-Gly-OH was allowed to react for one hour using several coupling conditions. The coupling reagents employed were DIC/HOBt (entry 1), PyBOP/HOBt (entry 2), TBTU/HOBt (entry 3) and activated amino acid by pentafluorophenyl (Pfp) ester with 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) (entry 4). In the case of PyBOP/HOBt, the reaction temperature and the amounts of the reactants were varied (entry 5; 50 °C and entry 6; 10 equiv). In this experiment, concentrations of Fmoc-Gly-OH derivatives were 40 mM. After the reaction, the resin was treated by TFA for 2 h and the solution (filtrate) was evaporated in vacuo. To determine the presence of any esterified product, each crude residue obtained was analyzed by ^{1}H NMR spectroscopy and then the corresponding spectrum was compared with that of authentic sample **10**, which was prepared without the Fmoc-Gly-OH coupling step.

The results of this study are summarized in Figure 5. If any esterification occurred, signals of the proton attached to the carbon bearing the esterified hydroxyl groups should shift to the downfield compared with that of authentic GlcNAc **10**. However, there was no obvious difference among their NMR spectra, which were very similar to that of authentic sample. It was found that whenever low concentrations of Fmoc-Gly-OH and coupling reagents were employed, there was little esterification by Fmoc-Gly-OH depending on both the reaction temperature and excess amounts of Fmoc-Gly-OH. These results suggest that this low concentration coupling condition can be useful for a solid-phase glycopeptide synthesis when oligosaccharyl Fmoc-asparagine having over 40 free-hydroxyl groups is used.

Synthetic strategy of a high molecular sialylglycopeptide (CTLA-4; 113–150): Native chemical ligation (NCL) is a powerful tool to obtain high molecular peptides and proteins. Accordingly, this technique should be applied to the synthesis of high molecular sialylglycopeptides. However, there has been no report on the use of N-linked sialylglycopeptide segments to a NCL reaction. Therefore, in order to examine the usefulness of our new findings mentioned above and to investigate whether complex-type sialylglycopeptides are applicable to NCL reaction, we planned the synthesis of a high molecular sialylglycopeptide. In this case, since benzyl ester form of sialic acid should be used, we should monitor whether this benzyl ester would be converted into thioester under excess of thiophenol and whether this thioester subsequently causes native chemical ligation as a side-reaction.

As mentioned in the introduction, we chose the CTLA-4 fragment (113–150) **13** as a target compound, which has originally two sialyloligosaccharides at Asn-113 and Asn-145. Although a sialyloligosaccharide at Asn-113 is far from the binding-motif, MYPPPY (134–139),[13] we wanted to attach a sialyloligosaccharide to the Asn-113, since this might enable the target glycopeptide to have resistance toward peptidase-digestion—a troublesome occurrence during peptide medication. On the other hand, a sialyloligosaccharide at Asn-145 is thought to be related with its binding process between CTLA4 and

Table 2. Experimental procedure and the coupling conditions of Fmoc-Gly-OH for verification of esterification toward sugar hydroxyl groups on the resin by Fmoc-Gly-OH.[a]



Entry		Coupling conditions Reagent	Additive	Base	T [°C]
1	Fmoc-Gly-OH (5)	DIPCDI (5)	HOBt (5)	–	RT
2	Fmoc-Gly-OH (5)	PyBOP (5)	HOBt (5)	DIPEA (10)	RT
3	Fmoc-Gly-OH (5)	TBTU (5)	HOBt (5)	DIPEA (10)	RT
4	Fmoc-Gly-OPfp (5)	–	Dhbt (1)	–	RT
5	Fmoc-Gly-OH (5)	PyBOP (5)	HOBt (5)	DIPEA (10)	50
6	Fmoc-Gly-OH (5)	PyBOP (10)	HOBt (10)	DIPEA (20)	RT

[a] The equivalents of each reagent to **9** are given in parentheses. In each reaction, DMF was used as a coupling solvent, and the concentration of Fmoc-Gly-OH was 40 mM.

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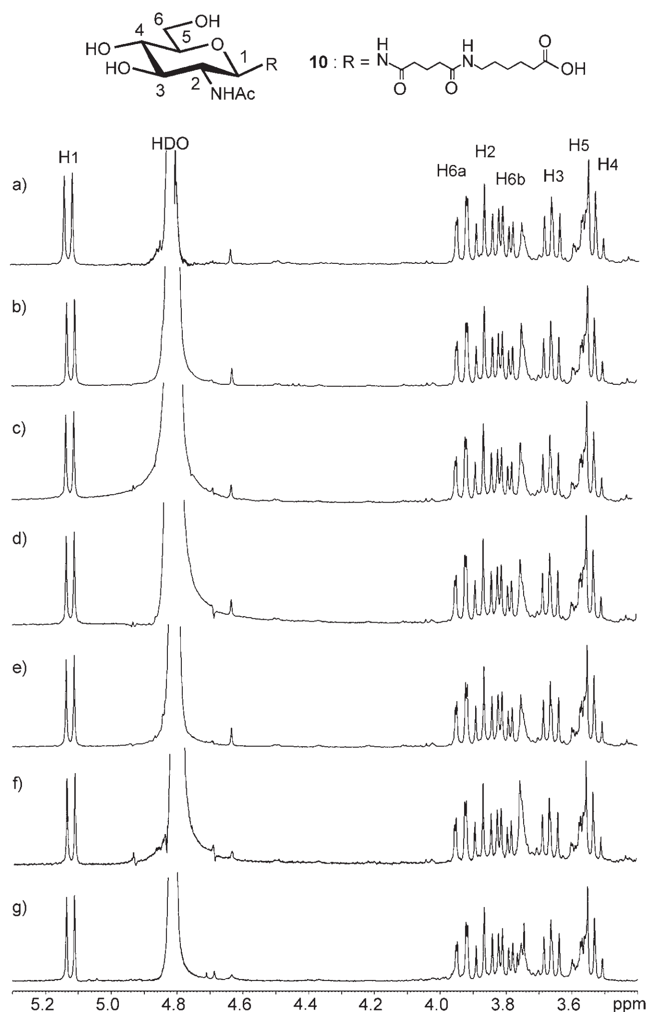

Figure 5. ^{1}H NMR spectra (δ 5.3–3.4 ppm) of crude samples of **10**; a) ^{1}H NMR spectrum of authentic sample. ^{1}H NMR spectra of b)–g) correspond with the crude sample treated by condition of entries 1–6, respectively.

its receptor, B7–2 on the APC. Therefore, we selected a peptide sequence ranging from 113 to 150. The synthetic strategy of CTLA-4 (113–150) **13** is shown in Figure 6.

The sequence of CTLA-4 (113–150) **13** was divided into two segments, **11** and **12**, at the Ile-128/Cys-129, which was chosen as a ligation site. However, it has been known that the NCL reaction between Ile-Cys does not effectively proceed.[30] Therefore, in this case, an isoleucine (Ile-128) was substituted by an alanine. Our synthetic plan was to synthesize sialylglycopeptides, **11** and **12**, by manual Fmoc solid-phase synthetic method employing our established conditions and then ligate both sialylglycopeptides by NCL reaction.[31] To the best of our knowledge, this examination is to be the first time when an N-linked sialylglycopeptide having two intact complex-type sialyloligosaccharides on its backbone has been synthesized using NCL reaction.

Solid-phase synthesis of a sialylglycopeptide with a Cys at the N-terminus, CTLA-4 fragment (129–150) 11: As shown in Figure 7, the solid-phase synthesis of the CTLA-4 fragment (129–150) **11** was performed manually on the HMPA-PEGA resin (2.0 μmol scale) using the Fmoc method. Fmoc-Asn(CHO)-OH **1** was introduced using DEPBT and DIPEA. The coupling yield was over 90 % judging from the Fmoc-fullvene estimation method and the HPLC analysis of the cleavage product. Further peptide elongation was carefully performed using 5.0 equivalents of Fmoc-amino acid derivatives to the resin with DIC/HOBt under the low concentration condition (40 mM). Each Fmoc-amino acid was allowed to react for 1 h and then the Kaiser test was performed to determine the coupling yield. After the complete assembly of the sequence (129–150), the resin was treated with 94 % TFA, 2.5 % TIPS, 2.5 % water, and 1 % 1,2-ethanedithiol (EDT) at room temperature for 2 h. The desired sialylglycopeptide **11** was obtained in good purity, as shown in Figure 7a. The crude product was then subjected to RP-HPLC purification. *cis–trans* Isomers at the proline residues seemed to overlap at the same elution time on HPLC profile under 50 °C, while these are well separated at the ambient temperature analysis. The pure compound **11** was obtained in 30 % isolated yield based on the first amino acid loaded on the resin and characterized by ^{1}H NMR spectroscopy (Figure 7c) and mass spectrometry (MALDI: m/z calcd for $[M+\mathrm{Na}]^+$ 4929.15, found 4928.34). The results demonstrate that our new methods are highly efficient in the solid-phase sialylglycopeptide synthesis.

Solid-phase synthesis of a sialylglycopeptide-thioester, CTLA-4 fragment (113–128) 12: There are many reports of the Boc solid-phase synthesis of peptide-thioester.[30,32] In the Boc method, HF is usually employed in the cleavage of a synthesized peptide from the resin. However, unfortunately it is not suitable in the case of a glycopeptide because HF also decompose the glycosidic linkage of the glycopeptide. Therefore, in order to obtain a glycopeptide-thioester using the solid-phase synthesis, the Fmoc method should be employed. In the Fmoc method, the most critical problem is thought to be the decomposition of the thioester linkage during treatment of 20 % piperidine solution, which is generally employed for removal of the Fmoc group. Recently, to overcome this problem, the Fmoc solid-phase synthesis of peptide-thioester in various approaches has been reported.[10,31,33] This time, to demonstrate the solid-phase synthesis of a sialylglycopeptide-thioester, CTLA-4 (113–128) **12**, we utilized the modified Fmoc strategy reported previously by the Aimoto group;[33b] this method was thought to be a relatively convenient procedure. As shown in Figure 8, at first, C-terminal Ala was introduced to PEGA-resin equipped with Rink linker using Fmoc-Ala-SCH_2CH_2COOH **14** by the DIC/HOBt method. The Fmoc group was then removed by the mixture of 1-methylpyrrolidine, hexamethyleneimine, and HOBt in NMP/DMSO,[33b] which effectively removes the Fmoc group without decomposition of the thioester linkage. Then to avoid the formation of diketopiperazine, which is a well-known side reaction, the combination of triisopropylsilyloxy carbonyl (Tsoc)-amino acid **15** and amino acid

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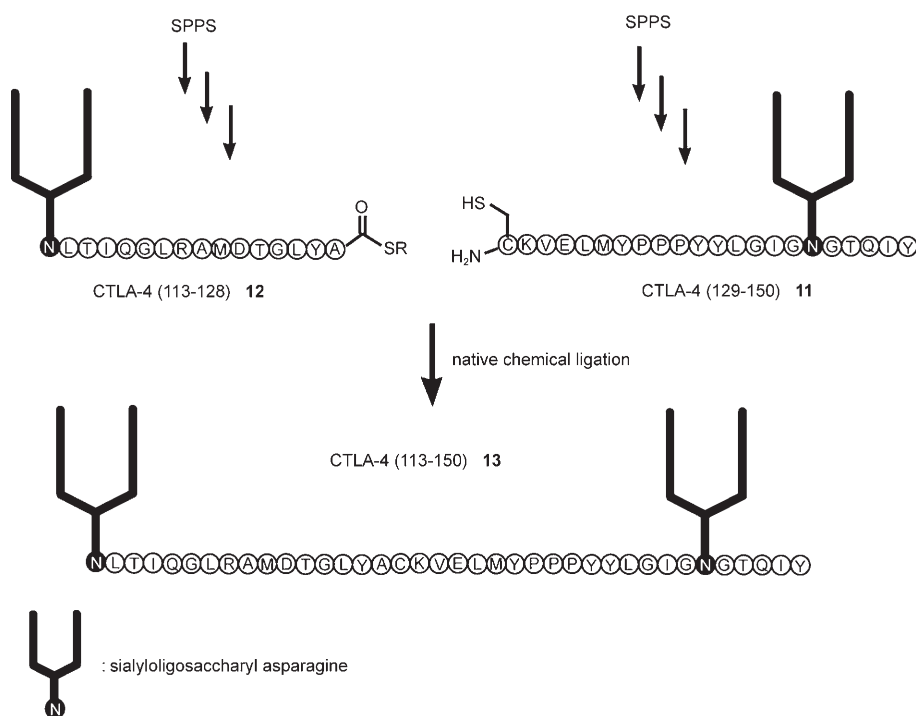

Figure 6. Synthetic strategy for CTLA-4 (113–150) **13** using native chemical ligation.

fluoride (Fmoc-Leu-F) was employed.[34] To the amino group of alanine on the resin, Tsoc-Tyr(*t*Bu)-OPfp **15** was first reacted and then the third amino acid, Fmoc-Leu-F,[35] was condensed in the presence of a catalytic amount of tetrabutylammonium fluoride (TBAF). This protocol adopted both deprotection of the Tsoc group and coupling of Fmoc-Leu-F simultaneously. Further elongation of the peptide chain was continued using DIC/HOBt as a coupling condition and the above-mentioned mixture to remove the Fmoc group. Then, Fmoc-Asn(CHO)-OH **1** was introduced using DEPBT and DIPEA. After removal of the Fmoc group, the resin was treated with 95% TFA, 2.5% TIPS

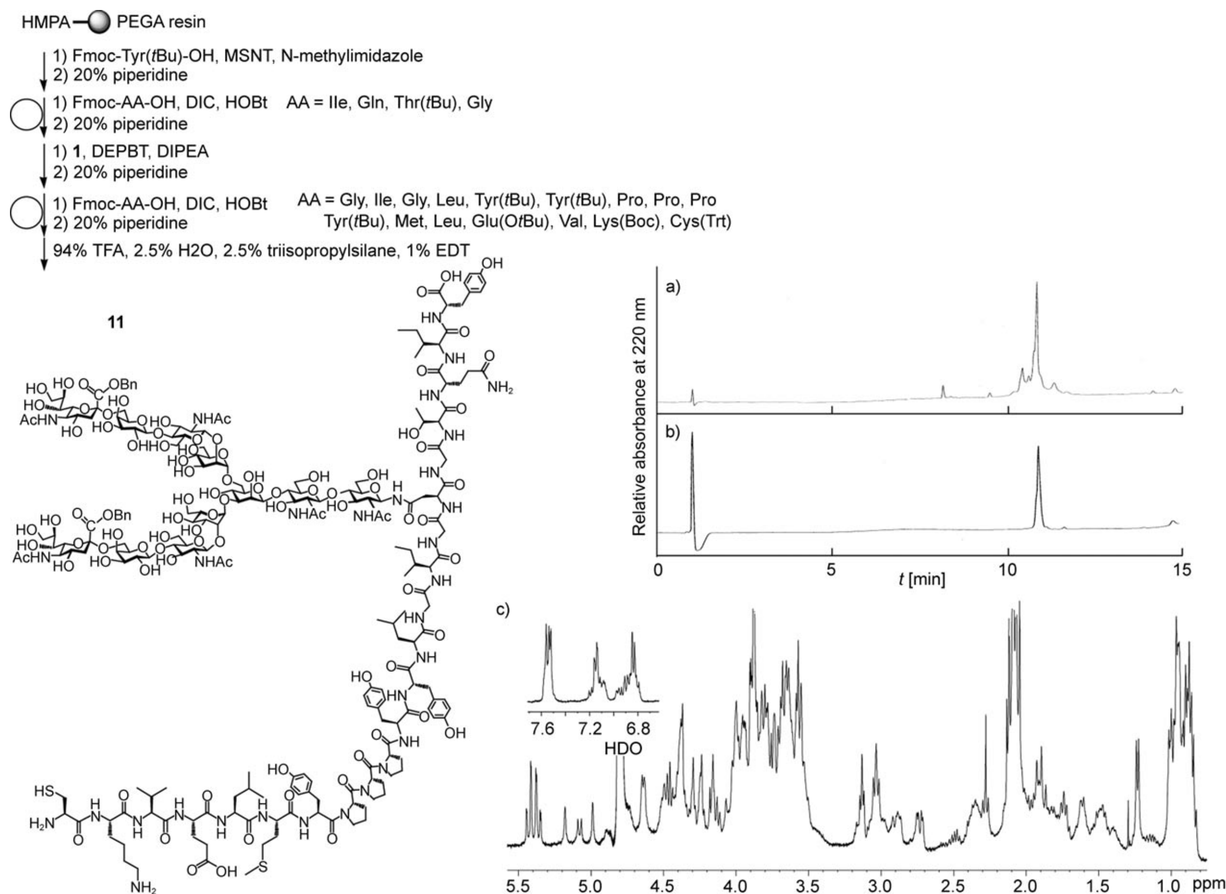

Figure 7. Synthesis of CTLA-4 (129–150) **11**. a) HPLC profile of crude sample after cleavage from the resin, b) HPLC profile of the sialylglycopeptide **11** after RP-HPLC purification, c) NMR spectrum of the sialylglycopeptide **11**. HPLC elution condition of (a) and (b): column, Cadenza CD-18 (3 μm, 4.6×75 mm) at a flow rate of 1.0 $mL\,min^{-1}$ at 50°C, linear gradient of 4.5→67.5% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 15 min.

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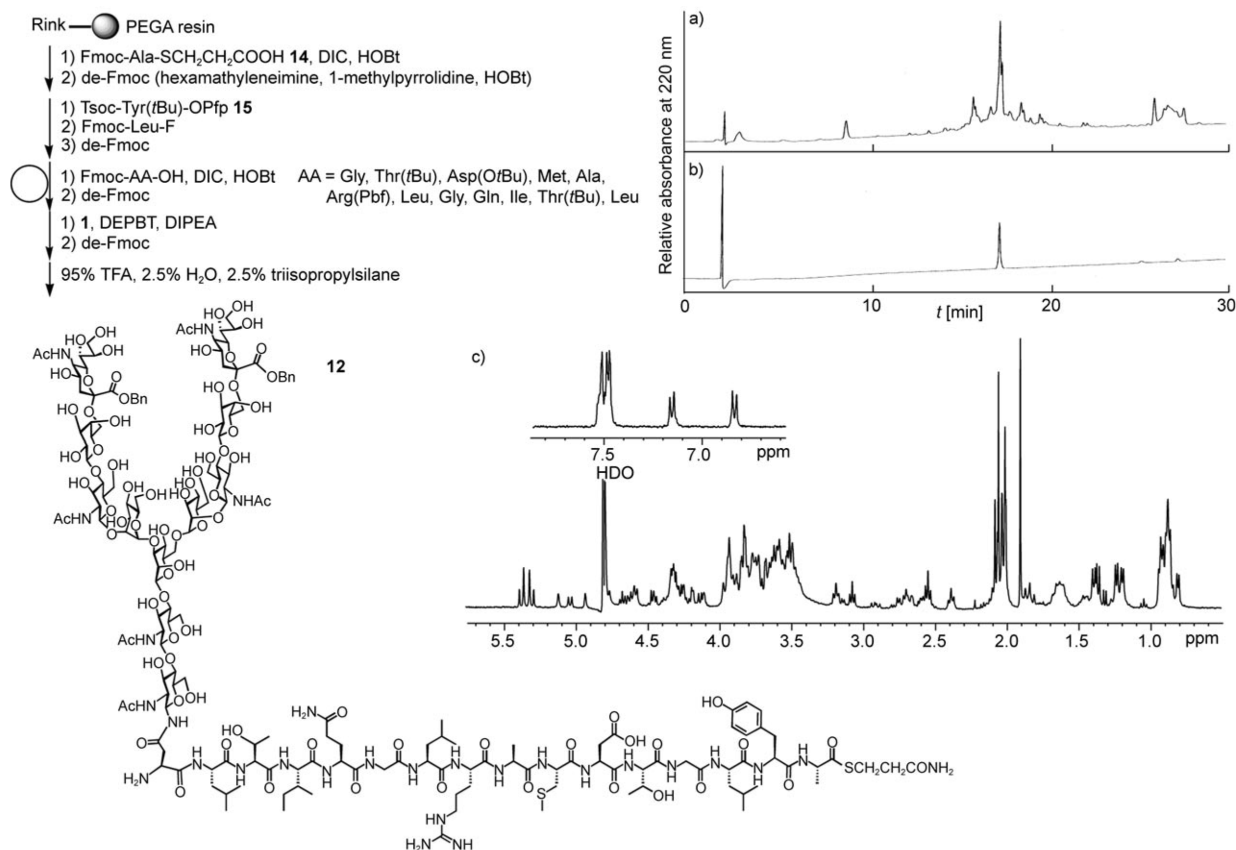

Figure 8. Synthesis of CTLA-4 (113–128) **12**. a) HPLC profile of crude sample after cleavage from the resin, b) HPLC profile of the sialylglycopeptide **12** after RP-HPLC purification, c) NMR spectrum of the sialylglycopeptide **12**. HPLC elution condition of a) and b): column, Inertsil ODS-2 (5 μm, 4.6 × 150 mm) at a flow rate of 1.0 mL min^{-1}, linear gradient of 4.5→67.5% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 15 min.

and 2.5% water at room temperature for 2 h. The crude product was then subjected to RP-HPLC purification (Figure 8a) and pure sialylglycopeptide-thioester **12** was obtained (Figure 8b). However, partial epimerization at the C-terminal amino acid is known to occur during the peptide elongation.[36] As far as we examined by RP-HPLC analysis using several elution conditions, we clearly identified epimerized product at the C-terminus by use of extensive mass analysis. The degree of the epimerization may depend on the treatment time with a mixture of 1-methylpyrrolidine, hexamethyleneimine, and HOBt in NMP/DMSO[36] and we observed at least 10–20% of the epimerized product. However, we obtained highly pure sialylglycopeptide-thioester as shown in Figure 8b. If we can not avoid a large amount of epimerization, we can adopt mutation by a glycine at the C-terminal thioester. This mutation has been used as a safety protocol to avoid epimerization and to enhance NCL reactivity. As shown in Figure 8c, the pure compound **12** was characterized by ^{1}H NMR spectroscopy and mass spectrometry (MALDI: *m*/*z* calcd for $[M+H]^+$ 4208.77, found 4208.94). Although the isolated yield is still low (under 10% based on the amino groups on the initial resin), we succeeded in synthesizing a pure sialylglycopeptide-thioester for the first time.

Native chemical ligation: The sialylglycopeptides **11** and **12** thus obtained were then applied to the NCL reaction (Figure 9). Sialylglycopeptide **11** and sialylglycopeptides-thioester **12** were dissolved in 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine·HCl, 1% (v/v) benzylmercaptane (BnSH), 1% (v/v) thiophenol (PhSH).[4f,31,37] The final sialylglycopeptide concentrations of **11** and **12** were 1 and 2 mM, respectively.

The reaction was performed at 37 °C and monitored by RP-HPLC (Figure 10). The exchange reaction of thioester at the C-terminus of **12** with BnSH was completed within 24 h (Figure 10b), and subsequent ligation reaction afforded a ligated product.[38] Although the ligation reaction did not occur quantitatively, it afforded the desired product, which was characterized by mass spectrometry (ESI: *m*/*z* calcd for $[M+4H]^{4+}$ 2252.7, $[M+5H]^{5+}$ 1802.4, found 2252.9, 1802.8; MALDI: *m*/*z* calcd for $[M+H]^+$ 9012.3, found 9012.4). Purification of the mixture by RP-HPLC afforded the pure ligation product (Figure 10c), and subsequently, the product was treated with 50 mM NaOH to remove the benzyl ester of

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12

11

1) 6M Gn/HCl, 0.1M Phosphate buffer (ph7.6), PhSH, BnSH
2) Purification

50mM NaOH

CTLA-4 (113-150) **13**

Figure 9. Synthesis of CTLA-4 (113–150) **13**.

NeuAc. As the result, the desired product, CTLA-4 (113–150) **13**, was obtained in excellent purity (Figure 10d), which was also characterized by mass spectrometry (ESI: *m*/*z* deconvolution: calcd for $C_{361}H_{570}N_{58}O_{177}S_3$ $[M+H]^+$ 8646.6, found 8645.6).

Thus, we succeeded in synthesizing a high molecular sialylglycopeptide having the intact structure of complex-type sialyloligosaccharides for the first time.

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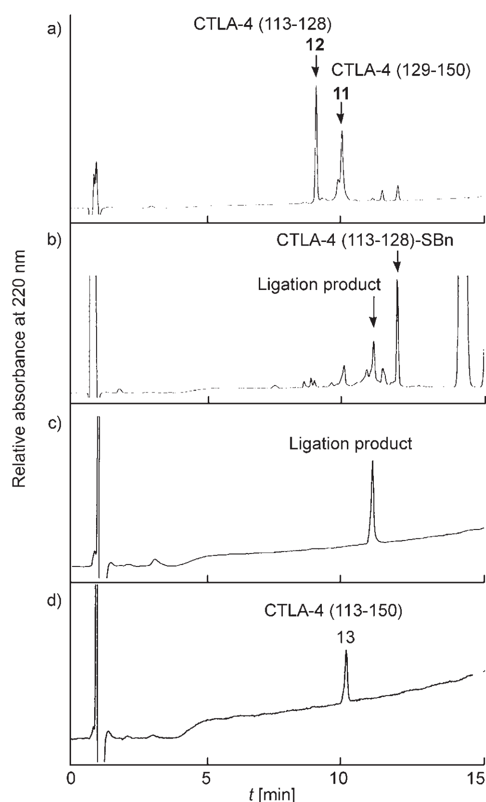

Figure 10. RP-HPLC profiles of the NCL reaction: a) at 0 h; b) after 24 h; c) the ligation product after HPLC purification; d) CTLA-4 (113–150) **13** purified after saponification of benzyl ester of ligation product. HPLC elution condition of a), b), c), and d): column, Cadenza CD-18 (3 μm, 4.6×75 mm) at a flow rate of 1.0 mL min^{-1} and at 40 °C, linear gradient of 18 → 54 % CH_3CN containing 0.09 % TFA in 0.1 % TFA_{aq} over 15 min.

Conclusion

For the preparation of glycopeptides and glycoproteins having mature pure oligosaccharides, currently only chemical synthesis may be efficient, because cell or bacterial expression system affords a glycoprotein having heterogeneous oligosaccharides. In order to examine an efficient synthesis of a complex-type sialylglycopeptide, synthetic methods for appropriate amounts of oligosaccharide, sialylglycopeptide, and its thioester should be essential. We systematically solved such several problems and we obtained a high molecular (9 kDa) sialylglycopeptide having two complex-type pure sialyloligosaccharides by use of a NCL reaction for the first time. This result suggests that the NCL reaction is useful for the synthesis of a high molecular sialylglycopeptide and, furthermore, it indicates that an approach to the chemical synthesis of an intact glycoprotein is possible. Currently, we are examining syntheses of several intact glycoproteins.

Experimental Section

NMR spectra were measured with Bruker Avance 400 (295 K, internal standard HOD = 4.81 ppm in case of D_2O, TMS = 0.00 ppm in case of $CDCl_3$) instrument. Fmoc-Asn(CHO)-OH **1** was prepared by our method previously reported.[8b] Fmoc-AA-OH derivatives, Fmoc-Rink linker and coupling reagents were commercially available except for Fmoc-Ala-SCH_2CH_2COOH **14** and Tsoc-Tyr(*t*Bu)-OPfp **15**. Wang resin, Trt-resin, and HMPA-PEGA resin and Amino-PEGA resin were purchased from Merck (USA). RP-HPLC analyses were carried out on a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using a Cadenza column (Imtakt Corp., 3 μm, 75×4.6 mm) or an Inertsil ODS-2 (GL Science, 5 μm, 150×4.6 mm) at a flow rate of 1.0 mL min^{-1}. ESI mass measurement was carried out on a Bruker Daltonics/Esquire3000 plus. MALDI mass measurement was performed on a Bruker Daltonics/Autoflex using a dihydroxybenzoic acid as a matrix.

Assay conditions for aspartimide formation using several coupling reagents (Figure 2): Fmoc-Asn(CHO)-OH (**1**) (1.0 mg, 0.36 μmol) was dissolved in DMF (10 μL) containing each coupling reagent (PyBOP, TBTU, HBTU, DEPBT, HATU, DIC/HOBt, 0.54 μmol). To each of these solutions, DIPEA (0.36 μmol or 1.08 μmol) was added, respectively, except for DIC/HOBt. After 5 and 30 min, 1μL of the reaction mixture was sampled and diluted with 30 % CH_3CN/H_2O (20 μL). This solution was analyzed by RP-HPLC (Inertsil ODS-2 column, linear gradient of 4.5→67.5 % CH_3CN containing 0.09 % TFA in 0.1 % TFA_{aq} over 30 min). The ratio of **1** and **2** was estimated based on these peak areas. The results are summarized in Figure 2.

Esterification experiment toward N(CHO)GTQIY-HMPA PEGA resin by Fmoc-Gly-OH: The preparation of N(CHO)GTQIY-HMPA PEGA resin was performed on 5 μmol scale, by the same procedure in the preparation of glycopeptide **3** and **4** (Supporting Information). The first amino acid, tyrosine was attached quantitatively to HMPA-PEGA resin by use of MSNT. The peptide elongation was performed by DIC/HOBt method. Fmoc-Asn(CHO)-OH **1** (14 mg, 5 μmol) was coupled to the resin with DEPBT (2.3 mg, 7.5 μmol) and DIPEA (0.65 mg, 5 μmol). In this case, coupling **1** with peptide-resin was arranged in 60 % yield to afford both N(CHO)GTQIY-resin and GTQIY-resin. After 16 h, the resin was washed with DMF and treated with 20 % piperidine in DMF. After the resin was washed with DMF, the resin was separated into five aliquots (1 μmol scale) and then used for each experiment. The resin was treated by Fmoc-Gly-OH for 1 h under the conditions described in Table 1. After these coupling reactions, the resin was washed with DMF (3 mL) and CH_2Cl_2 (3 mL), and was treated by a solution containing 95 % TFA, 2.5 % TIPS, and 2.5 % waster. The obtained mixture (filtrate) was evaporated in vacuo. The residues were analyzed by RP-HPLC, respectively. Saponification of ester group of sugar hydroxyl group was performed in NMR tube by use of 50 mM NaOH solution for 15 min monitoring by NMR measurement. After neutralized the solution with acetic acid, the reaction mixture was lyophilized then analyzed by RP-HPLC. These results are shown in Figure 4.

Esterification experiment toward GlcNAc derivative linked HMPA-PEGA resin by Fmoc-Gly-OH: The preparation of GlcNAc derivative linked HMPA-PEGA resin **9** and **10** is described in the Supporting Information (Scheme S1). To the resin **9** (3.7 μmol, based on the amino groups on the initial resin), Fmoc-Gly-OH (5.5 mg, 18.5 μmol) or Fmoc-Gly-OPfp (8.6 mg, 18.5 μmol) was added and reacted for 1 h under the conditions in Table 2 (entries 1–6). After 1 h, the resin was washed with DMF (5 mL) and CH_2Cl_2 (5 mL), and then was treated by a solution of 95 %TFA for 3 h. The obtained mixture (filtrate) was evaporated in vacuo and the residues were analyzed by NMR measurement. These results are shown in Figure 5.

Solid-phase synthesis of a sialylglycopeptide with Cys at the N-terminus, CTLA-4 fragment (129–150), 11: The synthesis of CTLA-4 fragment (129–150) **11** was performed on HMPA-PEGA resin (2.0 μmol scale) by same procedure in the preparation of **3** and **4**. Briefly, the first amino acid, Fmoc-Tyr(*t*Bu)-OH (2.8 mg, 6.0 μmol) was attached quantitatively to the resin using MSNT (1.6 mg, 6.0 μmol) and *N*-methylimidazole (0.45 mg, 5.5 μmol) in CH_2Cl_2 (60 μL). The elongation of peptide (G, T,

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Q, I) was performed using Fmoc-amino acid (10 μmol), DIC (10 μmol), and HOBt (10 μmol) in DMF (60 μL) and 20% piperidine in DMF (500 μL) was used for removal of Fmoc group. The coupling of Fmoc-Asn(CHO)-OH **1** (11 mg, 4.0 μmol) was performed using DEPBT (1.8 mg, 6.0 μmol) and DIPEA (0.52 μg, 4.0 μmol) in DMF (130 μL) for 24 h at room temperature. The resin was washed with DMF (3 mL) and was treated with 20% piperidine in DMF (500 μL) for 20 min. The remaining amino acids were introduced manually using Fmoc-amino acid (10 μmol), DIC (10 μmol) and HOBt (10 μmol) in DMF (250 μL). After completion of the chain assembly, the resin was treated by a solution containing 94% TFA, 2.5% TIPS, 2.5% water, and 1% EDT for 2 h at room temperature, and then the obtained mixture (filtrate) was evaporated in vacuo, the residue was lyophilized. Purification of the residue by RP-HPLC with Cadenza CD-18 (4.6×75 mm, linear gradient of 18 → 72% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 30 min) at a flow rate of 1.0 mL min^{-1} afforded the desired sialylglycopeptide **11** (3.0 mg, 30% yield based on first tyrosine attached). ^{1}H NMR (400 MHz, 296 K in D_2O): δ = 7.55 (m, 10H, *Ph*CH_2×2), 7.21–6.80 (m, 8H, Tyr-Ar×4), 5.43 (br d, 2H, PhCH_2×2), 5.37 (br d, 2H, PhCH_2×2), 5.18 (s, 1H, Man4-H-1), 5.18 (d, 1H, GlcNAc1-H-1), 4.99 (s, 1H, Man4′-H-1), 2.73 (br dd, 2H, NeuAc7,7′-H-3eq), 2.08–2.02 (6s, 18H, Ac×6), 1.90 (br dd, NeuAc7, 7′-H-3ax), 1.22 (d, 3H, Thr-βCH_2); ESI-MS: *m*/*z*: calcd for: 2452.5; found 2452.0 $[M+2H]^{2+}$, 1635.8 $[M+3H]^{3+}$; MALDI-MS: *m*/*z*: calcd for: 4928.15, found 4928.34 $[M+Na]^{+}$.

Solid-phase synthesis of a sialylglycopeptide-thioester, CTLA-4 fragment (113–128) 12: Fmoc-Rink-PEGA resin (50 μmol) was prepared manually by the coupling Fmoc-Rink linker (121 mg, 225 μmol) with PEGA resin (1 g, 50 μmol g^{-1}) using TBTU (72 mg, 225 μmol) and DIPEA (58 mg, 450 μmol) in DMF (200 mM). The resin was treated with 20% piperidine in DMF (3 mL) for 20 min and then washed with DMF (20 mL). Fmoc-Ala-SCH_2CH_2COOH **14** (73 mg, 180 μmol) was attached to the resin using TBTU (40 mg, 125 μmol) and DIPEA (16 mg, 210 μmol) in DMF (0.5 mL) for 3 h at room temperature. The resin was washed with DMF (5 mL) and was treated by the Fmoc de-blocking mixture [25% 1-methylpyrrolidine, 2% hexamethyleneimine, 2% HOBt in 1-methyl-2-pyrrolidinone (NMP)/DMSO 1:1, 1 mL] for 3 min and additionally 18 min. In this synthesis, this mixture was used for removal of Fmoc group. After the resin was washed with DMF (20 mL), Tsoc-Tyr(*t*Bu)-OPfp **15** (90 mg, 150 μmol) in tetrahydrofuran (0.5 mL) was reacted to the resin for 15 min. This reaction was repeated twice with the same amount of fresh Tsoc-Tyr(*t*Bu)-OPfp **15** but second reaction time was for 45 min. The resin was washed with CH_2Cl_2 (5 mL), and then Fmoc-Leu-F (36 mg, 100 μmol) in CH_2Cl_2 (1 mL), which prepared by reported method,[34] was added to the resin. The reaction was initiated by adding TBAF (1.6 mg, 5 μmol) to the mixture. After 2 h, the resin was washed with CH_2Cl_2 (10 mL) and DMF (10 mL). Further elongation of the peptide chain was carried out by the Fmoc method using Fmoc-amino acid (250 μmol), DIC (250 μmol), and HOBt (250 μmol) in DMF (0.5 mL). A part of this resin (50 μmol) was used for the introduction of Fmoc-Asn(CHO)-OH **1**. To the resin corresponding to 1-μmol scale, Fmoc-Asn(CHO)-OH **1** (5.5 mg, 2.0 μmol) was reacted using DEPBT (1.1 mg, 3.0 μmol) and DIPEA (0.26 mg, 2.0 μmol) in DMF (60 μL) for 24 h. After the resin was washed with DMF (3 mL) and CH_2Cl_2 (3 mL), removal of Fmoc group was performed. And then the resin was treated by a solution containing 95% TFA, 2.5% TIPS, and 2.5% water for 2 h at room temperature. The obtained mixture (filtrate) was evaporated in vacuo, and the residue was lyophilized. Purification of the residue by RP-HPLC with Cadenza CD-18 (4.6×75 mm, linear gradient of 18→72% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 30 min) at a flow rate of 1.0 mL min^{-1} afforded the desired sialylglycopeptide **12** (0.4 mg, 9.5% yield based on the amino groups on the initial resin). ^{1}H NMR (400 MHz, 296 K in D_2O): δ = 7.55 (m, 10H, *Ph*CH_2×2), 7.19 (d, 2H, Tyr-Ar), 6.83 (d, 2H, Tyr-Ar), 5.39 (d, 2H, PhCH_2×2), 5.32 (d, 2H, PhCH_2×2), 5.14 (s, 1H, Man4-H-1), 5.05 (d, 1H, GlcNAc1-H-1), 4.93 (s, 1H, Man4′-H-1), 2.73 (NeuAc7,7′-H-3eq), 2.08–2.02 (Ac×6), 1.85 (NeuAc7, 7′-H-3ax): ESI-MS: *m*/*z*: calcd for: 2104.4, 1403.3, found 2105.1 $[M+2H]^{2+}$, 1403.9 $[M+3H]^{3+}$; MALDI-MS: *m*/*z*: calcd for 4207.78, found 4208.94 $[M+H]^{+}$.

Native chemical ligation: Sialylglycopeptide **11** (0.2 mg, 40 nmol) and sialylglycopeptide thioester **12** (0.34 mg, 80 nmol) were dissolved into 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine (50 μL). To the solution, 1% (v/v) benzylmercaptane (BnSH), 1% (v/v) thiophenol (PhSH) was added to start the NCL reaction. The reaction was performed at 37 °C for 24 h. Direct purification of the reaction mixture by RP-HPLC with Cadenza CD-18 (4.6×75 mm, linear gradient of 18→72% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 30 min) at a flow rate of 1.0 mL min^{-1} afforded desired product in good purity. Then, this compound was treated by 50 mM NaOH (50 μL) for 15 min at room temperature. After neutralization of the reaction mixture by 50 mM acetic acid (50 μL), purification of the mixture by RP-HPLC with Cadenza CD-18 (4.6×75 mm, linear gradient of 18→72% CH_3CN containing 0.09% TFA in 0.1% TFA_{aq} over 30 min) afforded sialylglycopeptide **13** (ca. 20%) in high purity. The yield (20%) was estimated by the peak area corresponding to **13** on HPLC profile. ESI: m/*z*: calcd for 2882.9, 2162.4; found 2883.2 $[M+3H]^{3+}$, 2162.7$[M+4H]^{4+}$; deconvolution: calcd for $C_{361}H_{570}N_{58}O_{177}S_3$: 8646.6; found 8645.6 $[M+H]^{+}$.

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[38] The analysis of the byproduct in NCL reaction was performed by RP-HPLC and mass spectrometry in detail. The results were shown in the Supporting Information, Figure S6.

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