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Highly efficient synthesis of sialylglycopeptides overcoming unexpected aspartimide formation during activation of Fmoc-Asn(undecadisialyloligosaccharide)-OH

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Abstract—Oligosaccharyl Fmoc-asparagine in which amide nitrogen of the asparagine side chain attached to the anomeric position at the reducing end, is a versatile building block and has been used for various glycopeptide synthesis using Fmoc solid-phase peptide synthesis (SPPS). We found unexpected aspartimide formation between amide nitrogen at the reducing end and α-carboxyl acid of oligosaccharyl Fmoc-asparagine during activation of α-carboxyl acid and this side reaction resulted in low coupling yields of oligosaccharyl Fmoc-asparagine with peptide-resin. This aspartimide formed efficiently using conventional coupling reagents such as PyBOP and HATU, but DEPBT afforded little of the aspartimide derivative. Activation condition using DEPBT (3.0 equiv) and DIPEA (2.0 equiv) afforded excellent yield (97%) in coupling reaction between Fmoc-Asn(CHO)-OH and peptide-resin. Based on these results, we performed a synthesis of a sialylglycopeptide, HIV-gp120 (54–63) VVLLVN(CHO)VTENF, in high yield.

Glycoproteins and glycopeptides contain O- or N-linked oligosaccharides on their peptide backbone and play central roles in several biological events. N-Glycosylation of proteins is the predominant modification in eukaryotic cells.[1] In order to investigate the role of oligosaccharides, it is essential to synthesize glycoproteins and glycopeptides having a homogeneous oligosaccharide form for use as probes to study their biological roles.[2]

One approach to synthesize N-linked glycopeptides, a solid-phase synthetic technique has frequently been attempted. Currently, there are two approaches: (a) the convergent approach[3] and (b) the building-block approach.[4] The former is based on the coupling of a glycosylamine to the activated aspartic acid side chain in the protected peptide either in solution or on a solid phase.[3] However, this route affords side products depending on the reaction conditions. This problem is the intramolecular aspartimide formation with the proximal C-terminal amide nitrogen on activation of the aspartic acid side chains. Several approaches have been described to reduce the aspartimide formation,[3i,5] but this convergent approach still potentially faces the side reaction depending on the peptide sequence. Alternatively, because of no side reaction such as aspartimide formation, the building-block approach using oligosaccharyl Fmoc-asparagine has been thought to be a versatile and advantageous method for the synthesis of a large number of glycopeptides,[4] while this approach might give low coupling yields occasionally.

Recently, we have reported solid-phase synthesis of sialylglycopeptides using Fmoc-Asn(CHO)-OH **1** as the building block[4c,d] and undertook the synthesis of various sialylglycopeptides. In the course of this study using Fmoc-Asn(CHO)-OH **1**, we found a critical side reaction to form aspartimide (Fig. 1) leading to low coupling yield of Fmoc-Asn(CHO)-OH **1** to the peptide on solid-phase support, despite the building-block approach had been thought to have no side reaction

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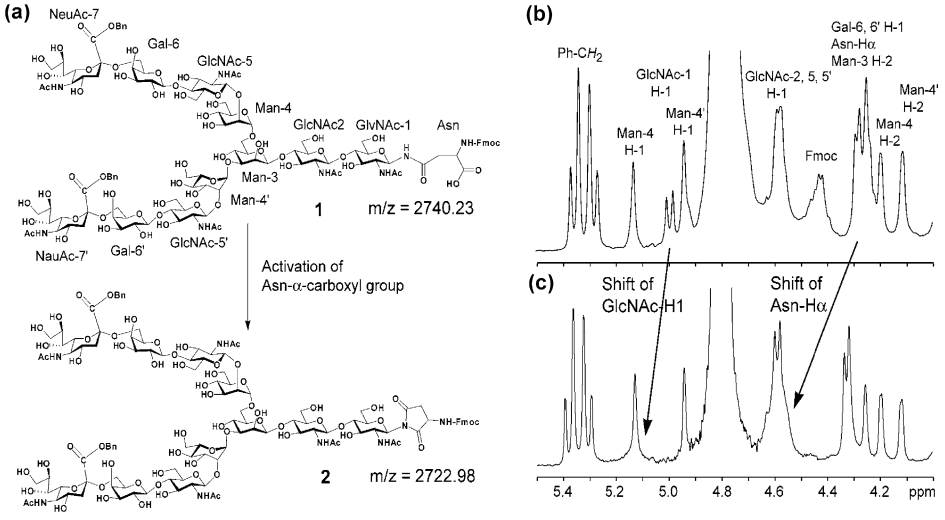


Figure 1. Unexpected formation of aspartimide: (a) structure and its mass observed; (b) ^{1}H NMR spectrum of Fmoc-Asn(CHO)-OH **1**; (c) ^{1}H NMR spectrum of aspartimide derivative **2**.

such as the aspartimide formation in the convergent approach. This critical aspartimide formation has not been found and discussed, and might have reduced the coupling yield of the desired glycopeptide bond in a number of glycopeptide syntheses.

In this letter, we describe in detail the conditions, which afforded the side reaction and the effect on the coupling reaction of Fmoc-Asn(CHO)-OH **1** with peptide-resin. In addition, we also describe both the suppression condition of aspartimide formation and highly efficient coupling condition of Fmoc-Asn(CHO)-OH **1** with peptide-resin.

Previously, we have reported that an equivalent of Fmoc-Asn(CHO)-OH **1** to peptide-resin was coupled using 2-(1*H*-9-azobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphonate (HATU)[6] and *N*,*N*′-diisopropylethylamine (DIPEA), but the coupling yield was 38% based on estimation of the cleaved Fmoc group.[4c,d] At first, we hypothesized the reason for the low coupling yields was due to the steric hindrance of the oligosaccharide chain. However, when we monitored a reaction mixture containing Fmoc-Asn(CHO)-OH **1** and an activation reagent in the presence of peptide-resin by RP-HPLC, we found generation of an unexpected new compound. Using both mass spectrometry and NMR spectrometry, we revealed this compound isolated to be aspartimide derivative **2**, which formed via intramolecular cyclization between amide nitrogen attached to the GlcNAc and activated the asparagine-α-carboxyl group. As shown in Figure 1b and c, the ^{1}H chemical shift value of anomeric H-1 of the Asn-linked GlcNAc in **1** shifted to the downfield by 0.1 ppm and Asn-Hα shifted to the downfield by 0.3 ppm compared to that of Fmoc-Asn(CHO)-OH **1**. These proton signals were assigned by extensive NMR studies such as 2D-HMQC, 2D-HMBC, and 2D-TOCSY. In the case of 2D-TOCSY experiment in 5%D_2O/H_2O, the proton signal of the amide group

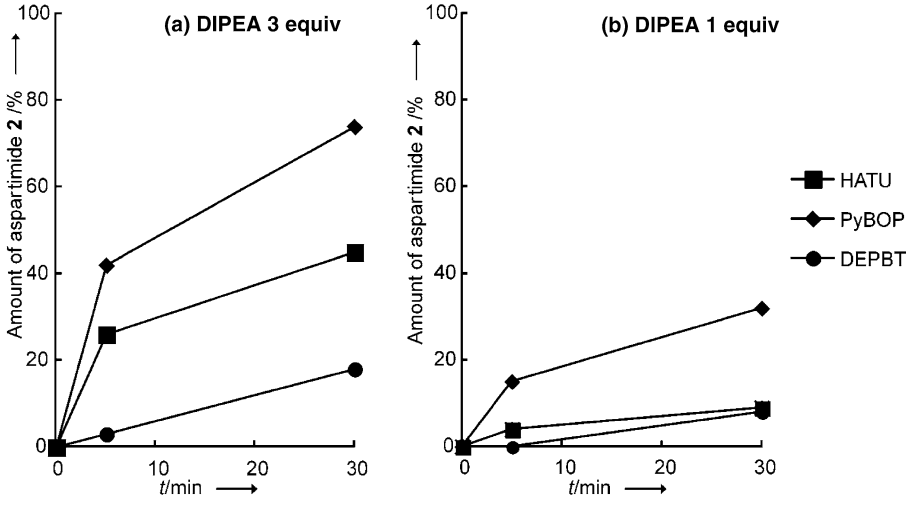


Figure 2. The amount of aspartimide **2** produced during activation of **1** with several coupling reagents (1.5 equiv) and DIPEA (a) 3.0 equiv or (b) 1.0 equiv.

linked at the anomeric position of the reducing end disappeared, although the corresponding proton signal of Fmoc-Asn(CHO)-OH **1** can be easily found in the same experiment.[7]

Next, we investigated whether this aspartimide derivative **2** is generally generated on activation of the asparagine-α-carboxyl group by use of several conventional activation conditions. Fmoc-Asn(CHO)-OH **1** was activated by 1.5 equiv of HATU, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphonate (PyBOP),[8] or 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT)[9] in the presence of DIPEA (1.0 or 3.0 equiv) and each reaction mixture was analyzed by RP-HPLC after 5 and 30 min, respectively. These results are summarized in Figure 2. When 3.0 equiv of DIPEA was used (Fig. 2a), aspartimide derivative **2** was already generated after 5 min under all conditions and nearly 80% of Fmoc-Asn(CHO)-OH **1** was converted into aspartimide derivative **2** after 30 min. On the other hand, the condition of DEPBT, DIPEA (1.0 equiv) afforded a minimum amount of aspartimide derivative **2** after 5 min (Fig. 2b).

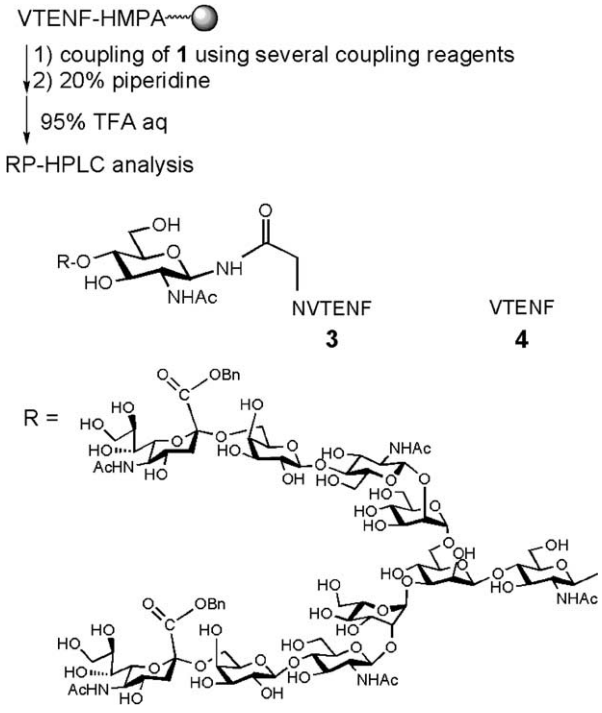


Figure 3. Experimental procedure for comparison of coupling conditions and coupling yields.

However, since these examinations were performed in the absence of peptide-resin, the competition rate between aspartimide formation and glycopeptide formation on the resin should be evaluated. In order to investigate whether aspartimide formation affects the coupling yield of Fmoc-Asn(CHO)-OH **1** to peptide-resin, we compared the coupling yield of Fmoc-Asn(CHO)-OH **1** with peptide-resin dependent on the coupling conditions under monitoring both glycopeptide formation on solid-phase and aspartimide formation in solution by RP-HPLC (Figs. 3 and 4).

The sequence N(CHO)VTENF we selected for this experiment was part of gp120,[10] one of the membrane proteins of human immunodeficiency virus (HIV). Toward VTENF-HMPA-PEGA-resin, which was prepared manually by Fmoc solid-phase peptide synthesis, an equivalent of Fmoc-Asn(CHO)-OH **1** to the resin was added with PyBOP or DEPBT. After 21 h, each reaction mixture was filtered out and then both the filtrates and the peptides on the resin were analyzed. Each crude material released from the resin was analyzed by RP-HPLC and the peak area of glycopeptide **3** was compared with that of peptide **4**, respectively.[11] The ratios of the desired glycopeptide **3** to peptide **4** are shown in Figure 4a and b. It was found that DEPBT in the presence of DIPEA gave higher yield (96%) (Fig. 4b) compared to that of PyBOP (84%) (Fig. 4a). Each solution phase in the reaction mixtures after 30 min was also analyzed by RP-HPLC. The results are shown in Figure 4c and d. Under the condition of PyBOP, Fmoc-Asn(CHO)-OH **1** in the solution phase was already converted into aspartimide derivative **2** within 30 min (Fig. 4c), while there was little aspartimide derivative **2** under the condition of DEPBT (Fig. 4d). After 21 h under this condition (DEPBT), 30% of Fmoc-Asn(CHO)-OH **1**, which

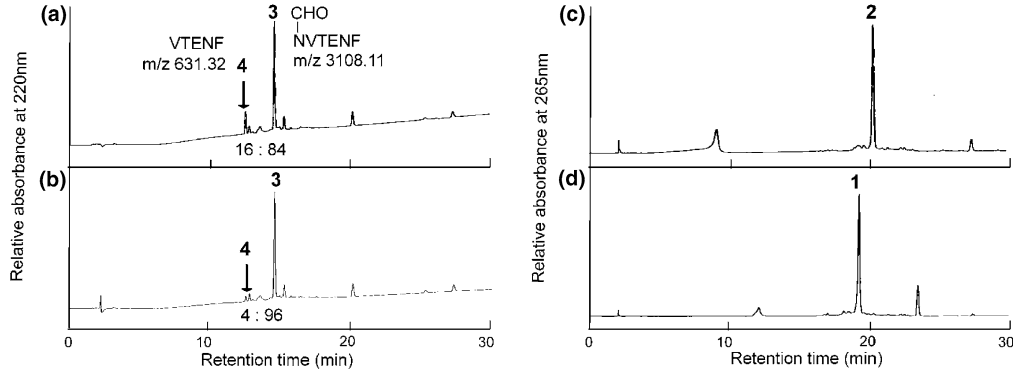
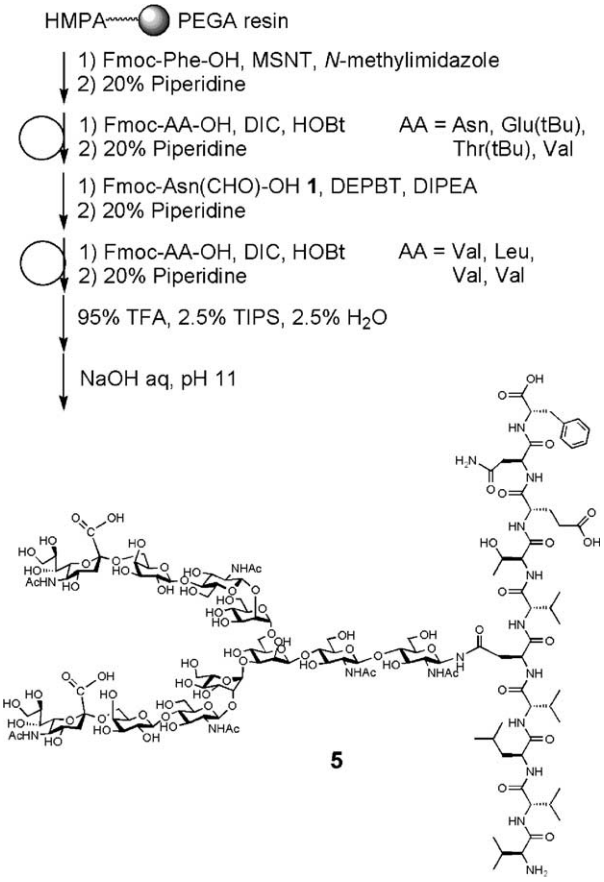


Figure 4. Comparison of coupling yield of **1** with VTENF-HMPA-PEGA resins using PyBOP or DEPBT. HPLC profiles of crude sample after cleavage from the resin; (a) **1** (1.0 equiv) was coupled by PyBOP (1.5 equiv), DIPEA (3.0 equiv) in DMF (30 mM); (b) **1** (1.0 equiv) was coupled by DEPBT (1.5 equiv), DIPEA (1.0 equiv) in DMF (30 mM). The ratios of **3** to **4** based on its peak area are shown below each HPLC profiles. HPLC profiles of each solution phase of reaction mixtures after 30 min are shown in (c) **1**, PyBOP, DIPEA, and (d) **1**, DEPBT, DIPEA.

remained in the solution phase was converted into aspartimide derivative **2**. These results indicate aspartimide formation competes with the desired peptide formation on solid-phase and reduces the coupling yield of Fmoc-Asn(CHO)-OH **1** to the peptide-resin. Therefore, it is necessary to employ at least a few excess of precious Fmoc-Asn(CHO)-OH **1** to peptide-resin in order to accomplish quantitative coupling and we further examined optimization of the coupling conditions employing the same peptide-resin (VTENF-HMPA-PEGA-resin) and activation reagents (PyBOP, DEPBT).

We examined two conditions: (1) two-time coupling of an equivalent of Fmoc-Asn(CHO)-OH **1** and (2) single-step coupling of 2.0 equiv of Fmoc-Asn(CHO)-OH **1**, and then the crude samples were analyzed by HPLC after cleavage from each resin. The ratio of **3** and **4** was estimated. When 2.0 equiv of Fmoc-Asn(CHO)-OH **1** was used under the PyBOP or DEPBT condition (Fig. 5a and b), it was found that the condition using Fmoc-Asn(CHO)-OH **1**, DEPBT, DIPEA (2:3:2, molar ratio) gave the best yield of 97% compared to that of PyBOP (**3**: 73%; **4**: 27%). On the other hand, two-time coupling of an equivalent of Fmoc-Asn(CHO)-OH **1** to peptide-resin using both PyBOP and DEPBT afforded excellent yields (over 91%, Fig. 5c and d).

Using N(CHO)VTENF-HMPA-PEGA resin prepared under the optimized condition (Fig. 5b), we attempted to elongate the peptide after introduction of oligosaccharyl asparagine to synthesize the sequence VVLVN-(CHO)VTENF (54–63) **5** (Scheme 1). Synthesis of N-linked sialylglycopeptide **5** was performed according to the procedure in Scheme 1.[12] After introduction of Fmoc-Asn(CHO)-OH **1**, further peptide elongation was achieved by use of DIC/HOBt method under low concentrations of Fmoc-amino acids to avoid esterification of sugar hydroxyl groups by activated Fmoc-amino acids. Cleavage/deprotection was performed with TFA, H_2O, TIPS (95:2.5:2.5, v/v/v) (Fig. 6b) and then removal of benzyl ester was achieved with 50 mM NaOH. Finally, RP-HPLC purification of the crude sample afforded the desired sialylglycopeptide **5** (Fig. 6b, 36% isolated yield). The sialylglycopeptide **5** was characterized by ^{1}H NMR spectroscopy and mass spectrometry as shown in Figure 6.



Scheme 1. Solid-phase synthesis of sialylglycopeptide **5**, gp120 (54–63).

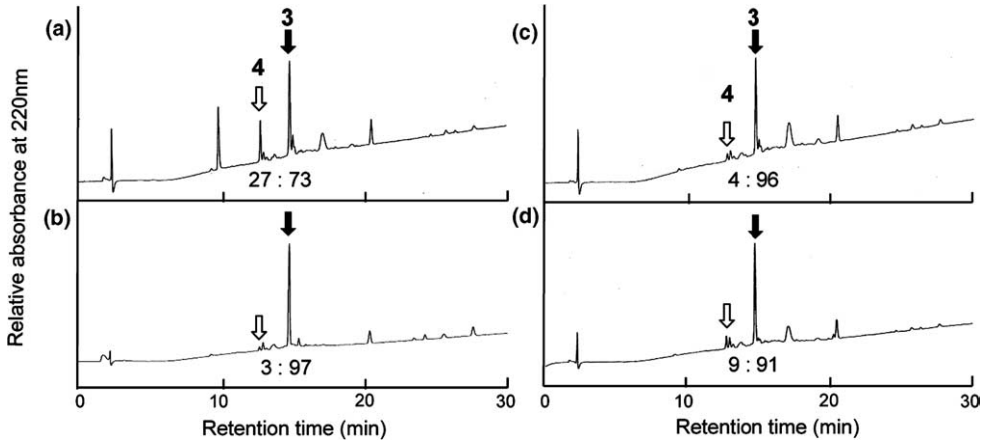


Figure 5. Comparison of coupling yields and coupling conditions. Black arrows indicate the desired glycopeptide **3**, open arrows indicate peptide **4** (Fig. 4). The ratios of **3** to **4** were estimated based on its peak area and are shown below each HPLC profile. (a) **1** (2.0 equiv) was coupled by PyBOP (3.0 equiv), DIPEA (6.0 equiv) in DMF (30 mM); (b) **1** (2.0 equiv) was coupled by DEPBT (3.0 equiv), DIPEA (2.0 equiv) in DMF (30 mM); (c) **1** (1.0 equiv) was coupled two times by PyBOP (1.5 equiv), DIPEA (3.0 equiv) in DMF (30 mM); (d) **1** (1.0 equiv) was coupled two times by DEPBT (1.5 equiv), DIPEA (1.0 equiv) in DMF (30 mM).

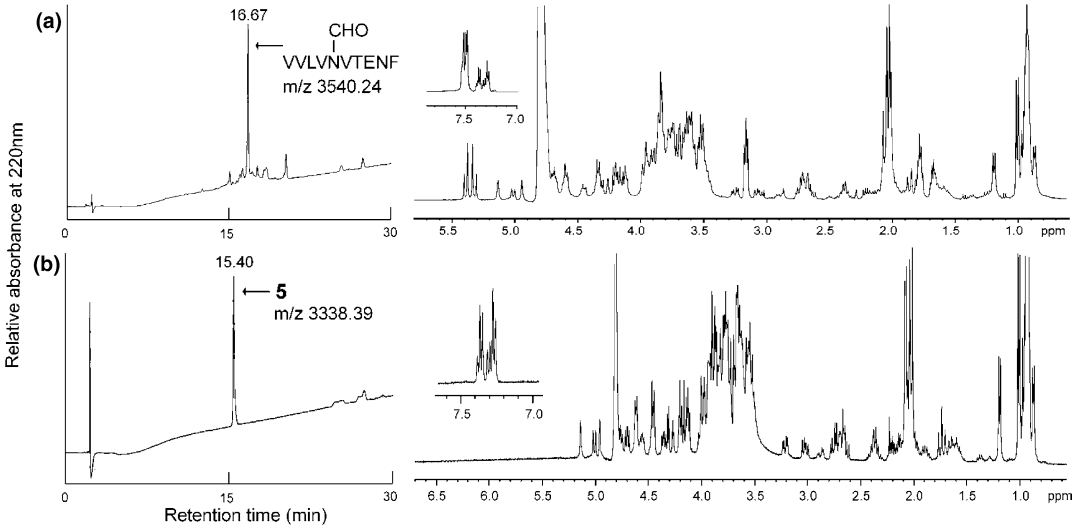


Figure 6. HPLC profiles at 220 nm and corresponding ^{1}H NMR spectra: (a) Crude sample of VVLVN(CHO)VTENF after cleavage from the resin with 95%TFA, 2.5% TIPS, and 2.5% H_2O. The desired product elutes at 16.67 min; (b) Sialylglycopeptide **5** purified by HPLC after NaOH treatment elutes at 15.40 min.

In conclusion, we have found the aspartimide formation as side reaction occurred on coupling Fmoc-Asn(CHO)-OH **1** to peptide-resin and this side reaction led to reduce the coupling yield of precious Fmoc-Asn(CHO)-OH **1**. These results indicate any glycosylated Fmoc-protected asparagine derivative may face this side-reaction dependent on the reactivity of peptide on the resin. In addition, we have found the condition, which suppressed the side reaction and achieved quantitative coupling of Fmoc-Asn(CHO)-OH **1** to peptide-resin. As a result, we found a highly efficient and concise high-yield synthetic method of a sialylglycopeptide.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.12.064.

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11. Compound **3** and **4** were characterized by mass spectrometry. Compound **3**; MALDI-MS: *m*/*z* calcd for $[M+H]^+$ 3108.19, $[M+Na]^+$ 3130.18, found 3107.99, 3129.78. Compound **4**; MALDI-MS: *m*/*z* calcd for $[M+H]^+$ 609.28, $[M+Na]^+$ 631.27, found 609.45, 631.42.
12. In order to confirm that the optimized condition for introduction of Fmoc-Asn(CHO)-OH **1** to a peptide-resin is generally useful, we also attempted to synthesize another sialylglycopeptide ALLVN(CHO)SS **6**, which is already synthesized in total yield 5.1% by use of HATU as a coupling reagent.[4d] As a result, we obtained sialylglycopeptide **6** in 32% isolated yield. The details of the synthesis of **6** are shown in supplementary.