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Convergent Synthesis of *N*-Linked Glycopeptides via Aminolysis of *ω*-Asp *p*-Nitrophenyl Thioesters in Solution

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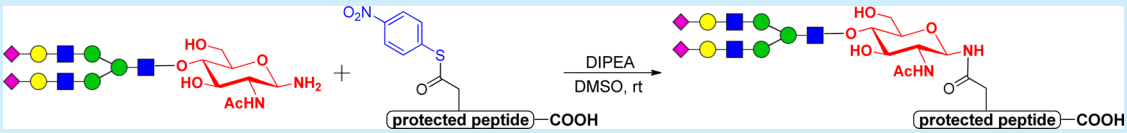
Supporting Information

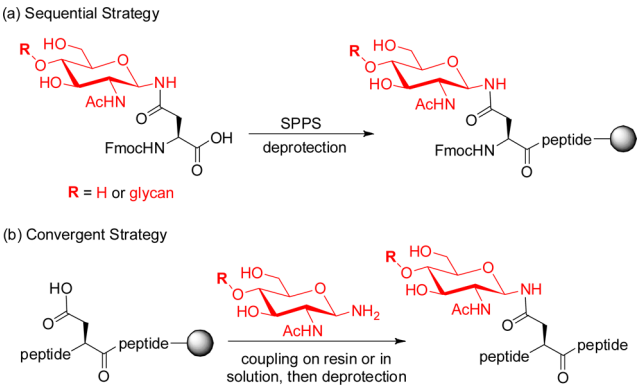


ABSTRACT: An efficient *N*-linked glycosylation reaction between glycosylamines and *p*-nitrophenyl thioester peptides has been developed. The reaction conditions are mild and compatible with the C-terminal free carboxylic acid group and the unprotected *N*-linked sialyloligosaccharide. By means of this convergent strategy, a versatile *N*-glycopeptide fragment containing an N-terminal Thz and a C-terminal thioester was readily prepared, which is available for the synthesis of long glycopeptides and glycoproteins using the protocol of native chemical ligation.

N-Linked glycosylation is the attachment of a glycan through *N*-acetylglucosamine by an amide bond at the side chain of asparagine within an Asn-X-Ser/Thr consensus sequence (where X is any natural amino acid except proline), and it is regarded as a kind of important co- and post-translational modification for many proteins.[1] This type of linkage influences various important biological processes, such as protein quality control and cell recognition, and its faulty structure may result in protein malfunction and diseases.[2] Thus, understanding the relationship between the structure and biological function of *N*-linked glycopeptides is essential in various biomedical projects. However, *N*-linked glycosylation in nature usually results in heterogeneous mixtures of glycoforms, which hinders the study of their diverse biological phenomena. Fortunately, chemical[3,4] or chemoenzymatic[5] methods provide an ideal platform for studying *N*-linked glycopeptides and glycoproteins with structure-defined glycans.

In recent years, various advances to chemically synthesize *N*-linked glycoproteins have been reported.[6] Chemical synthetic strategies can be encompassed into sequential or convergent approaches (Scheme 1). The synthesis on resin via either the sequential (Scheme 1a) or convergent (Scheme 1b) approach often suffers from loss of precious material and low reaction yield.[3] In contrast, the convergent method in solution ("Lansbury aspartylation"[3b,c]) can be advantageous, and pseudoproline dipeptides incorporated into these peptide substrates significantly suppressed the formation of undesired aspartimides during the *N*-linked glycosylation.[7] However, in the practical synthesis of glycoproteins via ligation between glycopeptides and other peptide fragments, some inconveniences still remained, such as the use of metal catalysts[3m] or protection of C-terminal carboxylic acids.[3l] In this regard, we decided to develop an efficient convergent method for *N*-linked glycopeptides. Herein we report an efficient *N*-linked glycosylation reaction between the glycosylamines and *p*-nitrophenyl thioester peptides in solution.

Scheme 1. Strategies for *N*-Linked Glycosylation Reaction



In previous studies, activated C-terminal peptides with thiol esters[8] and *o*-benzaldehyde esters[9] have been utilized to directly condense with peptide fragments in the absence of an N-terminal cysteine residue, thiol auxiliary, or exogenous activating reagent. Aminolysis of the activated thioesters to form amide bonds was conducted smoothly, leading to the ligation products in good yields. We decided to investigate the strategy of incorporating the glycosylamine moiety into an active thiol ester of an Asp residue of the peptide.

Initially, we investigated the influence of different leaving groups of activated esters during the reaction with glycosylamine **1a**[10,3j,m] (*β*-anomer; Figure S44) to obtain the glycosyl product **3** (Table 1 and Figure 1). The reaction processes were monitored and analyzed by HPLC (Figure S1). In our study,

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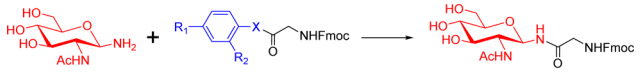
Table 1. *N*-Linked Glycosylation Reactions of Esters with Different Functional Groups[a]



1a 2 3

entry	ester	X	R_1	R_2	time (h)[b]	yield (%)[c]
1	**2a**	S	NO_2	H	4	73
2	**2b**	S	H	CHO	26	56
3	**2c**	S	CHO	H	42	42
4	**2d**	S	H	H	>60	15
5	**2e**	CH_2S	H	H	>60	2
6	**2f**	O	NO_2	H	60	34
7	**2g**	O	CHO	H	>60	3
8	**2h**	O	H	CHO	>60	7
9	**2i**	O	H	H	>60	<1

[a]Reaction conditions: **1a** (0.1 M), **2** (0.05 M), DIPEA (0.1 M), DMSO, rt. [b]Times for >95% conversion of the starting materials to the amide products at room temperature as determined by HPLC. [c]Yields were determined by HPLC at 60 h.

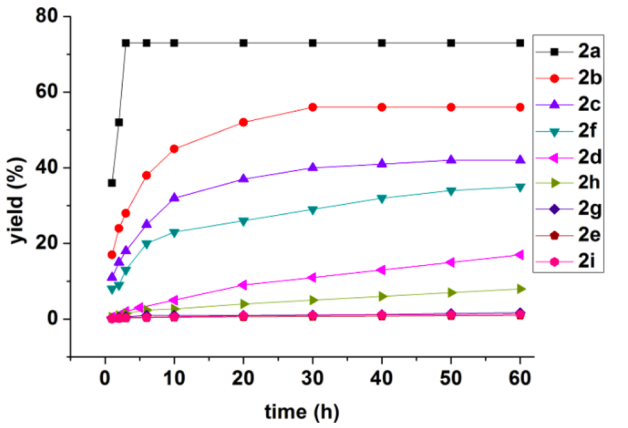
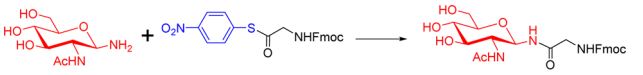


Figure 1. Yields of *N*-linked glycosylation product **3** vs time for different esters.

the reactivities of thioesters and oxoesters were compared; the influence of different substituents on the aromatic moiety was also investigated, i.e., *p*-nitro (electron-withdrawing group),[11] *o*-aldehyde (electron-withdrawing + neighbor-participating group),[9] and *p*-aldehyde (electron-withdrawing group)[9c] For thioesters (**2a**–**e**), **2a** containing a *p*-nitro group had the best reactivity and achieved the highest yield of product, which was followed by **2b** containing an *o*-aldehyde group and **2c** containing a *p*-aldehyde group; thioesters **2d** and **2e**, the common kind of activated esters usually employed to active C-terminal peptides in peptide and protein synthesis, have a lower reaction rate. For oxoesters (**2f**–**i**), the influence of different substituents on the reactivity has a similar tendency as for the thioesters (**2a**–**d**), but the reactivities of the oxoesters are too low (≥60 h for 95% conversion) to accommodate the *N*-linked glycosylation processes, even with prolonged time. Therefore, thioester **2a** containing a *p*-nitro group, which had the best reactivity and gave the highest yield of product, was the optimized activated ester used for *N*-linked glycosylation.

To further probe the efficiency of the *N*-glycosylation reaction, different conditions were investigated for thioester **2a** to react with **1a** (Table 2). In aqueous solution (entry 1), the predominant product was the hydrolysis product of **2a**. For different organic solvents (NMP, DMF, and DMSO), DMSO

Table 2. Optimization of the Reaction Conditions[a]



1a 2a 3

entry	1a	2a	solvent	base (equiv)	yield (%)[b]
1	2.0	1.0	DMSO/PB[c]	DIPEA (2.0)	6
2	2.0	1.0	NMP	DIPEA (2.0)	41
3	2.0	1.0	DMF	DIPEA (2.0)	60
4	2.0	1.0	DMSO	DIPEA (2.0)	73
5	2.0	1.0	DMSO	NMM (2.0)	61
6	2.0	1.0	DMSO	TEA (2.0)	64
7	2.0	1.0	DMSO	–	58
8	1.0	1.0	DMSO	DIPEA (2.0)	53
9	3.0	1.0	DMSO	DIPEA (2.0)	73

[a]Reaction conditions: **1a** (0.1 M), **2a** (0.05 M), base, rt, 4 h. [b]Yields were determined by HPLC. [c]PB = phosphate buffer (pH 7.4, 200 mM).

gave the best performance (entries 2–4). When diisopropylethylamine (DIPEA) was replaced with another base (4-methylmorpholine (NMM) or triethylamine (TEA)), the yield of product slightly decreased (entries 5 and 6). For the amount of DIPEA, 2.0 equiv relative to the thioester was suitable for the *N*-linked glycosylation reaction (Table S1, entries 4 and 7–10). For the amount of glycosylamine **1a**, the highest yield of **3** was obtained when 2.0 equiv of **1a** was added to thioester **2a** (Table 2, entries 4, 8, and 9). Therefore, the optimal conditions are to employ 2.0 equiv of glycosylamine **1a** and 2.0 equiv of DIPEA in DMSO at room temperature for 4 h.

With the optimized reaction conditions in hand, we prepared a range of peptides containing *ω*-Asp *p*-nitrophenyl thioesters (**4a**–**10a**) as the substrates for *N*-linked glycosylation. The peptides were prepared by solid-phase peptide synthesis and obtained in good yields after cleavage from the resin (the detailed procedure is available in the Supporting Information). As shown in Scheme 2, these substrates were conjugated with unprotected glycosylamines (**1a**–**c**)[10,12] to give *N*-linked glycopeptides (**4b**–**10b**, **4c**, **8c**, **9c**, **8d**, **9d**), and the desired coupling products bearing the amide bond linkage at *ω*-Asp were achieved in good yields. Notably, in this protocol just mixing two shelf-stable components affords the desired glycopeptide quickly; no other coupling reagents or catalysts are required, and the workup is simple. Furthermore, this coupling reaction is compatible with the free carboxylic acid group at the C-terminus of peptide substrates or the sialic acid of unprotected sialyloligosaccharides. The C-terminal free carboxylic group of glycopeptides can be directly converted into thioesters for further ligation with other peptide fragments. Using the unprotected complex-type sialyloligosaccharide[12] **1c** as the substrate greatly simplified the procedure for synthesizing naturally occurring *N*-linked glycopeptides and glycoproteins. The amino acid protecting groups in glycopeptides can be easily removed using the common trifluoroacetic acid deprotection protocol. For the orthogonal masking group Thz (**7b**), deprotection with methoxyamine hydrochloride gives free N-terminal Cys, which can be employed in the next reaction of native chemical ligation.

In light of these encouraging results, we continued to examine the applications of *N*-linked glycopeptide products by extending the glycopeptide sequence from either the N-terminus or the C-terminus via peptide fragment condensation (Scheme 3; boxed residues are potential ligation sites).

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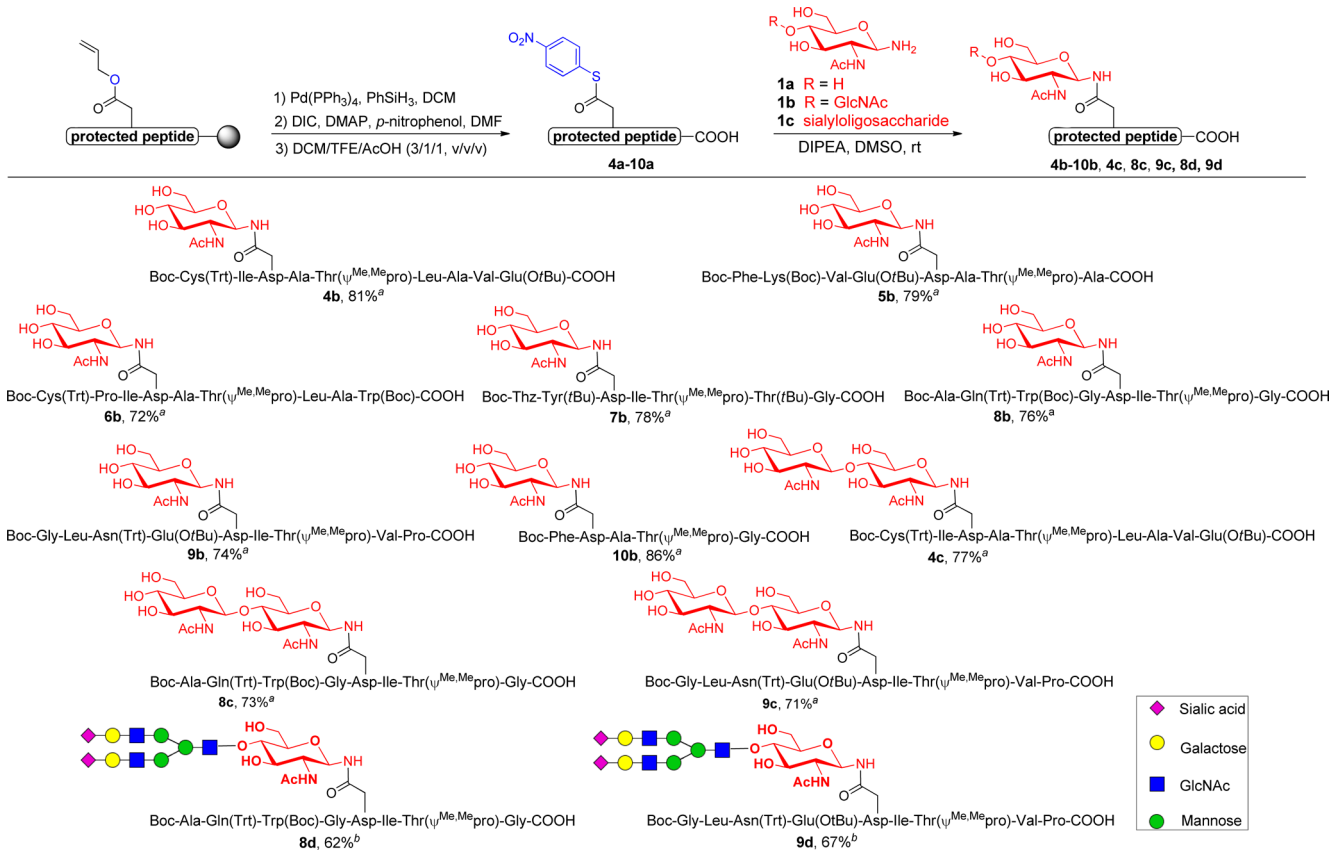
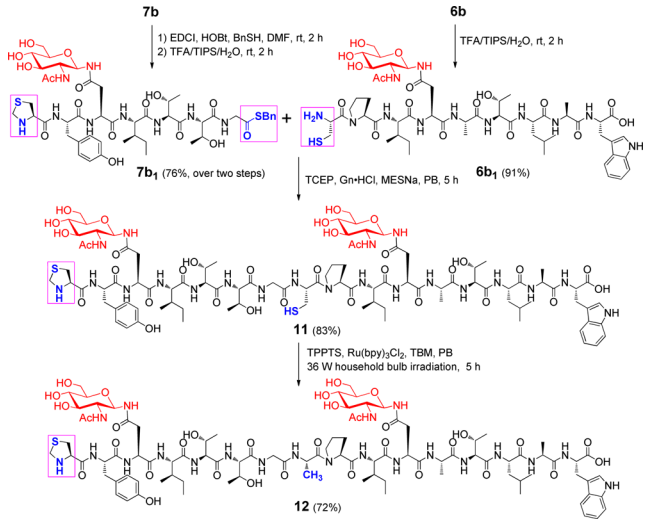
Scheme 2. Substrate Scope of the *N*-Glycosylation Reaction between Glycosylamines and *p*-Nitrophenyl Thioester Peptides[a]



[a]Reaction conditions: glycosylamine (**1a** or **1b**) (0.1 M), *p*-nitrophenyl thioester peptide (0.05 M), DIPEA (0.1 M), DMSO, rt, 4 h. [b]Reaction conditions: sialyloligosaccharide **1c** (2.7 mM), *p*-nitrophenyl thioester peptide (1.8 mM), DIPEA (3.6 mM), 4 Å MS, rt, 10 h. Yields of isolated products are shown.

Scheme 3. Condensation of *N*-Linked Glycopeptide Fragments through Ligation/Desulfurization



Glycopeptide $\mathbf{7b_1}$ was prepared from **7b** after conversion of the C-terminus into a thioester and deprotection; glycopeptide $\mathbf{6b_1}$ bearing an N-terminal Cys was prepared from **6b** after deprotection. Under standard native chemical ligation conditions, the C-terminus of $\mathbf{7b_1}$ and N-terminus of $\mathbf{6b_1}$ were selectively covalently joined to give the longer glycopeptide **11** in good yield (Scheme 3 and Figure S21). We recently developed a protocol of visible-light-induced specific desulfurization of cysteinyl peptides and glycopeptides at room temperature.[13] Under such conditions of visible light with $Ru(bpy)_3Cl_2$ and TPPTS, the thiol group of *N*-linked glycopeptide **11** was selectively removed to give glycopeptide **12** in good yield (Scheme 3 and Figure S22). It should be noticed that the orthogonal masking group Thz in **11** and **12** can be removed in the next step, endowing these glycopeptides with the ability to undergo further NCL from their N-terminus. In our vision, this *N*-linked glycosylation strategy can be extended to prepare various long biologically relevant glycopeptide and glycoprotein fragments.

In conclusion, we have demonstrated a convergent and facile synthesis of *N*-linked glycopeptides via combination of glycosylamines and *p*-nitrophenyl thioester peptides. This glycosylation protocol has the merits of simple operation and good yields. We believe that this method can be particularly interesting for the preparation of large *N*-linked glycopeptides and glycoproteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02288.

Detailed experimental procedures, HPLC traces, and ESI-MS and characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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