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Published on Web 02/16/2010

Study of On-Resin Convergent Synthesis of N-Linked Glycopeptides Containing a Large High Mannose N-Linked Oligosaccharide

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Abstract: Here we present a convergent on-resin glycosylamine coupling strategy for solid phase N-linked glycopeptide synthesis, and apply it to the synthesis of high mannose containing glycopeptides. In this strategy, the 2-phenylisopropyl protecting group is used as an orthogonal handle to create glycosylation sites on-resin after synthesis of nonglycosylated peptides. In addition to allowing selective deprotection of aspartic acid residues for creation of glycosylation sites, the 2-phenylisopropyl protecting group also efficiently suppresses aspartimide formation during peptide synthesis. The key step of on-resin glycosylamine coupling to an aspartic acid residue was first optimized for a small sugar, N-acetylglucosamine, and then applied to a much larger high mannose oligosaccharide, $Man_8GlcNAc_2$. Satisfying coupling yields were obtained for both small and large sugars. The use of on-resin glycosylamine coupling simplifies purification of N-linked glycopeptides, and also allows convenient recovery of unreacted valuable large oligosaccharides. This approach was applied to the solid phase synthesis of glycosylated forms of the 34 amino acid HIV-1 gp41 C34 glycopeptide, which is an HIV-1 entry inhibitor. The HIV-1 entry inhibition assay of synthesized glycopeptides showed the retention of bioactivity of high mannose $Man_8GlcNAc_2$−C34.

Introduction

Interest in understanding the specific effects of glycosylation and in developing glycopeptide-based vaccines and therapeutics stimulate the study of N-linked glycopeptide synthesis.[1] However, it is still a challenge to synthesize glycopeptides containing large N-linked oligosaccharides, not only because it is difficult to obtain sufficient amounts of the N-linked oligosaccharides themselves[2] but also because reactions coupling bulky N-linked oligosaccharides to peptides are often low yielding and prone to aspartimide formation.[3] Several strategies have been utilized for N-linked glycopeptide synthesis with each strategy having its own advantages and drawbacks.[4] Solid phase synthesis of N-linked glycopeptides using glycosylated asparagine (Asn) building blocks takes advantage of the benefits of solid phase peptide synthesis (SPPS), but is quite costly in terms of the amounts of oligosaccharides necessary to first synthesize the building blocks and then couple them to peptides.[5] Convergent strategies that utilize Lansbury aspartylation to couple N-linked glycosylamines[6a] to protected peptides in solution maximize the yield of glycopeptides produced based on the amount of N-linked oligosaccharide used.[6b−d] Enzymatic transglycosylation can also be used to synthesize N-linked glycopeptides in solution, although the preparation of the sugar oxazoline substrates is required for appreciable transglycosylation yields.[7] These in solution strategies allow N-linked oligosaccharides to be introduced at a late stage, but at the expense of introducing additional purification steps into the synthesis and eliminating some of the advantages of SPPS.

To combine the advantages of SPPS and convergent synthesis, researchers have utilized on-resin Lansbury aspartylation to couple glycosylamines to peptides on the solid phase. This strategy involves synthesizing a peptide by SPPS with orthogonally protected aspartic acid residues that can be selectively deprotected on-resin at the end of peptide synthesis. Subsequent on-resin coupling of a glycosylamine to the Asp side chain and resin cleavage results in the desired glycopeptide (Figure 1). This on-resin Lansbury aspartylation strategy has only previously been applied to the synthesis of glycopeptides containing

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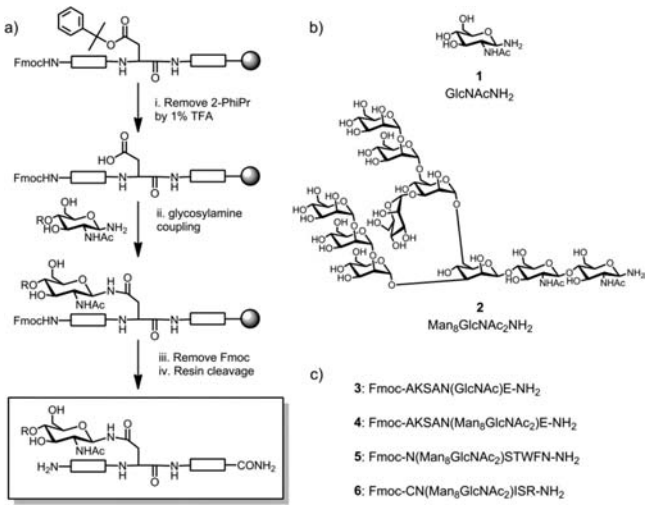

Figure 1. N-linked glycopeptide synthesis using on-resin Lansbury aspartylation. (a) synthetic strategy, (b) glycosylamines, and (c) glycopeptides.

monosaccharides and disaccharides, and can suffer from aspartimide formation difficulties during peptide synthesis.[8] Herein we report studies of on-resin convergent synthesis of N-linked glycopeptides containing a large high mannose oligosaccharide. To optimize the coupling of the costly N-linked oligosaccharide we utilized the aspartimide-prone peptide AKSANE, first working out conditions on the monosaccharide N-acetylglucosamine (glycosylamine ($GlcNAcNH_2$, **1**) and then applying those conditions to a high mannose oligosaccharide glycosylamine ($Man_8GlcNAc_2NH_2$, **2**).

Experimental Section

Materials and Equipment. All normally used amino acid building blocks and coupling reagents 3-(Diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT), *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′ tetramethyluronium Hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBt) for solid phase peptide synthesis were purchased from AAPPTEC. Methyl sulfoxide (DMSO, 99.9%), *N*-acetyl-D-glucosamine and *N*,*N*-Diisopropylethylamine (DIEA) were purchased from Sigma-Aldrich. Rink Amide PEGA resin (50−100 mesh, 0.20−0.50 mmol/g) and special Aspartic acid building blocks, Fmoc-Asp(O-2-PhiPr)−OH and Fmoc-Asp-(OAll)−OH were purchased from EMD Biosciences. The Pierce Economy Mini-Spin Column (0.8 mL resin capacity) from Thermo Scientific was used for on-resin coupling of glycosylamines. Solid phase peptide synthesis was carried out following standard Fmoc/tBu chemistry protocols on the Applied Biosystems 433A Peptide Synthesizer. Preparative RP−HPLC systems (hyper prep, 120 C18 8u; length, 250 mm; I.D., 10 mm) were used to purify peptides. Isolated yields refer to chromatographically pure compounds. Theoretical yields for peptide synthesis were calculated based on the first amino acid loadings of the resins, which were determined by the UV monitoring of the Fmoc removal at 301 nm ($\epsilon = 7800$ $cm^{-1}M^{-1}$).[15] Purity of purified peptides were determined by analytical HPLC at wavelength of 214 nm (0−80% B, 10 min, Beckman SGB 0.46 × 5 cm Zorbax C8 column, A buffer: Water, 0.1% TFA; B buffer: 90% Acetonitrile 10% Water 0.1% TFA). Lyophilization was carried on VirTis Sentry 2.0 lyophilizer. 60−200 mesh silica gel 62 (EMD) was employed for flash chromatography. TLC analysis was on silica gel plates (EMD Chemical, Si 60 F254) and stained by vanillin (15 g vanillin in 250 mL ethanol and 2.5 mL concentrated sulfuric acid.). The 400 and 500 MHz ^{1}H NMR was carried out on a Varian Inova NMR Spectrometer. Masses of peptides were determined either by a Bruker Autoflex III MALDI-TOF or an API III ESI Mass spectrometer. HRESI-TOF−MS of $Man_8GlcNAc_2$ was performed on a Waters/Micromass LCT Mass spectrometer.

General Procedure for the Synthesis of Glycosylamines. A total of 20−200 mg free sugar (N-acetyl-D-glucosamine or $Man_8GlcNAc_2$) were dissolved in 25 mL saturated NH_4HCO_3 in water and stirred for 5−6 days at room temperature. The reaction solution was diluted with water and rotary evaporated to dryness. This procedure was repeated several times to remove the residual ammonium hydrogen carbonate. Then, the sample was dissolved in water and lyophilized. The lyophilization was repeated until the sample reached constant mass. ^{1}H NMR was employed to monitor the conversion of free sugars into their corresponding β-D-glycosylamines.

General Procedure for Peptide Synthesis. Five equivalents of Fmoc-amino acids, DEPBT, and 3 equiv. of DIEA were used for manually loading of Rink Amide PEGA resin. Synthesis of fully protected peptides was carried out following standard Fmoc/tBu chemistry protocols on the Applied Biosystems 433A Peptide Synthesizer. For the glycosylation site, Fmoc-Asp(O-2-PhiPr)−OH or Fmoc-Asp(OAll)−OH was used. All of the other amino acids side chains were protected by most commonly used protecting groups, including *t*Bu for Tyr, Ser, Thr, Glu, and Asp, Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) for Arg, trt (trityl) for Gln, Asn and Cys, His, Boc for Trp. The Fmoc protecting group was removed by 20% piperidine in DMF (10 min × 3). The loading for the amino acid was determined by UV monitoring of the Fmoc removal at 301 nm ($\epsilon = 7800$ $cm^{-1}M^{-1}$).[15] The capping of unreacted amino groups was carried out using a large excess of acetic anhydride with 3 equivalents of *N*-methylmorpholine in DCM. The cleavage of resin was accomplished with neat TFA containing 1% TIS scavenger for 3 h. The crude peptide was precipitated and washed twice by diethyl ether anhydrous before RP-HPLC purification.

General Procedure for on-Resin Coupling of Glycosylamines. In a typical coupling reaction, fully protected peptides with Asp(O-2-PhiPr) at the N-glycosylation site of Asn were synthesized on Rink Amide PEGA resin. The PhiPr protecting group was selectively removed by the treatment of 94:1:5 DCM/TFA/TIS, 2 min repeated 4 times. After washing with DCM and DMF thoroughly, the resin was transferred into the reaction column and preswelled with DMSO. Different and various equivalents of glycosylamines, corresponding equivalents of coupling reagents and bases in DMSO were added. The total reaction volume is typically less than one-third of the column capacity. The reaction column was attached to a rotator (30 rpm) and the coupling was conducted typically for 12 h. For analytical HPLC analyzed trials, the N-terminus Fmoc was left on in order to absorb at wavelength of 254 nm. For C34 analog production, the N-terminus Fmoc was cleaved by 20% piperidine in DMF (10 min × 3). Peptides then were cleaved from resin by treatment with neat TFA containing 1% TIS scavenger for 3 h. Crude peptides after resin cleavage were precipitated and washed twice by diethyl ether anhydrous and then analyzed by analytical HPLC or purified by RP−HPLC.

General Procedure for Recovery of Unreacted High Mannose Glycan. Reaction solutions in DMSO from different $Man_8GlcNAc_2NH_2$ on-resin coupling synthesis trials were pooled together and precipitated by diethyl ether anhydrous. The precipitate was then redissolved in a loading buffer (10:3:3 ethyl acetate/methanol/water) and purified by silica gel flash chromatography (ethyl acetate/methanol/ water, 10:3:3 to 5:3:3). Fractions containing glycan were detected positively by phenol-sulfuric acid test,[16] then pooled together and lyophilized to give $Man_8GlcNAc_2$, yield 78% (based on the extra equivalents of $Man_8GlcNAc_2$ in the pooled reactions).

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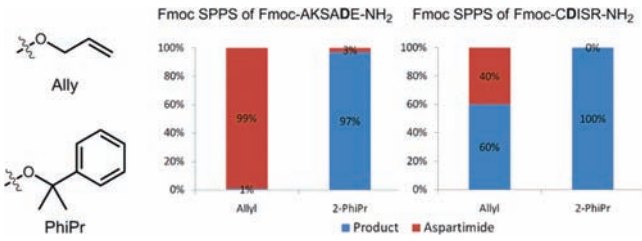

Figure 2. Aspartimide formation during Fmoc SPPS of peptides Fmoc-AKSADE-NH_2 and Fmoc-CDISR-NH_2 using either Allyl or PhiPr orthogonal protecting groups to protect Asp residues.

Analytical HPLC for on-Resin Glycosylation Yields. Analytical HPLC (0−80% B, 10 min, Beckman SGB 0.46 × 5 cm Zorbax C8 column, A buffer: Water, 0.1% TFA; B buffer: 90% Acetonitrile 10% Water 0.1% TFA, detection at 254 nm). Individual peaks were identified by mass spectrometry and product distribution (glycopeptide: uncoupled peptide: aspartimide) was determined by the integrated analytical HPLC absorption signal using Peaksimple 2000 version 2.83 (SRI Instruments).

In Vitro HIV-1 Entry Inhibition Assay. TZM-bl and HL2/3 cells were obtained from the NIH AIDS Research and Reference Reagent Program.[13] A luciferase-based cell−cell fusion assay was performed as previously described.[13a] The target cells, TZM-bl cells, express CD4 and CCR5 and are cotransfected with a luciferase reporter gene linked to the HIV-1 promoter which can be activated by HIV-1 Tat from the effector HL2/3 cells. Cells were cultured in DMEM medium containing 10% FBS, 100 units of Penicillin and 0.1 mg/mL Streptomycin at 37 °C, 5% CO_2. In the assay, serial dilutions of C34 peptide analogs (20 μL per well) were added to a 96-well Microplate (Perkin-Elmer, Isoplate-96 TC) containing TZM-bl cells (40 μL per well, 2.5×10^4 cells per well, seeded and incubated in 37 °C, 5% CO_2 overnight). Then, the HL2/3 cells (40 μL per well, 2.5×10^4 cells per well) were added and the plate was incubated for 6 h at 37 °C, 5% CO_2. After incubation, LucLite luminescence substrate reagent (100 μL per well, Perkin-Elmer) was added and the plate was shaken for 3 min at 600 rpm before reading. Light output was measured on MicroBeta-1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). IC_{50} values were calculated by Origin software (OriginLab).

Results and Discussion

In our initial studies of on-resin convergent synthesis of N-linked glycopeptides, we attempted to use the allyl protecting group for selective on-resin deprotection of Asp side chains, but as others[8] have reported, we observed significant aspartimide formation during Fmoc SPPS (Figure 2 and Figure S1−4 of the Supporting Information). The amount of aspartimide formation that occurs during Fmoc SPPS renders the allyl protecting group unsuitable for on-resin N-linked glycopeptide synthesis without amide backbone protection.[8] Since amide backbone protection can introduce additional complications into peptide syntheses, we sought a protecting group that could be used for on-resin N-linked glycopeptide synthesis without backbone protection, but which would also be compatible with backbone protection if desired. It has been previously noted by Offer et al.[8] that the sterically hindered *t*-butyl ester suppresses aspartimide formation under conditions where the allyl protecting group does not. Because of this, we explored the use of the sterically hindered 2-phenylisopropyl (PhiPr)[9] protecting group for this application, which can be selectively deprotected unlike *t*-butyl esters. Although a previous work reported that this more bulky protecting group was slightly more susceptible to aspartimide formation than standard *t*-butyl esters in their aspartimide prone peptide syntheses,[9] the PhiPr protecting group performed very well in our trial peptide syntheses. The PhiPr protecting group even suppressed aspartimide formation in the highly aspartimide prone AKSADE peptide (Figure 2 and Figure S1−2 of the Supporting Information), and allowed selective, on-resin deprotection in 1% trifluoroacetic acid (TFA). These properties make PhiPr an excellent orthogonal handle for on-resin introduction of N-linked glycosylation.

The other important element for glycopeptide synthesis, glycosylamines **1** and **2**, were prepared from the corresponding free sugars, *N*-acetyl-D-glucosamine and the high mannose N-linked oligosaccharide $Man_8GlcNAc_2$, by treatment with ammonium hydrogen carbonate as described previously.[6a] Repetitive rotary evaporation and lyophilization were utilized to remove the residual ammonium hydrogen carbonate, which if left in the sample could disrupt the glycosylation reaction. ^{1}H NMR spectra (Figure S5−6 of the Supporting Information) showed the conversion of free sugars into their corresponding β-D-glycosylamines with close to 100% yields.

Next, the glycosylation reaction was optimized for glycosylamines **1** and **2** using the aspartimide-prone sequence AKSANE. In order to quantify the glycosylation yields in these experiments, N-terminal Fmoc protecting groups were left on at the end of peptide synthesis to aid UV detection. After resin cleavage from each of the synthesis trials, crude peptides were precipitated and washed twice with anhydrous diethyl ether, and then analyzed by analytical HPLC. Individual peaks detected at 254 nm were identified by mass spectrometry and product distribution (glycopeptide: uncoupled peptide: aspartimide) was determined by the integrated analytical HPLC absorption signals. Example analytical HPLC traces from different synthesis trials are shown in Figure 3, and the results of synthesis trials analyzed in this manner are summarized in Table 1.

Using the approach described above, PEG-polyacrylamide-based (PEGA) and polystyrene-based (PS) rink amide resins were tested as solid supports for glycopeptide synthesis (entries 1, 2, 9, and 10, Table 1). Excellent glycosylation yields were achieved on both PS and PEGA resins with the monosaccharide glycosylamine **1** (80% and 87%, respectively). In contrast, for coupling with the high mannose oligosaccharide **2**, no glycosylation at all occurred on PS resin and a 43% glycosylation yield was achieved on the more hydrophilic PEGA resin.[5a,10] Because of this, PEGA resin was utilized as the solid support for on-resin glycosylamine coupling in the rest of this study. Due to the poor solubility of large oligosaccharides in most organic solvents, we explored the use of DMSO/water mixtures in addition to the neat DMSO that has been reported previously for in solution glycosylamine coupling reactions.[6] Unfortunately, DMSO/water mixtures resulted in a significant increase in aspartimide formation for monosaccharide **1** and no product formation at all for oligosaccharide **2** (see entries 2, 4, 11, and 12 of Table 1), therefore neat DMSO was utilized in the remainder of this study. The selection of coupling reagent is also crucial to achieve high glycosylation yields. We found that DEPBT, 3-(Diethoxyphospho-ryloxy)-1,2,3-benzotriazin-4(3H)-one, was more efficient than HBTU (O-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium Hexafluoro-phosphate) and slightly

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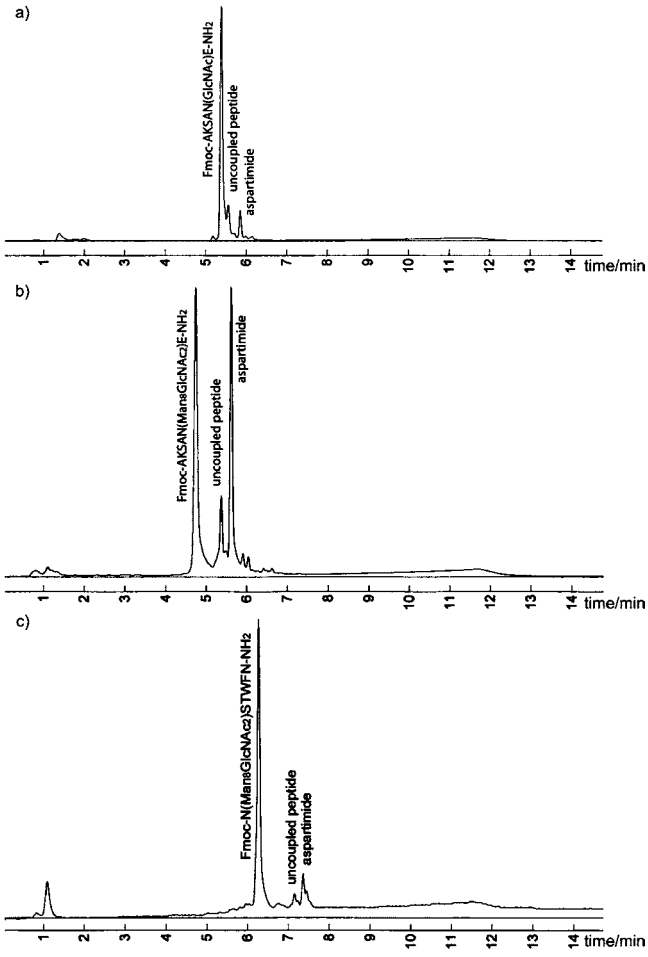

Figure 3. Analytical HPLC trace examples for on-resin glycosylation (detection at 254 nm); (a) glycosylamine **1** coupling to produce glycopeptide **3**, crude sample from synthesis entry 5, Table 1; (b) glycosylamine **2** coupling to produce glycopeptide **4**, crude sample from synthesis entry 10, Table 1; and (c) glycosylamine **2** coupling to produce glycopeptide **5**, crude sample from synthesis entry 16, Table 1.

more efficient than HBTU/HOBt(1-hydroxybenzotriazole) in on-resin glycosylation of glycosylamine **1** (see entries 2, 7, and 8 of Table 1). Because DEPBT has also been reported as a coupling reagent selective for amide bond formation with great resistance to racemization,[11] DEPBT was chosen as the on-resin coupling reagent for use with the large glycosylamine **2**. We also investigated the effect of the presence of base on glycosylation yield since it has been reported that omission of base can reduce aspartimide formation.[8] The omission of base during coupling of glycosylamine **1** (Table 1 entry 3) did not have much of an effect on glycosylamine coupling efficiency, but removing base from the coupling of glycosylamine **2** (Table 1, entries 11, 13, 14, and 15) resulted in significant reduction in coupling efficiency making it desirable to include base in reactions containing the large glycosylamine **2**. Investigation into the amount of glycosylamines utilized in on-resin coupling reactions suggested that changing the amount of glycosylamine **1** (entries 2, 5, and 6) did not have significant effects on coupling yields (87%, 84%, and 83% respectively). The amount of glycosylamine **2**, however, did appear to have a significant effect on glycopeptide yield, with 1.5 and 3 equiv. of glycosylamine **2** resulting in coupling yields of 49% and 58%, respectively (Table 1 entries 11 and 14). This result agrees with studies of in-solution coupling of bulky, large sugar glycosylamines,[3c] where the aspartimide formation side reaction has been found to compete with glycosylation to reduce overall glycosylation yields. Using the optimized reaction conditions developed here, shown in Table 1 entry 14, an isolated yield[12] of 50% was achieved for the synthesis of the aspartimide prone, high mannose N-linked glycopeptide **4**.

To explore how these on-resin glycosylation conditions worked with other small glycopeptide sequences, we applied this on-resin coupling strategy to the synthesis of high mannose containing glycopeptides **5** and **6** (Table 1, entries 16 and 17) obtaining excellent glycosylation yields of 86% and 53% respectively. Taken together, under similar reaction conditions we have observed glycosylamine coupling yields ranging from 49% to 86% for different peptide sequences, indicating that the coupling efficiency of large glycosylamines may be affected by sequence.

An advantage of the on-resin approach taken here is the possibility of easy recovery of valuable unreacted oligosaccharides after on-resin coupling. This is possible because of the hydrophilic nature of oligosaccharides, which allows them to be easily distinguished from the base and coupling reagents used

Table 1. Optimization of on-Resin Glycosylation with Glycosylamines **1** and **2**

entry[a] (glycopeptides)	resin	solvent	sugar-NH_2 (equiv)	coupling reagents (equiv)	DIEA (equiv)	product distribution %[b] glycopeptide: uncoupled peptide: aspartimide
1 (**3**)	PS	DMSO	**1** (5)	DEPBT (3)	1.5	80: 7: 13
2 (**3**)	PEGA	DMSO	**1** (5)	DEPBT (3)	1.5	87: 9: 4
3 (**3**)	PEGA	DMSO	**1** (5)	DEPBT (3)	0	88: 9: 3
4 (**3**)	PEGA	DMSO/water (4/1)	**1** (5)	DEPBT (3)	1.5	69: 5: 26
5 (**3**)	PEGA	DMSO	**1** (3)	DEPBT (3)	1.5	84: 9: 9 (75%[c])
6 (**3**)	PEGA	DMSO	**1** (1)	DEPBT (3)	1.5	83: 3: 14
7 (**3**)	PEGA	DMSO	**1** (5)	HBTU (3)	1.5	79: 4: 17
8 (**3**)	PEGA	DMSO	**1** (5)	HBTU/HOBt (3/3)	1.5	84: 6: 10
9 (**4**)	PS	DMSO	**2** (1)	DEPBT (3)	1.5	0: 39: 61
10 (**4**)	PEGA	DMSO	**2** (1)	DEPBT (3)	1.5	43: 8: 49 (36%[c])
11 (**4**)	PEGA	DMSO	**2** (1.5)	DEPBT (3)	1.5	49: 3: 48
12 (**4**)	PEGA	DMSO/water (4/1)	**2** (1.5)	DEPBT (3)	1.5	0: 28: 72
13 (**4**)	PEGA	DMSO	**2** (1.5)	DEPBT (3)	0	31: 4: 65
14 (**4**)	PEGA	DMSO	**2** (3)	DEPBT (3)	1.5	58: 7: 35 (50%[c])
15 (**4**)	PEGA	DMSO	**2** (3)	DEPBT (3)	0	43: 3: 53
16 (**5**)	PEGA	DMSO	**2** (1.5)	DEPBT (3)	1.5	86: 5: 9 (76%[c])
17 (**6**)	PEGA	DMSO	**2** (1.5)	DEPBT (3)	1.5	53: 11: 36 (43%[c])

[a] Synthesis: scale 1−5 μmol; coupling reagent and glycosylamine equivalents based on loading values reported by the resin manufacturer; DIEA equivalents based on amounts of glycosylamine added; reaction time $\approx$ 12 h. [b] Product distributions for crude peptides were determined by integrated analytical HPLC absorbance signals at 254 nm. [c] Isolated yields based on Fmoc quantification for the first amino acid loaded.

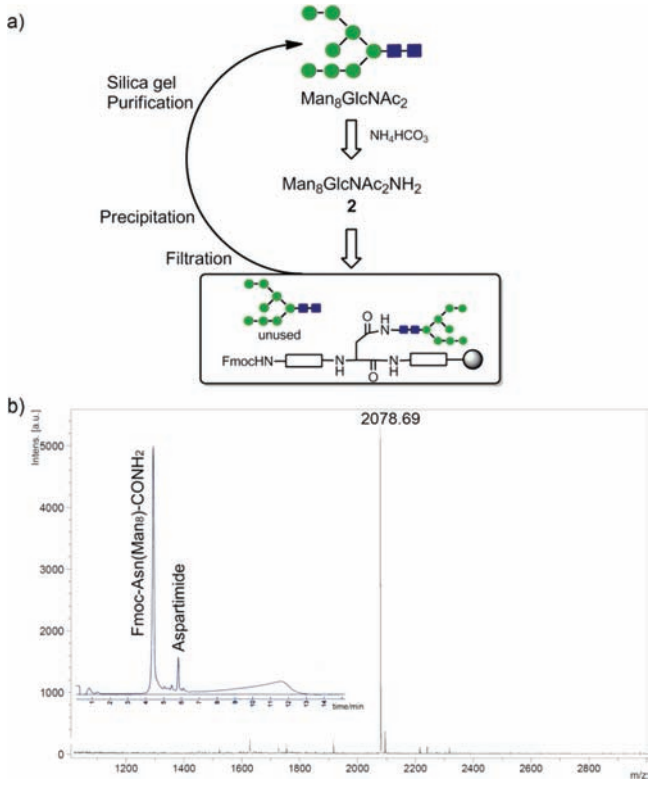

Figure 4. Recovery and reuse of the unreacted, excess high mannose oligosaccharide $Man_8GlcNAc_2$ after on-resin coupling. (a) Process scheme; (b) MALDI-TOF−MS and analytical HPLC for crude sample from on-resin coupling of recovered and reproduced glycosylamine **2** to rink amide PEGA resin bound Fmoc-Asp, expected mass for Fmoc-Asp($Man_8GlcNAc_2$)-$CONH_2$, $[M + Na]^+ = 2078.71$.

in on-resin coupling reactions. We have investigated the recovery of unused high mannose oligosaccharide **2** from our syntheses. After on-resin coupling of glycosylamine **2**, the coupling solution containing unused **2** was collected by filtration from the reaction column, precipitated and washed twice with anhydrous diethyl ether. The resulting precipitate was then purified by silica gel flash chromatography, providing high mannose oligosaccharide $Man_8GlcNAc_2$ with the approximate yield of 78% of the excess glycosylamine **2** utilized in on-resin coupling reactions. The ^1H NMR spectra (shown in Figure S7 of the Supporting Information) of the recovered high mannose oligosaccharide was identical to the starting material $Man_8GlcNAc_2$ (also shown in Figure S7 of the Supporting Information), indicating the success of recovery, and also the conversion of the glycosylamine back into the free sugar form during the purification process. To demonstrate reuse of the recovered material, we further converted it back into glycosylamine **2** using ammonium hydrogen carbonate, and tested its coupling reactivity with rink amide PEGA resin bound Fmoc-Asp and Fmoc-Glu with free side chain carboxylic acids. As shown in Figure 4 and Figure S8 of the Supporting Information, the recovered glycosylamine **2** provided excellent coupling yields, above 85% for both on-resin couplings. Using this approach we have routinely been able to recover and reuse valuable unreacted oligosaccharides from on-resin glycosylation reactions. This allows the use of excess oligosaccharide glycosylamines to drive on-resin glycosylation reactions without complete loss of the unreacted, excess oligosaccharide.

To demonstrate that this approach works for large N-linked glycopeptides as well as small glycopeptides, it was utilized to synthesize different glycosylated forms of the 34 amino acid HIV-1 gp41 C34 glycopeptide which is active as a HIV entry inhibitor (Figure 5). The on-resin glycosylamine coupling step was conducted in DMSO using 3 equiv. of DEPBT, 1.5 equiv. of DIEA, and 3 equiv. of glycosylamine **1** or **2** respectively for the synthesis of GlcNAc-C34 **8** and $Man_8GlcNAc_2$−C34 **9**. Excellent isolated yields[12] of 17% and 10% were obtained for GlcNAc-C34 **8** and $Man_8GlcNAc_2$−C34 **9** respectively (Figure 5). To determine if the C34 glycopeptides retained their anti-HIV activities they were tested by a luciferase-based cell−cell fusion assay[13] (Figure 6). The estimated IC_{50} values for C34 **7**, GlcNAc-C34 **8**, and $Man_8GlcNAc_2$−C34 **9** were 3.7, 5.2, and 8.2 nM, respectively. Although the inhibitory activity of C34 was reduced slightly by glycosylation, these results are similar to what has been observed in previous studies of glycosylated analogues of C34.[14]

Conclusions

In conclusion, we have successfully developed an efficient convergent glycosylamine coupling approach for solid phase

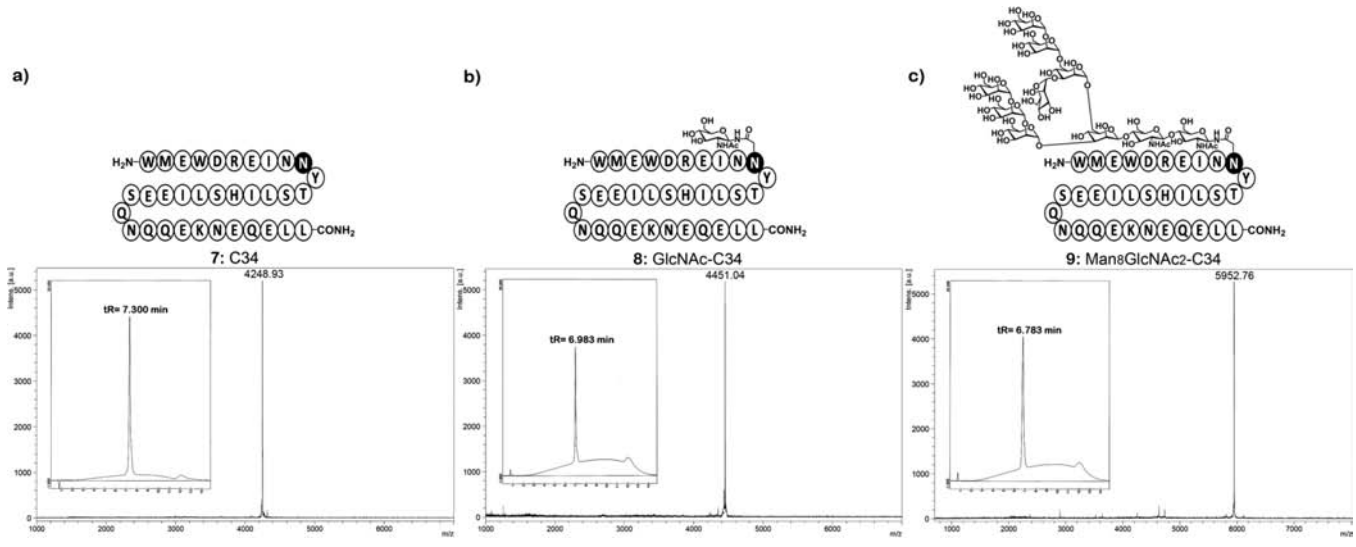

Figure 5. MALDI-TOF−MS and analytical HPLC for Synthetic HIV-1 gp41 C34 Analogues: (a) C34, **7**, expected mass:$[M + H]^+ = 4248.58$; (b) GlcNAc-C34, **8**, expected mass: $[M + H]^+ = 4451.77$; (c) $Man_8GlcNAc_2$−C34, **9**, expected mass: $[M + H]^+ = 5952.09$.

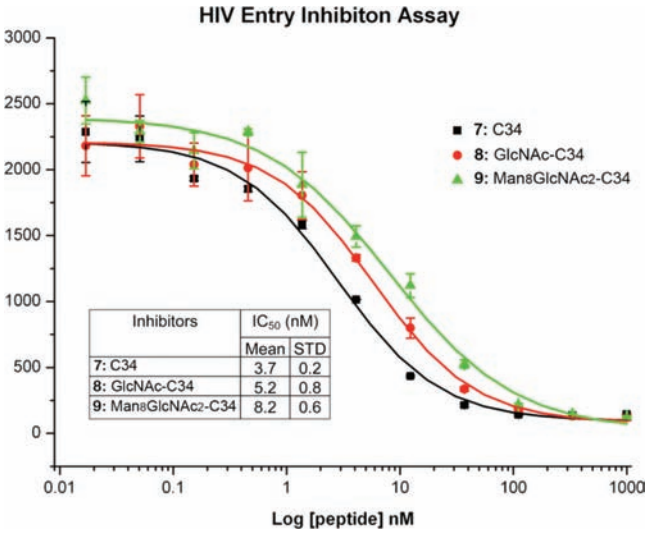

Figure 6. HIV-1 entry inhibition assay (luciferase-based cell−cell fusion assay[13]) and estimated IC_{50} values for nonglycosylated and glycosylated HIV-1 gp41 C34 analogs.

synthesis of N-linked glycopeptides that utilizes PhiPr protected side chains to generate N-linked glycosylation sites on-resin. The PhiPr protecting group is ideal for on-resin N-linked glycopeptide synthesis, suppressing aspartimide formation during peptide synthesis and allowing selective deprotection of Asp side chains for glycosylamine coupling. On-resin glycosylamine coupling allows glycosylation reactions to be driven by an excess of glycosylamines, with the possibility of high-yield recovery of unused valuable oligosaccharides. We have observed significant differences between on-resin coupling behavior of the monosaccharide **1** and the large oligosaccharide **2**, emphasizing that care must be taken when adapting reaction conditions worked out on monosaccharides to large oligosaccharides. This strategy allows the rapid and efficient synthesis of biologically active N-linked glycopeptides by SPPS such as the 34 amino acid C34 HIV entry inhibitor glycopeptides shown here.

Acknowledgment. This work was supported by Indiana University. We thank Dr. Richard DiMarchi for his generous support for SPPS. We also thank Mr. Junpeng Xiao for helping with the HIV-1 entry inhibition assay. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HL2/3 from Dr. Barbara K. Felber and Dr. George N. Pavlakis; TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc.

Supporting Information Available: Effects of Asp orthogonal protecting groups on the quality of Fmoc SPPS,^{1}H NMR for glycosylamine conversions, recovery of unreacted $Man_8GlcNAc_2$, characterizations of peptides and high mannose glycan $Man_8GlcNAc_2$. This material is available free of charge via the Internet at http://pubs.acs.org.

JA9104073

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