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A Practical, Convergent Method for Glycopeptide Synthesis

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Abstract: Glycopeptides are useful compounds to model the conformational effects of the biosynthetic glycosylation of asparagine (N) residues in glycoproteins. We report herein a practical, convergent method for the synthesis of N-glycopeptides. The key reaction involves the acetylation of a β-glycosyl amine with a partially protected peptide. Commercially-available protected amino acids and peptide-synthesis resin are used. The β-glycosyl amine can be derived from any reducing sugar by a simple procedure. Optimized experimental protocols are included for each step. Several glycosylations involving complex and acid-sensitive oligosaccharides are reported, including the coupling of a heptasaccharide (**8**) with a pentapeptide (**14**) in 55% purified yield.

Introduction

Many secreted and cell-surface proteins are modified by the covalent attachment of a carbohydrate to an asparagine (Asn, N) residue via a β-N-glycosidic linkage (Figure 1).[2] The structures of N-linked oligosaccharides fall into three basic types: high-mannose, complex, and hybrid oligosaccharides. All of these contain the common pentasaccharide core (Man)$_3$-(GlcNAc)$_2$ but differ in the nature of the outer residues.[3-5] The effects of these sugars both on the folding of the protein and on its final structure constitute areas of significant interest.[4,6-10] N-glycopeptides are often used as models for studying these interactions,[11-15] and therefore, a convenient route to these compounds would be of great value.

Synthesis of N-glycopeptides has been carried out most often by the stepwise approach, in which a glycosyl amine is coupled to a suitably-protected Asp derivative to give an Asn(Sug) derivative, which is then deprotected and elongated to give the desired glycopeptide.[16-18] There are several solid-phase methods available which utilize this approach.[19-25] These methods suffer from two major disadvantages related to the introduction of the sugar at an early stage in the synthesis. First, some of the *O*-glycosidic bonds present in complex oligosaccharides are not completely stable to the acidolytic deprotection conditions normally used in peptide synthesis. Although the methods mentioned above have been designed to minimize the exposure of the glycosidic bonds to acid, they all require a trifluoroacetic acid (TFA) treatment step for resin cleavage or side-chain deprotection. While this may be acceptable for certain oligosaccharides, it is likely to lead to *O*-glycosidic bond cleavage in some cases, particularly for the more sensitive linkages. Second, the early introduction of the sugar means that several equivalents of sugar are needed and that, because the sugar must survive additional amino acid coupling steps and deprotection, the overall yield from the oligosaccharide to the glycopeptide is low. This may be acceptable for monosaccharides or oligosaccharides which are available in large amounts but may be an impediment to the synthesis of glycopeptides containing complex oligosaccharides which are available from synthetic or natural sources in only small quantities. In the convergent strategy reported herein, the sugar must endure, at the most, two mild deprotection steps.

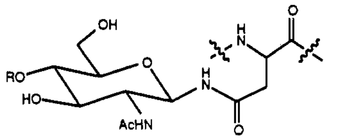

Figure 1. Structure of the carbohydrate–Asn linkage of N-glycoproteins. R = (Man)$_5$(GlcNAc) plus outer residues. In some cases, a fucose is α-linked to the 6-OH of the "inner" GlcNAc.

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(2) Standard one- and three-letter abbreviations for amino acids and protective groups are used. Other abbreviations are noted in the text, with the following exceptions: AAA, amino acid analysis; CIMS, chemical ionization mass spectrometry; Cl_2Bn, 2,6-dichlorobenzyl; DCHA, dicyclohexylammonium; DIEA, *N,N'*-diisopropylethylamine; DMF, *N,N'*-dimethylformamide; DMSO, dimethyl sulfoxide; DNP, dinitrophenyl; equiv, equivalent(s); FABMS, fast atom bombardment mass spectrometry; Fuc, fucose; GlcNAc, 2-acetamido-2-deoxyglucopyranose; $GlcNAcNH_2$, 2-acetamido-1-amino-1,2-dideoxyglucopyranose); HPLC, high-performance liquid chromatography; Man, mannose; PDMS, plasma desorption mass spectrometry; Sug, sugar (general); Tos, *p*-toluenesulfonyl.

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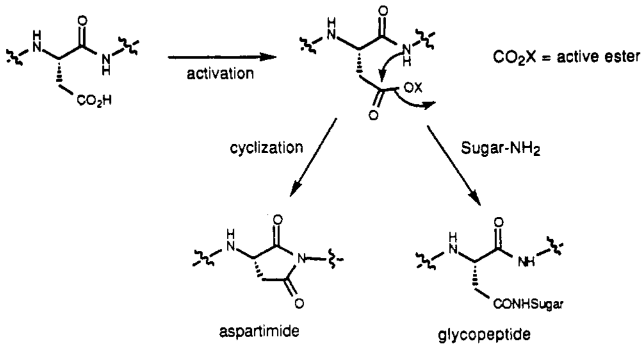

Figure 2. Aspartimide formation from an activated peptidyl Asp side chain. The rate of this reaction depends on the adjacent (*C*-terminal) amino acid.[31-33]

As an alternative to these methods, we have proposed a convergent approach, based on the coupling of the carbohydrate amine to an Asp-containing, partially-protected peptide.[11,12,26-29] The introduction of the sugar in a late step requires less material and avoids exposure of the oligosaccharide to acidic conditions. In addition, the convergent approach allows the synthesis of a series of glycopeptides containing different oligosaccharides, without the need to resynthesize the peptide for each individual case.

In order to make the convergent approach a viable alternative to the stepwise strategy, three problems must be solved.[30] First, since glycosylation of a peptide is expected to be slower and more difficult than glycosylation of an amino acid, especially when the sugars are large, a potent coupling reaction is needed in order to carry out a high-yield glycosylation of a peptide. Second, when a peptidyl Asp side chain is activated for glycosylation, there is the potential for a competing, relatively-facile intramolecular reaction, namely, cyclization to the succinimide[31-33] (Figure 2); this side reaction must be minimized. Third, a protective group scheme must be developed which allows selective deprotection of one Asp residue, with other protective groups remaining intact. After the glycosylation, the other protective groups must be removed in a mild manner.

In a previous paper,[26] we began to deal with the first two of these issues by reporting the optimization of a simple glycosylation to minimize succinimide formation and maximize yield. The present paper discusses the synthesis of appropriately-protected peptides for glycosylation as well as the synthesis of the other partner in the glycosylation reaction, the β-glycosyl amine. In addition, the optimization of several complex glycosylation reactions is reported as well as the utilization of the convergent approach for the synthesis of a variety of glycopeptides, including glycopeptides containing acid-sensitive and precious oligosaccharides, compounds which would be particularly difficult to synthesize by the stepwise approach. The glycopeptides which have been synthesized according to our strategy are among the most complex members of this class yet prepared by chemical synthesis.

Results and Discussion

Synthesis of β-Glycosyl Amines. β-Glycosyl amines have generally been synthesized by reduction of the corresponding azides.[28,34-36] More recently, several groups[24,37-39] have begun to use the much simpler approach introduced by Kochetkov,[40] in which the reducing oligosaccharide is treated for an extended period of time with saturated aqueous ammonium bicarbonate to afford exclusively the β-isomer of the corresponding amine. In addition to the glycosyl amine, the crude product of this reaction usually contains some starting material and, in some cases, side products, such as the diglycosyl amine.[24,37,40,41] Because of the instability of the glycosyl amine, purification is undesirable and, thus, this crude product has been used directly in glycosylations.[24,37]

It is important to determine the amount of glycosyl amine present in the crude reaction mixture, especially in the case of precious sugars. In addition, there is a need for a way to confirm that all the NH_3 from the reaction mixture has been removed, since any that remains will produce the undesired Asn-containing peptide. We have developed an HPLC assay which meets both these needs. After workup, a measured amount of the crude glycosyl amine is coupled to a known amount of Boc-Asp-OBn, using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[42] as the coupling reagent. Quantitation by HPLC of the ratio of Boc-Asn(Sug)-OBn, Boc-Asn-OBn, and Boc-Asp-OBn provides an estimate of the amount of glycosyl amine and the amount of ammonia present in the crude product. This HPLC assay is an indirect measure whose success depends on the efficiency of the glycosylation of Boc-Asp-OBn (we have, in several cases, measured 80–90% conversion to glycosyl amine).

With this analytical method in hand, we set out to prepare several glycosyl amines for glycopeptide synthesis. The desired glycosyl amines are shown in Figure 3. $GlcNAcNH_2$ (**1**) is commercially available. Chitobiose constitutes the disaccharide core of N-linked sugars; the peracetylated compound **2** is commercially available. Fucα1-6GlcNAc was of interest to us because the addition of a fucose to the interior GlcNAc of the core structure is a biological event whose consequences are unclear.[3-5] In addition, the Fucα1-6 linkage is a very acid-sensitive one, so that glycopeptides containing this sugar cannot be easily prepared by nonconvergent methods.[43] The peracetylated disaccharide **5** was synthesized from L-fucose and GlcNAc-OBn by a modification[30] of the literature procedure.[44-46] The heptasaccharide $(Man)_5(GlcNAc)_2$ (**7**), which occurs in many high-mannose glycoproteins, was made available to us by Dr. Christopher Warren of Massachusetts General Hospital. This compound is isolated from the urine of sheep with swainsonine-induced α-mannosidosis, a disease in which the catabolism of mannose-containing gly-

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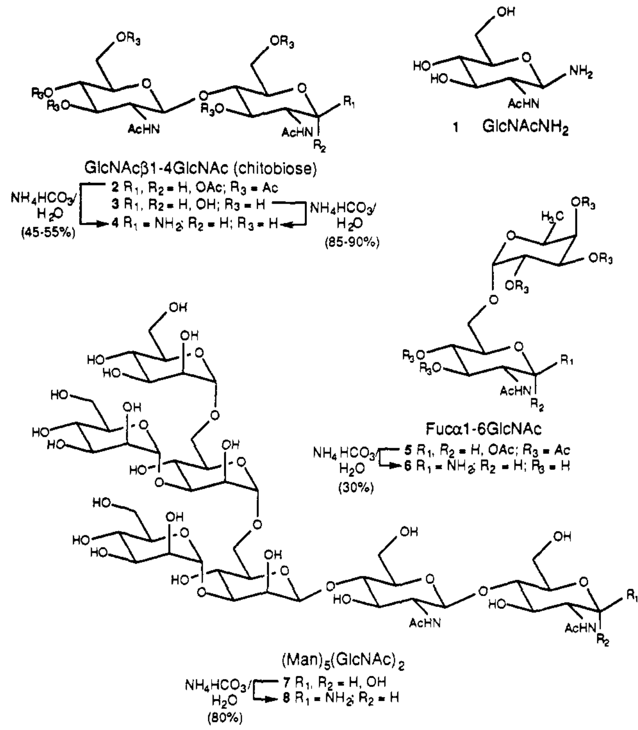

Figure 3. Glycosyl amines used in the glycopeptide synthesis. Numbers in parentheses are conversions to glycosyl amine, as measured by the HPLC assay described here.

coproteins is impaired, causing the buildup of mannose-containing oligosaccharides.[47,48]

These sugars were converted to the amines using the Kochetkov reaction. In some cases, the peracetylated compounds were treated directly without prior deacetylation, with the expectation that the ammonia would remove the protective groups as well as form the amine.[23,37] After a simple workup consisting of drying *in vacuo* to a constant weight (to remove NH_3), the samples were analyzed by the HPLC assay described above. The results are shown in Figure 3. Conversion to glycosyl amine was considerably less efficient in the cases where peracetylated sugar starting materials were used. This may be due to the formation of SugNHAc as a side product. In any case, these results indicate that *peracetylated sugars should be deprotected (e.g., by Zemplen hydrolysis) before use in the Kochetkov amination reaction.* For unprotected sugar starting materials (e.g., **3** and **7**), this reaction provides good yields of glycosyl amines. The β stereochemistry of these amines was confirmed by NMR analysis of the stereochemistry of the sugar–Asn linkage after glycosylation ($J_{H2,H1} \simeq 9$ Hz); no evidence of the α-anomer was found in any of the glycopeptides synthesized.

Synthesis of Appropriately-Protected Peptides. In order to synthesize complex glycopeptides, a double-deprotection scheme was required. A first deprotection step, performed after peptide synthesis but before glycosylation, should deprotect the Asp to be glycosylated but leave other Asp residues and other reactive side chains protected (Figure 4, step 2). A second, mild deprotection step, performed after glycosylation, should remove the remaining protective groups (Figure 4, steps 4 and 5).

In our approach (Figure 4), the peptide is synthesized using Boc amino acids on the methylbenzhydrylamine (MBHA, produces C-terminal peptide amide) resin, which is cleaved by strong acid (HF). Certain residues which are generally protected during peptide synthesis do not need to be protected during glycosylation and are therefore protected with protective groups which are removed during the cleavage from the resin; these residues are Tyr, Ser, Thr, and Arg (see Figure 4, step 2). The Asp to be glycosylated is likewise protected with an acid-labile group; the cyclohexyl ester (cHex) is preferable to the benzyl ester (Bn) in order to minimize aspartimide formation during peptide synthesis. The other reactive amino acids are protected with groups which are stable to the HF cleavage but can be removed under mild conditions after glycosylation. For Lys, Glu, and Asp (other than the one to be glycosylated), protective groups are used which can be removed by treatment with piperidine (or the even milder base morpholine), namely, the fluorenylmethoxycarbonyl (Fmoc) carbamate group (for Lys) and the fluorenylmethyl ester (Fm) group (for Glu and Asp). For Cys and His, protective groups are used which can be removed by treatment with thiophenol, namely, the ethyl disulfide group (for Cys) and the dinitrophenyl (DNP) group (for His). All of the protected amino acids required for this scheme are commercially available.

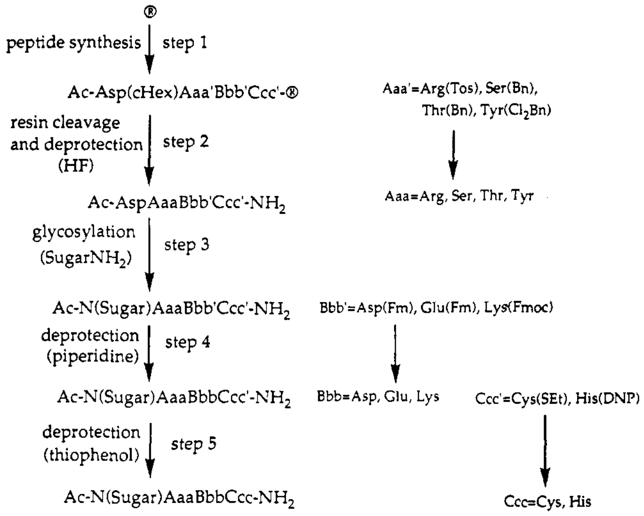

Figure 4. Glycopeptide synthesis scheme. Ⓡ = MBHA resin; Aaa′, Bbb′, and Ccc′ represent three different classes of protected amino acids which are deprotected in steps 2, 4, and 5, respectively, to provide the unprotected amino acids Aaa, Bbb, and Ccc.

9, Ac-E(Fm)DASK(Fmoc)A-NH_2

10, Ac-C(SEt)DH(DNP)TRA-NH_2

11, Ac-AE(Fm)AAAK(Fmoc)E(Fm)DASK(Fmoc)E(Fm)AAAK(Fmoc)A-NH_2

12, Ac-AE(Fm)AAAK(Fmoc)E(Fm)AAAK(Fmoc)E(Fm)DASK(Fmoc)A-NH_2

13, Ac-E(Fm)E(Fm)K(Fmoc)YDLTSVL-NH_2

14, Ac-YDLTS-NH_2

Figure 5. Peptides used in the glycopeptide synthesis by our convergent approach.

Several peptides were synthesized using this protection strategy (Figure 5). The peptides Ac-E(Fm)DASK(Fmoc)A-NH_2 (**9**) and Ac-C(SEt)DH(DNP)TRA-NH_2 (**10**) were designed to test this protective group scheme. Ac-AE(Fm)AAAK(Fmoc)E(Fm)-AAAK(Fmoc)E(Fm)DASK(Fmoc)A-NH_2 (**11**) and Ac-AE-(Fm)AAAK(Fmoc)E(Fm)DASK(Fmoc)E(Fm)AAAK(Fmoc)A-NH_2 (**12**) have sequences based on the helix-forming peptide of Marqusee and Baldwin[49-51] and were designed to test the effect of the sugar on the peptide conformation. Ac-E(Fm)E(Fm)K-(Fmoc)YDLTSVL-NH_2 (**13**) comprises residues 288–297 of ovalbumin and is glycosylated, *in vivo*, with a high-mannose-type oligosaccharide. These peptides were synthesized on the MBHA resin using the protective groups shown in Figure 4 and cleaved from the resin with HF. In the case of Ac-E(Fm)DASK(Fmoc)A-

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Table I. Effect of the Amounts of HBTU and HOBt on the Outcome of the Glycosylation of Peptide 9 with Glycosyl Amine 1

						product distribution (%)[a]		
	$GlcNAcNH_2$ (equiv)	DIEA (equiv)	HOBt (equiv)	HBTU (equiv)	[peptide] (mM)	glyco-peptide	pep-tide	imide
1	2	0	6	3	17	65	7	28
2	2	0	6	6	17	69	5	26
3	2	0	6	9	17	71	0	29
4	1	1	0	3	17	31	*b*	69
5	1	1	1	3	17	37	*b*	63
6	2	0	0	3	14	55	*b*	45
7	2	0	1	3	14	60	*b*	40
8	2	0	3	3	14	65	*b*	35
9	2	0	3	3	7.7	61	*b*	39
10	2	0	6	3	7.7	66	*b*	34
11	2	0	18	3	17	56	20	24

[a] Product distribution was determined by measurement of HPLC peak heights. [b] Peptide was present at ≤5% and was not included in calculating the product distribution.

Table II. Effect of the Amounts of Glycosyl Amine and DIEA on the Outcome of the Glycosylations

					product distribution (%)[a]		
	peptide	sugar	$(Sug)NH_2$ (equiv)	DIEA (equiv)	glyco-peptide	peptide	imide
1	**9**	**1**	1	0	30	33	37
2	**9**	**1**	1	1	31	*b*	69
3	**9**	**1**	2	0	55	*b*	45
4	**14**	**8**	2	0	73	25	2
5	**14**	**8**	1	1	44	53	3
6	**14**	**8**	1	2	81	12	7

[a] Product distribution was determined by measurement of HPLC peak heights. [b] Peptide was present at ≤5% and was not included in calculating the product distribution.

Table III. Glycosylations Carried Out Using Our Convergent Approach

peptide	sugar	$(Sug)NH_2$ (equiv)	DIEA (equiv)	HBTU (equiv)	HOBt (equiv)	yield (%)[a]
9	**1**	2	0	9	5	29 (step 3)[c]
10[b]	**1**	2	1	5	0	43 (step 3),[b] 48 (step 5)[d]
11[b]	**1**	2	0	5	5	11 (steps 3 and 4)[b]
12[b]	**1**	3	0	5	5	12 (steps 3 and 4)[b]
13[b]	**1**	3	0	5	5	39 (steps 3 and 4)[b]
14	**1**	2	0	3	1	61 (step 3)
14	**4**	2	2	3	0	88 (step 3)
14	**6**	1	2	5	5	54 (step 3)
14	**8**	1	2	5	5	55 (step 3)

[a] Yield shown is the purified yield for the indicated step(s) (see Figure 4). [b] These peptides were not purified prior to glycosylation. Yields are based on the amount of starting peptide as determined by amino acid analysis. [c] Deprotection (step 4) was quantitative by HPLC. [d] The unprotected glycopeptide was isolated as the disulfide-bonded dimer.

NH_2, the peptide was purified by HPLC before glycosylation. For the other peptides, the glycosylation was carried out on the crude peptide. In addition, the peptide Ac-YDLTS-NH_2 (**14**), comprising residues 291–295 of ovalbumin, was synthesized. Since this peptide contains no residues which require protection during the glycosylation, it was synthesized using a standard peptide synthesis protocol rather than the approach described above. This peptide was purified by HPLC before glycosylation.

Optimization of the Glycosylation. We reported previously that three factors are important in achieving the glycosylation of peptides.[26] First, the unprotected glycosyl amine was found to give higher yields than the amine in which the hydroxyls were protected as acetyl esters. Second, HBTU was found to be the most useful coupling reagent of those examined. Third, the minimization of base in the reaction medium was found to be important for reducing the amount of aspartimide formed. On the basis of these results, we proposed the following optimal conditions: 2 equiv of unprotected glycosyl amine, 3 equiv of HBTU, and 1 equiv of hydroxybenzotriazole (HOBt). Note that the coupling reaction requires 1 equiv of base to deprotonate Asp; under these conditions, that base is provided by the glycosyl amine, which serves both as a nucleophile and a base. Using these conditions, we were able to successfully glycosylate the peptide Ac-DGF-NH_2, which is expected to be quite aspartimide-prone.[32]

In subsequent experiments, we found that for some peptides, these conditions still led to significant aspartimide formation. For example, for the glycosylation of peptide **9** with glycosyl amine **1** using the above conditions, *ca.* 40% of the product was the aspartimide. A series of optimization experiments were therefore carried out in an attempt to maximize the yield of this glycopeptide. These experiments involved small-scale glycosylations in which the amounts of DIEA, HOBt, HBTU, and glycosyl amine were varied as well as the solvent, temperature, and concentration of the reactants.

Table I shows the results of varying the amount of HOBt and HBTU used in the coupling of **9** and **1**. Increasing the amount of HBTU from 3 to 6 equiv gave increased glycosylation (line 2 vs line 1); a further increase to 9 equiv had a smaller effect (line 3). In addition, we found that adding HOBt to the reaction mixture caused a decrease in aspartimide formation. We saw approximately a 5% reduction in aspartimide (e.g., from 45% to 40%) for each increase in HOBt from 0 equiv to 1 equiv to 3 equiv to 6 equiv (lines 4–10); addition of 18 equiv, however, led to incomplete reaction (line 11).

The effects of temperature, solvent, and concentration were also examined (data not shown).[30] Lower temperatures caused a decrease in aspartimide formation, but the effect was small (≤5% decrease for a reduction from 21 to 0 °C). Varying the percentage of DMF in DMSO as the solvent had little or no effect on the outcome of the glycosylation. Increasing the concentration of the peptide and/or the glycosyl amine caused a decrease in aspartimide formation relative to intermolecular coupling (a 30% drop in aspartimide for a 10-fold concentration increase). Because of the limited solubility of unprotected glycosyl amines in other solvents, we recommend the use of neat DMSO as the solvent in order to achieve the most concentrated reaction mixtures. This precludes the use of lower temperatures, the effect of which is small in any case.

The amount of glycosyl amine needed was also explored. We were particularly interested in whether 1 equiv of glycosyl amine could be used rather than 2 equiv. This would be particularly desirable, of course, in the case of complex, precious sugars, which may only be available in small quantities. The results of these experiments are shown in Table II. For the coupling of **9** and **1**, the use of only 1 equiv of glycosyl amine with no additional base resulted in a significant amount of unreacted starting material (line 1) because an equivalent of base is needed to deprotonate the Asp. When that equivalent of base was provided in the form of DIEA (line 2), large amounts of aspartimide were formed. However, when the second equivalent of base was instead provided in the form of an additional equivalent of glycosyl amine (line 3), the yield of glycopeptide became more acceptable. Thus, in the case of aspartimide-prone peptides, the use of 2 equiv of glycosyl amine seems to be necessary to achieve reasonable yields of glycopeptide.

Most peptides are likely to be considerably less aspartimide-prone than peptide **9**.[31–33] Therefore, in the case of glycosylation of peptide **14** with glycosyl amine **8** (lines 4–6), 1 equiv of glycosyl amine could be used without high levels of aspartimide formation. In fact, the use of 1 equiv of glycosyl amine, 2 equiv of DIEA, 5 equiv of HOBt, and 5 equiv of HBTU seemed to be the optimal set of conditions.

Glycosylations Carried Out Using the Convergent Approach. Several glycosylations were carried out using the peptides and glycosyl amines whose syntheses are described above. The conditions and yields of the glycosylations are shown in Table III. Note that for peptides **10–13**, the glycosylation was carried out on crude material from the HF cleavage; in these cases, the yields given represent the combined yields for peptide synthesis, glycosylation, and—except for peptide **10**—deprotection. In most of the glycosylations, **2** or **3** equiv of glycosyl amine was used. For the sugars $(Man)_5(GlcNAc)_2$ and Fucα1-6GlcNAc, only 1 equiv was used, but good yields of glycopeptide were still obtained.

Conclusions

We have reported here a systematic method for the synthesis of glycopeptides by a practical, convergent approach. We have demonstrated the generality of this method by using it to synthesize a variety of complex glycopeptides. The syntheses carried out include the first convergent syntheses both of glycopeptides containing a variety of functionalized amino acids and glycopeptides containing complex branched oligosaccharides of the type which characterize glycoproteins.

Glycosyl amines for this approach can be synthesized by a simple reaction using aqueous ammonium bicarbonate; acetylated sugars should be deprotected before conversion to the amine. An HPLC assay can be used to determine the amount of glycosyl amine present in the crude product and to confirm that excess NH_4HCO_3 has been removed. Partly-protected peptides for the glycosylation can be synthesized using the protective group scheme outlined here; final deprotection of the glycopeptides can be achieved under mild conditions. The precise optimal conditions for the glycosylation may vary slightly from case to case, but two types of conditions can be identified. For aspartimide-prone peptides (Gly, Ser, or Ala C-terminal to Asp[31–33]) or for glycosyl amines which are readily available, 2 or 3 equiv of glycosyl amine should be used along with 5 equiv of HBTU and 5 equiv of HOBt. For less aspartimide-prone peptides and for precious glycosyl amines, 1 equiv of glycosyl amine and 1–2 equiv of DIEA can be used along with 5 equiv of HBTU and 5 equiv of HOBt.

In stepwise solid-phase methods of glycopeptide synthesis,[19–25] the sugars are exposed to at least one acidic step (TFA). As a result, the danger of *O*-glycosidic bond cleavage is always present, and special precautions must be taken to avoid cleavage of the more sensitive linkages. In our convergent approach, on the other hand, the sugars are never exposed to acidic conditions but instead are only subjected to mild deprotection with piperidine or thiophenol. This allowed us to achieve the synthesis, in good yield, of a glycopeptide containing the very acid-sensitive linkage Fucα1-6GlcNAc.

Stepwise methods of glycopeptide synthesis[19–25] also require the use of several equivalents of sugar at an early step in the synthesis, resulting in low conversion from the oligosaccharide to the glycopeptide. This makes difficult or impossible the synthesis of glycopeptides containing oligosaccharides which are available only in small quantities. In our convergent approach, on the other hand, the peptide can be purified and then glycosylated on a small scale, using, in many cases, only 1 equiv of glycosyl amine. This allowed us to prepare, in good yield, a glycopeptide containing the heptasaccharide $(Man)_5(GlcNAc)_2$, of which only *ca.* 35 μmol was available to us.

Thus, this approach makes available glycopeptides containing a variety of oligosaccharides. Using this approach, a series of glycopeptides (for example, **14 + 1**, **14 + 4**, **14 + 6**, and **14 + 8**; see Table III) can readily be synthesized in order to analyze the effects of different sugars on the structure of the peptide.

Experimental Section

Equipment, Materials, and Methods. 4-Methylbenzhydrylamine (MBHA) resin was purchased from AminoTech. 4-Methoxy-4′-alkoxybenzhydrylamine resin was purchased from Bachem Bioscience. Protected amino acids were purchased from Fisher, Calbiochem, Bachem, and Bachem Bioscience. $GlcNAcNH_2$ and $Ac_3GlcNAc\beta 1\text{-}4(Ac_2)$-GlcNAc-OAc were purchased from Sigma. $(Man)_5(GlcNAc)_2$ was a gift from Dr. Christopher Warren of Massachusetts General Hospital. All other chemicals were purchased from Aldrich.

Reverse-phase HPLC was carried out on a Waters 600E system, using a single-wavelength or diode array spectrophotometer for detection. For analytical HPLC, Waters Deltapak C4 100- or 300-Å columns (3.9 mm × 30 cm) were used, while for semipreparative HPLC, the columns used were Waters Deltapak C4 100- or 300-Å columns (19 mm × 30 cm) or a YMC C18 300-Å column (30 mm × 30 cm). Analytical TLC was performed using Baker-flex silica gel sheets (1B-F).

^1H NMR spectra were obtained on a Varian XL-300, a Varian Unity 300, or a Varian VXR-500. Only assigned peaks are reported; copies of spectra can be obtained from the authors upon request. PD mass spectra were obtained by Paul Weinreb and Joseph Jarrett of the Lansbury laboratory. FAB mass spectra were obtained by Dr. Andrew Tyler and Laura Romo of the Harvard Mass Spectrometry Facility.

Synthesis of Glycosyl Amines. General Procedure for the Synthesis of Glycosyl Amines Using NH_4HCO_3. The sugar was dissolved in water in a concentration of 30–60 mM. Ammonium bicarbonate was added to saturation, and the reaction was stirred at room temperature in an uncovered flask. The reaction mixture was kept saturated by the addition of NH_4HCO_3 as needed. The reaction was monitored by TLC, and when complete or nearly complete conversion to $(Sug)NH_2$ was seen (usually ~1 week), the solution was lyophilized to give a large amount of solid, which was mostly ammonium bicarbonate. The flask was kept under vacuum (lyophilizer or vacuum pump), and the weight of the solid was measured daily. Every several days, water (usually half of the original reaction volume) was added and the solution was immediately frozen and lyophilized. This treatment was continued until the solid reached a constant weight (near the weight expected). Then, the material was analyzed by TLC and by the HPLC assay (see below) and used in glycosylations.

General Procedure for Determining the Composition of Products from the NH_4HCO_3 Reaction. TLC. A small portion of the product was dissolved in methanol and analyzed by TLC.

TLC (chitobiose): 4:3:2 EtOAc:MeOH:H_2O. **3**: $R_f = 0.52$. **4**: $R_f = 0.29$.

TLC (Fucα1-6GlcNAc): 4:3:1 EtOAc:MeOH:H_2O. Fucα1-6GlcNAc-OH: $R_f = 0.53$. **6**: $R_f = 0.20$.

TLC ($(Man)_5(GlcNAc)_2$): 2:3:3 EtOAc:MeOH:H_2O. **7**: $R_f = 0.69$. **8**: $R_f = 0.52$ (streak).

HPLC Assay. A small portion (0.3–1.5 mg) of the product was weighed into an Eppendorf tube and treated with either (a) 1 equiv of Boc-Asp-OBn (50 mM solution in DMSO), 3 equiv of DIEA (5% in DMF), and 5 equiv of HBTU (100 mM in DMSO) or (b) 2 equiv of Boc-Asp-OBn, 6 equiv of DIEA, and 10 equiv of HBTU. After reacting overnight, the samples were analyzed by HPLC (see below for conditions) and the heights of the Boc-Asn(Sug)-OBn, Boc-Asn-OBn, and Boc-Asp-OBn peaks were measured at 254 nm. If the conditions in (a) were used, then the percent of $(Sug)NH_2$ in the sugar sample was calculated as the ratio of the Boc-Asn(Sug)-OBn peak height to the sum of the heights of all three peaks, while the percent of NH_3 in the sample was calculated as the ratio of the Boc-Asn-OBn peak height to the sum of all three peaks. If the conditions in (b) were used, then the values obtained from these calculations were multiplied by 2.

HPLC: analytical, 2 mL/min, 80/20–30/70 over 15 min (H_2O/CH_3CN (0.1% TFA)). Boc-Asp-OBn: 10.8 min. Boc-Asn-OBn: 9.4 min. Boc-Asn(GlcNAcβ1-4GlcNAc)-OBn: 7.7 min. Boc-Asn(Fucα1-6GlcNAc)-OBn: 7.5 min. Boc-Asn($(Man)_5(GlcNAc)_2$)-OBn: 6.8 min.

Peptide Synthesis. Synthesis of Peptide 9. Ac-E(Fm)D(cHex)AS-(Bn)K(Fmoc)A-Ⓡ was synthesized on the MBHA resin using the standard Boc synthesis protocol.[52] The resin was cleaved, in several batches, with 90% HF/5% thioanisole/5% *m*-cresol at 0 °C for 45 min, using the standard HF cleavage protocol.[52] HPLC purification of the major peak [semiprep (YMC): 30 mL/min, 52/48 (H_2O/CH_3CN (0.1% TEA)), $RV = 310$ mL] gave Ac-E(Fm)DASK(Fmoc)A-NH_2 (**9**) in 21% yield based on Ala-Ⓡ. This was apparently contaminated with small amounts of a benzylated impurity (by MS) and of the aspartimide ($\leq 5\%$ by MS and HPLC). PDMS: 1061.3 $(M + H)^+$ (calcd MW = 1060.4), small peaks at 1044.3 (aspartimide) and 1152.3 $(M + Bn + H)^+$.

Synthesis of Peptide 10. Ac-C(SEt)D(cHex)H(DNP)T-(Bn)R(Tos)A-Ⓡ was synthesized on the MBHA resin using the standard Boc synthesis protocol. The Cys derivative was purchased and stored as its DCHA salt (Boc-Cys(SEt)-$O^-H_2N^+(cHex)_2$) and was converted to the acid form immediately prior to use. The resin was cleaved with HF/5% *m*-cresol/5% anisole using the standard HF cleavage protocol. A portion of the crude peptide was dissolved in TFA and then dried down and redissolved in DMSO. The DMSO solution was purified by HPLC [semiprep: 15 mL/min, 82/18 (H_2O/CH_3CN (0.1% TFA)), $R_v = 110$

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(52) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984.

mL] to give 91.8 mg (84.8 μmol, 34% yield based on A-Ⓡ) of Ac-C(SEt)DH(DNP)TR$^+$(CF$_3$CO$_2^-$)A-NH$_2$ (FABMS: 969 (M + H)$^+$ (calcd MW = 968.3)). The glycosylation was carried out on the crude peptide.

Synthesis of Peptide 11. Ac-AE(Fm)AAAK(Fmoc)E(Fm)-AAAK(Fmoc)E(Fm)D(cHex)AS(Bn)K(Fmoc)A-Ⓡ was synthesized on the MBHA resin using the standard Boc synthesis protocol. The resin was cleaved, in several batches, with 90% HF/5% thioanisole/5% *m*-cresol at 0 °C for 60 min, using the standard HF protocol. AAA of the crude: A 9.0 (9), E 3.0 (3), K 2.1 (3), D 1.1 (1), S 1.0 (1); the peptide comprised 70% of the weight of the crude. The crude peptide could not be dissolved in any of the solvents attempted except for TFA. Some of the peptide was HPLC-purified (from TFA solution) [semiprep (YMC): 30 mL/min, 15/85 (H_2O/CH_3CN (0.1% TFA)), R_v = 500 mL] to give Ac-AE(Fm)AAAK(Fmoc)E(Fm)AAAK(Fmoc)E(Fm)DASK(Fmoc)A-NH$_2$ (**11**) (FABMS: 2875.3 (M + H)$^+$ (calcd MW = 2872.3), small peak at 2857.3 ((M + H)$^+$ for aspartimide). However, since the HPLC purification was extremely tedious and gave a low recovery of the pure peptide, the crude peptide was used in glycosylations.

Synthesis of Peptide 12. Ac-AE(Fm)AAAK(Fmoc)E(Fm)D-(cHex)AS(Bn)K(Fmoc)E(Fm)AAAK(Fmoc)A-Ⓡ was synthesized on the MBHA resin using the standard Boc synthesis protocol. One batch of the resin was cleaved with 90% HF/5% thioanisole/5% *m*-cresol at 0 °C for 60 min, using the standard HF cleavage protocol. Deprotection of an aliquot of the crude peptide, using piperidine, allowed HPLC analysis of the unprotected peptide, which showed the presence of two peaks of approximately equal size; the earlier of these peaks was the correct peptide (FABMS: 1672.9 (M + H)$^+$ (calcd MW = 1671.8)), and the later peak was a benzylated impurity (FABMS: 1762.9 (M + Bn + H)$^+$). A second batch of resin was cleaved using the low–high HF protocol.[53] AAA of the crude: A 9.0 (9), E 3.1 (3), K 2.3 (3), D 1.1 (1), S 1.0 (1); the peptide comprised 77% of the weight of the crude. Deprotection of an aliquot of the crude peptide, using piperidine, allowed HPLC analysis of the unprotected peptide, which showed primarily one peak corresponding to the correct peptide (PDMS: 1675 (M + H)$^+$, 1697 (M + Na)$^+$, 1719 (M + 2Na – H)$^+$ (calcd MW = 1671.8)). The protected peptide was used in glycosylations without purification.

Synthesis of Peptide 13. Ac-E(Fm)E(Fm)K(Fmoc)Y(Cl$_2$Bn)D-(cHex)LT(Bn)S(Bn)VL-Ⓡ was synthesized on the MBHA resin using the standard Boc synthesis protocol. One batch of resin was cleaved with 90% HF/5% thioanisole/5% *m*-cresol at 0 °C for 60 min, using the standard HF protocol. AAA of the crude: E 2.0 (2), K 0.6 (1), Y 0.6 (1), D 1.1 (1), L 1.8 (2), T 1.0 (1), S 0.9 (1), V 0.9 (1); the peptide comprised 63% of the weight of the crude. HPLC purification of a portion of the crude peptide [semiprep: 15 mL/min, 50/50–10/90 over 15 min (H_2O/CH_3CN (0.1% TFA))] gave 8.5 mg of Ac-E(Fm)E(Fm)K(Fmoc)-YDLTSVL-NH$_2$ (**13**) (FABMS: 1816 (M + H)$^+$, 1837 (M + Na)$^+$ (calcd MW = 1814.8)) and 5.7 mg of a benzylated impurity (FABMS: 1930 (M + Bn + Na)$^+$). A second batch of resin was cleaved using the low–high HF protocol. AAA of the crude: E 2.0 (2), K 0.8 (1), Y 0.9 (1), D 1.0 (1), L 1.9 (2), T 0.9 (1), S 0.9 (1), V 0.9 (1); the peptide comprised 57% of the weight of the crude. Analytical HPLC showed that the crude consisted mostly of the correct material, with very little, if any, of the benzylated impurity. Two impurities which eluted earlier than the correct material on HPLC were slightly larger in this cleavage than in the first cleavage, though still <10%; these both proved to be missing one of the Fm protective groups (PDMS: 1661 (M – Fm + Na)$^+$). The peptide was used in glycosylations without purification.

Synthesis of Peptide 14. Ac-Y(tBu)D(tBu)LT(tBu)S(tBu)-Ⓡ was assembled on the 4-methoxy-4′-alkoxy-benzhydrylamine resin using the standard Fmoc synthesis protocol. The resin was cleaved, and the peptide was simultaneously deprotected by treatment with Reagent K.[54] The major peak was purified by HPLC [semiprep: 15 mL/min, 87/13 (H_2O/CH_3CN (0.1% TFA)), R_v = 90 mL] to give Ac-YDLTS-NH$_2$ (**14**). FABMS: 638.8 (M + H)$^+$, 661.0 (M + Na)$^+$, 677.0 (M + K)$^+$ (calcd MW = 638.3). ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.83 (2 overlapping d, $J \simeq 7$ Hz, 6H, Leu δMe's), 1.01 (d, $J = 5.9$ Hz, 3H, Thr γMe), 1.73 (s, 3H, Ac), 3.5–3.7 (m, 2H, Ser βH's), 4.0 (m, 1H, Thr βH), 4.2 (m, 1H, Ser αH), 4.3 (m, 1H, Thr αH), 4.4 (m, 2H, αH's), 4.5 (m, 1H, αH), 6.63 (d, $J = 8.3$ Hz, 2H, Tyr H3, H5), 7.02 (d, $J = 7.9$ Hz, Tyr H2, H6), 7.18 (s, 1H, C-term NH$_2$), 7.21 (s, 1H, C-term NH$_2$), 7.78 (d, $J = 7.8$ Hz, 1H, NH), 7.9 (m, 2H, NH's), 8.08 (d, $J = 8.0$ Hz, 1H, NH), 8.42 (d, $J = 7.6$ Hz, 1H, NH).

Optimization of Glycosylation. Procedure for HPLC Experiments Testing Different Conditions for the Glycosylation of Peptide 9 with Glycosyl Amine 1. **9** and **1** were allowed to react on a small scale (~0.5–1.0 μmol) under the various conditions described in the text. When the reaction had gone to completion, the amounts of glycopeptide, peptide, and aspartimide were assessed by measuring their respective peak integrations on analytical HPLC. The reactions were analyzed at 65/35 isocratic ((H_2O/CH_3CN (0.1% AcOH), 2 mL/min), with Ac-E(Fm)N-(GlcNAc)ASK(Fmoc)A-NH$_2$ eluting at 8–9 min, Ac-E(Fm)DASK-(Fmoc)A-NH$_2$ eluting at 12–13 min, and Ac-E(Fm)DimideASK-(Fmoc)A-NH$_2$ eluting at 16–17 min. The peaks were identified by their elution times, which were quite reproducible, or by coinjection with authentic samples.

Procedure for HPLC Experiments Testing Different Conditions for the Glycosylation of Peptide 14 with Glycosyl Amine 8. **14** and **8** were allowed to react on a small scale (~0.3 μmol) under the various conditions described in the text. When the reaction had gone to completion, the amounts of glycopeptide, peptide, and aspartimide were assessed by measuring their respective peak integrations at 225 nm on analytical HPLC. The gradient used was 95/5–0/100 over 20 min ((H_2O/CH_3CN (0.1% TFA), 2 mL/min), with Ac-YN((Man)$_5$(GlcNAc)$_2$)LTS–NH$_2$ eluting at 7.9 min, Ac-YDLTS-NH$_2$ eluting at 8.4 min, and Ac-YDimideLTS-NH$_2$ eluting at 9.1 min. The latter two peaks were identified by comparison with authentic samples, while the glycopeptide peak was identified by FABMS (1854 (M + H)$^+$ (calcd MW = 1853.7)).

Glycosylations. Glycosylation of Peptide 9 with Glycosyl Amine 1. **9** (29.2 mg, 27.5 μmol) and HOBt (18.6 mg, 138 μmol) were dissolved in DMSO, and **1** (12.1 mg, 55 μmol) and HBTU (94.1 mg, 248 μmol) were added. After the mixture was stirred overnight at 21 °C, HPLC analysis (as described for the small-scale optimization experiments) showed glycopeptide:aspartimide = 70:30. The solution was filtered and purified by HPLC [semiprep: 20 mL/min, 69/31 (H_2O/CH_3CN (0.1% AcOH)), R_v = 200 mL] to give 10.1 mg (7.99 μmol, 29% yield) of Ac-E(Fm)N-(GlcNAc)ASK(Fmoc)A-NH$_2$. PDMS: 1263.4 (M + H)$^+$ (calcd MW = 1262.6).

Test Deprotection of Ac-E(Fm)N(GlcNAc)ASK(Fmoc)A-NH$_2$. Ac-E(Fm)N(GlcNAc)ASK(Fmoc)A-NH$_2$ was dissolved in DMSO and treated with an equal volume of piperidine. After the mixture had been stirred at 21 °C for 30 min, the piperidine was evaporated *in vacuo*. HPLC analysis [analytical: 2 mL/min, 98/2 isocratic for 3 min followed by a 17-min gradient to 0/100 (H_2O/CH_3CN (0.1% TFA))] showed only three peaks, two of which (14.0 and 17.2 min) corresponded to piperidine–Fmoc adducts (CIMS: 264 (calcd MW = 263)); the third peak (3.1 min) corresponded to Ac-EN(GlcNAc)ASKA-NH$_2$ (FABMS: 863 (M + H)$^+$ (calcd MW = 862.4), small peak at 959).

Glycosylation of Peptide 10 with Glycosyl Amine 1. Crude Ac-C(SEt)-DH(DNP)TR$^+$(CF$_3$CO$_2^-$)A-NH$_2$ (161.3 mg, 149 μmol based on AAA) was dissolved in DMSO and treated with **1** (66 mg, 298 μmol) in DMSO (4 mL), HBTU (283 mg, 745 μmol), and DIEA (25 μL, 149 μmol). After being stirred for several days at 22 °C, the reaction mixture was filtered and purified by HPLC [semiprep: 15 mL/min, 81/19 (H_2O/CH_3CN (0.1% TFA)), R_v = 125 mL] to give 88.2 mg (68.6 μmol, 43% yield) of Ac-C(SEt)N(GlcNAc)H(DNP)TR$^+$(CF$_3$CO$_2^-$)A-NH$_2$. The β stereochemistry of the GlcNAc–Asn linkage was confirmed by NMR (J_{H1} = 9.1 Hz). FABMS: 1171 (M + H)$^+$ (calcd MW = 1170.4). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.06 (d, $J = 6.4$ Hz, 3H, Ala Me), 1.2 (m, 6H, Thr Me, SCH$_2$C*H*$_3$), 1.81 (s, 3H, Ac), 1.85 (s, 3H, Ac), 4.78 (t, $J = 9.1$ Hz, 1H, GlcNAc H1).

Deprotection of Ac-C(SEt)N(GlcNAc)H(DNP)TR$^+$(CF$_3$CO$_2^-$)A-NH$_2$. Ac-C(SEt)N(GlcNAc)H(DNP)TR$^+$(CF$_3$CO$_2^-$)A-NH$_2$ (34.7 mg, 27.0 μmol) was dissolved in DMF (5 mL) under an Ar atmosphere and treated with thiophenol (695 μL, 6.75 μmol). After being stirred for 23 h at 22 °C, the reaction mixture was precipitated from diethyl ether. The pellet, isolated by centrifugation, was washed with ether, dried, and redissolved in DMF (4 mL). HPLC analysis [analytical: 2 mL/min, 100/0 isocratic for 5 min and then 100/0–0/100 over 15 min (H_2O/CH_3CN (0.1% TFA))] showed mostly Ac-CN(GlcNAc)HTRA-NH$_2$ (11.6 min) but also ~20% of the dimer (12.2 min) and ~13% of Ac-C(SPh)N(GlcNAc)-HTRA-NH$_2$ (14.0 min). Water (2 mL) was added, and the solution was stirred in an unstoppered flask in order to oxidize the glycopeptide to the disulfide. After several days, HPLC indicated that the mixture was mostly dimer. The solution was purified by HPLC [semiprep: 15 mL/min, 95/5 (H_2O/CH_3CN (0.1% TFA)), R_v = 80 mL] to give 15.2 mg (6.48 μmol, 48% yield) of the tetra-TFA salt of the disulfide-bonded dimer (FABMS: 1887 (M + H)$^+$, 945 (M + 2H)$^{2+}$ (calcd MW = 1886.8), small peak at 2002).

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[53] Tam, J. P.; Heath, W. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1983**, *105*, 6442–6455.

[54] King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255–266.

Glycosylation of Peptide 11 with Glycosyl Amine 1 and Deprotection of the Glycopeptide. Crude **11** (151.2 mg, 52.5 μmol by AAA) was dissolved in DCM (~6 mL) containing a small amount (<0.5 mL) of TFA; after dissolution of the peptide to give a clear yellow solution, the solvent was evaporated *in vacuo*. The solid was redissolved in methylene chloride (DCM, ~15 mL), and the solvent was then evaporated *in vacuo*. This process was repeated several times, after which the solid was dried overnight on a vacuum pump. The solid was dissolved in 5% DIEA/DCM (10 mL), which was then evaporated *in vacuo*. This was repeated, and then, DCM was added and evaporated several times. Finally, the solid was dried overnight on a vacuum pump and dissolved in methylene chloride (1.7 mL). To this solution were added HOBt (35.5 mg, 262 μmol), **1** (24.3 mg, 105 μmol) in DMSO (3 mL), HBTU (99.6 mg, 262 μmol), and 1 mL each of DCM and DMSO. After being stirred for 22 h at 22 °C, the reaction mixture was precipitated from water. The pellet isolated by centrifugation was washed with water several times and lyophilized to give 153 mg. This solid was treated with 15 mL of piperidine, giving a cloudy solution. After the mixture was stirred for 1 h at 22 °C, the piperidine was evaporated off and water (22 mL) was added, resulting in the formation of a white precipitate. After sonication, this mixture was filtered through 0.22-μm filters. The resulting aqueous solution was dried to 143 mg of a solid, which was redissolved in water (10 mL) and purified by HPLC [semiprep: 15 mL/min, 88/12 (H_2O/CH_3CN (0.1% TFA)), R_v = 125 mL] to give 11.3 mg (6.0 μmol, 11% yield based on AAA of the crude) of Ac-AEAAAKEAAAKEN(GlcNAc)ASKA-NH_2. PDMS: 1876.9 $(M + H)^+$ (calcd MW = 1873.9).

Glycosylation of Peptide 12 with Glycosyl Amine 1 and Deprotection of the Glycopeptide. Crude **12** (175.8 mg, 47.1 μmol of peptide by AAA) was prepared for glycosylation in a manner similar to that described for **11**. It was then dissolved in DMSO (5 mL) and treated with HOBt (31.9 mg, 236 μmol), **1** (32.8 mg, 141 μmol), in DMSO (4 mL), and HBTU (89.5 mg, 236 μmol). After being stirred for 2 days at 22 °C, the reaction mixture was precipitated from water. The pellet isolated by centrifugation was washed with water several times and then lyophilized to give 160 mg. This solid was treated with 20 mL of piperidine, giving a cloudy solution. After the mixture was stirred for 1 h at 22 °C, the piperidine was evaporated off and water (40 mL) was added, resulting in the formation of a white precipitate. After sonication, this mixture was filtered through 0.22-μm filters. The resulting aqueous solution was dried to 125 mg of a solid, which was redissolved in water (4 mL) and purified by HPLC [semiprep: 15 mL/min, 90/10 (H_2O/CH_3CN (0.1% TFA)), R_v = 110 mL] to give 11.0 mg (5.9 μmol, 12% yield based on AAA of the crude) of Ac-AEAAAKEN(GlcNAc)ASKEAAAKA-NH_2. PDMS: 1877.0 $(M + H)^+$, 939.0 $(M + 2H)^{2+}$ (calcd MW = 1873.9).

Glycosylation of Peptide 13 with Glycosyl Amine 1 and Deprotection of the Glycopeptide. Crude **13** from the low–high HF cleavage (151.7 mg, 45.9 μmol based on AAA) was dissolved in DMSO (2.5 mL), giving a cloudy mixture. HOBt (31.1 mg, 230 μmol), **1** (32.0 mg, 138 μmol) in DMSO (5 mL), and HBTU (87.3 mg, 230 μmol) were added, and the mixture was sonicated, giving a solution which was still slightly cloudy. The reaction was stirred at 22 °C overnight. The peptide was then deprotected by the addition of piperidine (8 mL) directly to the reaction mixture. After the mixture was stirred for 75 min, the piperidine was evaporated *in vacuo* and water (20 mL) was added, resulting in the formation of a while precipitate. The mixture was sonicated and then filtered through 0.22-μm filters, which were washed with water (10 mL). The filtrate and washes were combined, and the water was evaporated *in vacuo*, giving a clear yellow DMSO solution. HPLC purification [semiprep: 15 mL/min, 78/22 (H_2O/CH_3CN (0.1% TFA)), R_v = 115 mL] gave 25.6 mg (17.8 μmol, 39% yield) of Ac-EEKYN(GlcNAc)-LTSVL-NH_2. PDMS: 1441 $(M + H)^+$, 1463 $(M + Na)^+$ (calcd MW = 1438.7).

Glycosylation of Peptide 14 with Glycosyl Amine 1. To **14** (31.9 mg, 50 μmol) in DMF (500 μL) were added **1** (22.0 mg, 100 μmol) in DMSO (1.35 mL), HBTU (56.9 mg, 150 μmol) in DMF (1 mL), and HOBt (6.8 mg, 50 μmol) in DMF (0.5 mL). After being stirred for 3.5 h at 22 °C, the reaction mixture was filtered and purified by HPLC [semiprep: 15 mL/min, 88/12 (H_2O/CH_3CN (0.1% AcOH))] to give 3.8 mg (5.9 μmol), 12% yield) of starting material (**14**; R_v = 180 mL; for spectral data, see above) and 25.6 mg (30.4 μmol, 61% yield) of Ac-YN(GlcNAc)LTS-NH_2 (R_v = 145 mL). The β stereochemistry of the GlcNAc–Asn linkage was confirmed by NMR (J_{H1} = 9.3 Hz). FABMS: 841.5 $(M + H)^+$ (calcd MW = 840.4) ^{1}H NMR (500 MHz, DMSO-d_6): δ 0.84 (d, J = 6.3 Hz, 3H, Leu δMe), 0.86 (d, J = 6.6 Hz, 3H, Leu δMe), 1.04 (d, J = 6.3 Hz, 3H, Thr γMe), 1.74 (s, 3H, N-term Ac), 1.81 (s, 3H, GlcNAc Ac), 3.09 (d, J = 5.4 Hz, 2H, GlcNAc H6′s), 4.02 (m, 1H, Thr βH), 4.18 (m, 1H, Ser αH), 4.26 (m, 1H, Thr αH), 4.4 (m, 2H, Tyr αH, Leu αH), 4.54 (m, 1H, Asn αH), 4.81 (t, J = 9.3 Hz, 1H, GlcNAc H1), 6.63 (d, J = 8.2 Hz, 2H, Tyr H3, H5), 7.02 (d, J = 8.2 Hz, 2H, Tyr H2, H6), 7.19 (m, 2H, C-term NH_2), 7.73 (d, J = 7.6 Hz, 1H, Ser NH), 7.8 (m, 3H, Leu NH, Thr NH, GlcNAc N*H*Ac) 8.04 (d, J = 8.2 Hz, 1H, Tyr NH), 8.21 (d, J = 9.2 Hz, 1H, Asn δNH), 8.33 (d, J = 7.9 Hz, 1H, Asn NH), 9.15 (br s, 1H, Tyr OH).

Glycosylation of Peptide 14 with Glycosyl Amine 4. **4** was prepared by treatment of **3** with NH_4HCO_3 using the general procedure described above. After workup as described above, analysis by the HPLC assay indicated that no ammonia was present and that glycosyl amine constituted 55% of the product.

14 (32.4 mg, 50.7 μmol) was dissolved in DMSO (7 mL) and treated with **4** (78.1 mg, 101 μmol based on 55% glycosyl amine), HBTU (57.7 mg, 152 μmol) in DMSO (3 mL), and DIEA (17.2 μL, 101 μmol). After being stirred overnight, the reaction mixture was purified by HPLC [semiprep: 20 mL/min, 93/7 (H_2O/CH_3CN (0.1% AcOH)), R_v = 120 mL] to give 46.8 mg (44.8 μmol, 88% yield) of Ac-YN(GlcNAcβ1-4GlcNAc)LTS-NH_2. The β stereochemistry of the GlcNAc–Asn linkage was confirmed by NMR (J_{H1} = 9.2 Hz). FABMS: 1066 $(M + Na)^+$ (calcd MW = 1043.5). ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.83 (2 overlapping d, $J \simeq 7$ Hz, Leu δMe's), 1.02 (d, J = 6.0 Hz, 3H, Thr γMe), 1.72 (s, 3H, Ac), 1.79 (s, 3H, Ac), 1.81 (s, 3H, Ac), 4.0 (m, 1H, Thr βH), 4.2 (m, 1H, Ser αH), 4.2 (m, 1H, Thr αH), 4.83 (t, J = 9.2 Hz, 1H, GlcNAc H1), 6.61 (d, J = 8.1 Hz, 2H, Tyr H3, H5), 7.00 (d, J = 8.6 Hz, 2H, Tyr H2, H6), 7.17 (br s, 2H, C-term NH_2), 7.7–7.8 (overlapping d, 5H, NH's), 8.02 d, J = 8.6 Hz, 1H, NH), 8.27 (d, J = 9.4 Hz, 1H, Asn δNH), 8.34 (d, J = 7.2 Hz, 1H, NH).

Glycosylation of Peptide 14 with Glycosyl Amine 6. **6** was prepared by treatment of **5** with NH_4HCO_3 using the general procedure described above. After workup as described above, the HPLC assay showed that this was 30% glycosyl amine (of the amount expected) and that it also contained ~15% ammonia.

6 (50.8 mg, 30.6 μmol of **6** and 22.2 μmol of ammonia based on the HPLC assay) and **14** (33.7 mg, 52.8 μmol) were dissolved in DMSO (2 mL). HOBt (35.7 mg, 264 μmol), DIEA (18.0 μL, 106 μmol), and a solution of HBTU (100 mg, 264 μmol) in DMSO (3 mL) were added. After being stirred overnight at 22 °C, the reaction mixture filtered and purified by HPLC [semiprep: 15 mL/min, 90/10 (H_2O/CH_3CN (0.1% TFA)), R_v = 115 mL] to give 16.3 mg (16.5 μmol, 54% yield) of Ac-YN(Fucα1-6GlcNAc)LTS-NH_2. The β stereochemistry of the GlcNAc–Asn linkage was confirmed by NMR (J_{H1} = 9.3 Hz). FABMS: 1009 $(M + Na)^+$ (calcd MW = 986.4). ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.84 (2 overlapping d, $J \simeq 6$ Hz, 6H, Leu δMe's), 1.04 (2 overlapping d, $J \simeq 7$ Hz, 6H, Thr γMe, Fuc Me), 1.73 (s, 3H, Ac), 1.80 (s, 3H, Ac), 4.61 (d, J = 2.2 Hz, 1H, Sug H1'), 4.79 (t, J = 9.3 Hz, Sug H1), 6.61 (d, J = 8.5 Hz, 2H, Tyr H3, H5), 7.00 (d, J = 8.5 Hz, Tyr H2, H6), 7.18 (br s, 2H, C-term NH_2), 7.72 (d, J = 8.0 Hz, 1H, NH), 7.80 (m, 3H, NH's), 8.02 (d, J = 8.1 Hz, 1H, NH), 8.19 (d, J = 8.9 Hz, 1H, NH), 8.31 (d, J = 7.9 Hz, 1H, NH).

Glycosylation of Peptide 14 with Glycosyl Amine 8. **8** was prepared by treatment of **7** with NH_4HCO_3 using the general procedure described above. After workup as described above, the HPLC assay indicated that the product contained no ammonia and was ~80% glycosyl amine.

8 (20.7 mg, 16.8 μmol) and **14** (10.7 mg, 16.8 μmol) were dissolved in DMSO (1 mL) and treated with HOBt (11.4 mg, 84.0 μmol), DIEA (5.7 μL, 33.6 μmol), and HBTU (31.9 mg, 84.0 μmol) in DMSO (1 mL). After the solution was stirred overnight at 22 °C, HPLC showed a glycopeptide:peptide:aspartimide ratio of 80:13:7. The reaction mixture was filtered and purified by HPLC [semiprep: 15 mL/min, 90/10 (H_2O/CH_3CN (0.1% TFA)), R_v = 110 mL] to give 17.1 mg (9.22 μmol, 55% yield) of Ac-YN($(Man)_5(GlcNAc)_2$)LTS-NH_2. The β stereochemistry of the GlcNAc–Asn linkage was confirmed by NMR (J_{H1} = 9.4 Hz). PDMS: 1878.9 $(M + Na)^+$ (calcd MW = 1853.7). ^{1}H NMR (300 MHz, D_2O): δ 0.71 (d, J = 5.5 Hz, 3H, Leu δMe), 0.78 (d, J = 5.6 Hz, 3H, Leu δMe), 1.05 (d, J = 6.4 Hz, 3H, Thr γMe), 1.82 (s, 3H, Ac), 1.84 (s, 3H, Ac), 1.90 (s, 3H, Ac), 4.1 (m, 1H, Thr βH), 4.71 (d, $J \leq$ 2 Hz, 1H, H1 of one of the Man), 4.75 (d, $J \leq$ 2 Hz, 1H, H1 of one of the Man), 4.85 (d, J = 9.4 Hz, 1H, term GlcNAc H1), 4.93 (d, $J \leq$ 2 Hz, 2H, H1's of two of the Man), 6.68 (d, J = 8.2 Hz, 2H, Tyr H3, H5), 6.97 (d, J = 8.2 Hz, 2H, Tyr H2, H6).

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