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Parallel solid-phase synthesis of mucin-like glycopeptides☆

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Abstract—The glycopeptide, Ac-Pro-Thr(α-D-GalNAc)-Thr(α-D-GalNAc)-Thr(α-D-GalNAc)-Pro-Leu-Lys-NH_2 (**1**), which features three consecutive O-glycosylated Thr residues and mimics a portion of mucin 2, has been prepared by solid-phase synthesis. Seven related, partially glycosylated peptides (**2**–**8**) were synthesized as well. This suite of molecules allowed a systematic analysis of synthetic protocols. N^{α}-(9-Fluorenylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine pentafluorophenyl ester [Fmoc-L-Thr(Ac_3-α-D-$GalN_3$)-OPfp] was used as a building block that coupled efficiently when used in a relatively low molar excess, that is, ~1.5 equiv, with *N*,*N*-dimethylformamide (DMF) as the solvent. For conversion of the azido group to the N-acetyl function, direct treatment with thioacetic acid was preferred over a two-step procedure involving reduction with dithiothreitol (DTT) followed by N-acetylation. Effective O-deacetylation of **1**–**8** in solution was achieved by treatment with sodium methoxide (10–15 mM; ~5 equiv) in methanol. On-resin deacetylation techniques were also examined, using sodium methoxide (6–10 mM) in DMF–methanol (17:3) (for **4** and **11**) or hydrazine (70 mM) in methanol (for **8**). The more convenient on-resin technique in DMF–methanol gave yields similar to solution conditions, and promises to be widely useful for solid-phase glycopeptide synthesis. HPLC profiles showed that free glycopeptides elute earlier than the corresponding O-acetylated derivatives, and that retention times vary systematically with the number of sugar moieties. ^{1}H NMR studies carried out in water showed an increase in conformational organization of glycopeptides with increased density of glycosylation.

Keywords: Azido functional group; Deacetylation; Glycopeptide; Sodium methoxide; Solid-phase glycopeptide synthesis; Thioacetic acid; Nuclear magnetic resonance

1. Introduction

A variety of biological functions have emerged for mucin-like glycoproteins and glycoprotein domains of secreted molecules and of cell-surface glycoproteins, and there is interest in gaining structural insights into the properties of these species.[1] These considerations come into play even at the initial stage of protein glycosylation, as manifested in the way particular transferase enzymes incorporate initial and subsequent GalNAc residues onto multiple Ser and Thr sites. Biochemical transformations by several of these enzymes on a mucin 2 (MUC2) derived sequence, PTTTPLK, devoid of glycosylation or with partial occupancy of GalNAc on Thr residues, have been reported.[2] Correlating these data with structural characteristics will lead to an understanding of the role of structure in controlling the rates and positions of glycosylation, and thus, the glycosylation patterns of mucins which are an element of their diversity. Such information, taken together with the recent first report of a GalNAc transferase X-ray structure,[3] can provide a more complete picture of the process.

The study of mucin-derived glycopeptides presents particular challenges, because of the size of native materials and the microheterogeneity associated with their glycosylation.[4] To experimentally address

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conformational properties, well-defined homogeneous material is needed. This can be provided with considerable versatility through chemical synthesis of mucin–glycopeptide constructs with a variety of glycosylation patterns.[5–9] In pursuing our efforts to relate the conformations of glycopeptide substrates to their modification by glycotransferases, and with the idea toward eventually incorporating into these glycoconjugates isotope labels as NMR probes, we describe herein iteratively optimized solid-phase synthesis of a series of glycopeptides (**1**–**8**) based on the MUC2 sequence segment (Fig. 1). In addition, we describe syntheses of aglycosylated heptapeptides **9** and **10**, and of glycoamino acid derivative **11**, all as reference compounds (Fig. 1).

Even with significant progress in the synthesis of complex glycopeptides in recent years,[6,8] further advances are being made. While elaboration of efficient solution-phase methods allows access to large quantities of the desired target compounds,[6] the development of strategies for solid-phase glycopeptide synthesis provides a broadly accessible approach to mucin glycopeptide preparation, particularly when simpler carbohydrate components like α-O-GalNAc (T_N) antigen are required.[5,7] Mucin glycopeptides with relatively complex glycan side chains have also been synthesized in the solid-phase mode.[10,11] Building blocks suitable for incorporation of glycosylated serine and threonine are prepared with appropriate protecting and activating groups[9,12] and are then introduced into the peptide chain by stepwise solid-phase synthesis. Protocols centered on 9-fluorenylmethyloxycarbonyl (Fmoc) N^{α}-amino protection, which is removed under mild basic conditions, are used compatibly with these building blocks. After chain assembly is complete, exposure to acid, typically 1–95% trifluoroacetic acid (TFA), depending on the precise resin and linkage, is used to release the glycopeptide from the resin. Use of acid concentrations at the high end of the range also serves to simultaneously remove acid-labile protecting groups for amino acid residue side chains, but additional chemical steps are needed to obtain the final desired mucin glycopeptides. For one, 2-azido groups must be converted to the corresponding acetamido groups; on-resin treatment with thioacetic acid (prior to acidolytic cleavage) is used for this purpose. O-Acetyl protecting groups on GalNAc must be removed as well, as usually achieved by transesterification featuring a catalytic amount of sodium methoxide in solution. Alternatively, hydrazine can be used for on-resin deacetylation. A significant portion of the present work entails extensive investigations of the experimental details and relative outcomes of the various strategies for manipulation of the glycan portion of the target glycopeptides.

2. Results and discussion

2.1. Solid-phase glycopeptide synthesis

Solid-phase synthesis of glycopeptides **1**–**7**, as outlined in Scheme 1, followed a variety of literature precedents.[5,7,9] Starting with a commercially available polyethylene glycol–polystyrene (PEG-PS) graft support[13,14] containing a tris(alkoxy)benzylamide (PAL) linker, the common tripeptide-resin intermediate H-Pro-Leu-Lys(Boc)-PAL-PEG-PS was assembled smoothly by Fmoc chemistry carried out on a continuous-flow peptide synthesizer. Remaining chain-elongation steps were carried out manually. For glycopeptide targets **1**–**7**, the appropriate protected building block, Fmoc-Thr-(Ac_3-α-D-$GalN_3$)-OPfp[12] (1.5 equiv, 30 mM), prepared in our laboratories, was incorporated as needed in the presence of 1-hydroxybenzotriazole (HOBt, 1.5 equiv, 30 mM) and *N*,*N*-diisopropylethylamine (DIEA, 1.5 equiv, 30 mM) in *N*,*N*-dimethylformamide (DMF). It should be noted that for all of the experiments reported in this paper, the Fmoc-Thr-(Ac_3-α-D-$GalN_3$)-OPfp building block used included ~10% of the unwanted β-anomer, but this did not represent a serious drawback, since later purifications [off-resin, protected, and another after final deprotection] of the full-length synthetic glycopeptides readily removed contaminants thus derived, to provide enantio-

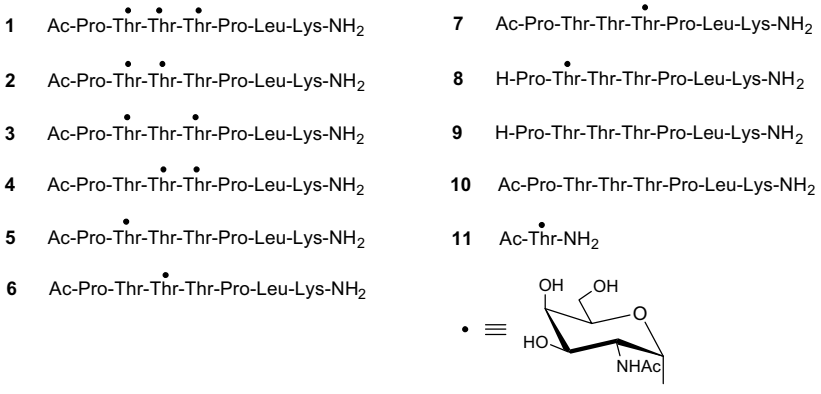
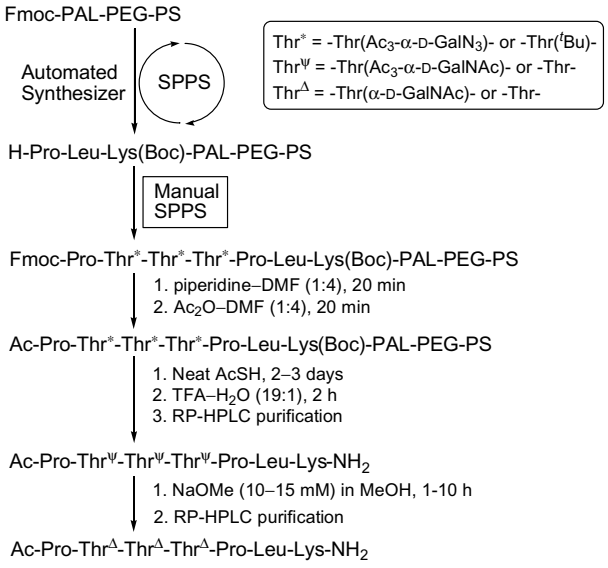


Figure 1. Mucin-like glycopeptides, aglycopeptides, and a glycosylated threonine derivative synthesized in this work.



Scheme 1. General parallel solid-phase peptide synthesis method of mucin-like glycopeptides **1**–**7**.

pure final products. Paulsen et al.[15] have made a similar observation. Nevertheless, since enantiopure building block can be isolated using preparative normal-phase high-performance liquid chromatography,[12] we would recommend this for any future experiments.

Fmoc-protected non-glycosylated amino acids (4 equiv) were incorporated, both manually and automated, following activation by 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/HOBt/DIEA (1:1:1, 70 mM each, manual), or HBTU (0.45 M in DMF)/HOBt (0.45 M in DMF)/DIEA (1.0 M in NMP) (1:1:2, automated). Following the completion of chain assembly, peptide resins were treated for 2–3 days with neat thioacetic acid to convert the azido functions to acetamido groups. Next, treatment with TFA–H_2O (19:1) was used to cleave glycopeptides from the supports, and concurrently remove *tert*-butyl (^{t}Bu) and *tert*-butyloxycarbonyl (Boc) protecting groups, respectively, from threonine and lysine side chains. The crude products were purified by reversed-phase HPLC (RPHPLC), following which removal of O-acetyl groups was carried out by using a solution of sodium methoxide in methanol [see next paragraph for details, and Section 2.3 for mechanistic references, as well as for an alternative strategy with on-resin deacetylation].

The aforementioned solution deacetylation reactions were analyzed by HPLC and electrospray-ionization mass spectrometry (ESIMS). The concentration of sodium methoxide was 10–15 mM, or 5 equiv with respect to total O-acetyl groups [this despite the literature view[16] that methoxide acts as a catalyst]. With the higher methoxide concentration, the 'apparent pH' (from blotting onto wet litmus paper) was 10–11, and deacetylation reactions for glycopeptides **2**, **3**, and **5**–**7** (of which the estimated concentrations were almost the same) were complete within 3 h. At lower 'apparent pH' of 9–10, a longer deacetylation time (8 h) was necessary (this was the case with glycopeptide **4**). For deacetylation of the precursor to glycopeptide **1**, the lower methoxide concentration reduced the 'apparent pH' to 8–9; in this case, partially deacetylated mixtures were detected by ESIMS and RPHPLC after 5 h. In order to drive the reaction to completion, more methoxide was added to bring the 'apparent pH' to 9–10, and an additional 5 h was sufficient. In all cases, RPHPLC purification gave pure glycopeptides (Fig. 2).

The glycopeptide **8**, which has a free N^{α}-amino group, was made by a somewhat different procedure. The single glycosylated amino acid was incorporated using commercially available N^{α}-(9-fluorenylmethoxycarbonyl)-O-(3,4,6-tri-O-acetyl-2-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine [Fmoc-Thr(Ac_3-α-D-GalNAc)-OH] as the building block, which was activated by HBTU/HOBt/DIEA (1:1:1, 1.5 equiv each). Therefore, the AcSH step (Scheme 1) was not necessary. In addition, the on-resin N-acetylation after terminal Fmoc removal was omitted. Finally, the aglycosylated controls **9** and **10** were prepared by straightforward solid-phase peptide synthesis.

Purified protected glycopeptides were characterized by RPHPLC [Table 1, which also presents the overall isolated yield and the RPHPLC retention time for each]

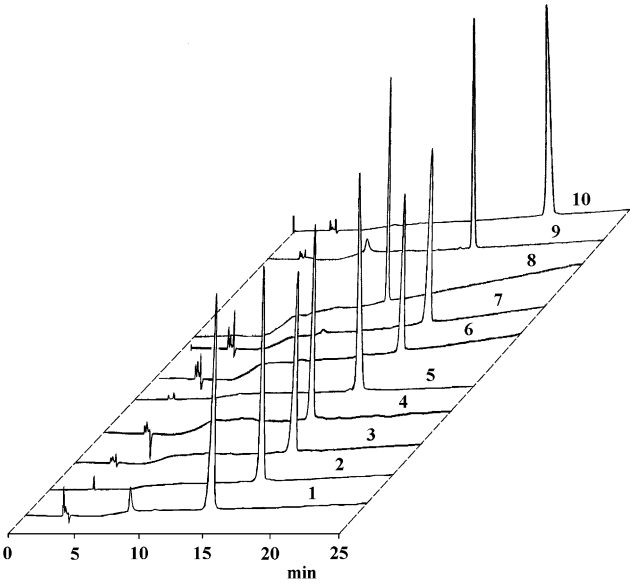


Figure 2. RPHPLC profiles of purified glycopeptides **1**–**8** and aglycopeptides **9**–**10**. Structures of these peptides are given in Figure 1. Analytical RPHPLC used a C_{18} column (4.6 × 250 mm) with detection at 220 nm and elution with 0.1% aq TFA (buffer A)–0.1% TFA in CH_3CN (buffer B), linear gradient from 0 to 40% buffer B, at a flow rate of 1 mL/min for 40 min (chromatograms shown above are truncated after 25 min, since no additional peaks were noted when running for the full 40 min).

Table 1. Glycopeptides **1**–**8** (unprotected) and corresponding per(O-acetylated) glycopeptide derivatives, along with heptapeptides **9** (free NH_2 terminal) and **10** (N-acetylated)

Glycopeptides	Sequence[a]	Molecular formula	Calcd.	Obsd. (*m*/*z*)[b]	Sugar number	Free OH	t_R (min)[c]	Yield[d] (%)
O-Acetylated **1**	Ac-PT$^{\Psi}$T$^{\Psi}$T$^{\Psi}$PLKNH$_2$	$C_{78}H_{120}N_{12}O_{35}$	1784.8	1786.1	3	0	35.3	—
O-Acetylated **2**	Ac-PT$^{\Psi}$T$^{\Psi}$TPLKNH$_2$	$C_{64}H_{101}N_{11}O_{27}$	1455.7	1457.0	2	1	30.4	—
O-Acetylated **3**	Ac-PT$^{\Psi}$TT$^{\Psi}$PLKNH$_2$	$C_{64}H_{101}N_{11}O_{27}$	1455.7	1457.2	2	1	29.8	—
O-Acetylated **4**	Ac-PTT$^{\Psi}$T$^{\Psi}$PLKNH$_2$	$C_{64}H_{101}N_{11}O_{27}$	1455.7	1457.0	2	1	30.8	—
O-Acetylated 5	Ac-PT$^{\Psi}$TTPLKNH$_2$	$C_{50}H_{82}N_{10}O_{19}$	1126.6	1127.7	1	2	26.0	—
O-Acetylated **6**	Ac-PTT$^{\Psi}$TPLKNH$_2$	$C_{50}H_{82}N_{10}O_{19}$	1126.6	1127.8	1	2	25.2	—
O-Acetylated **7**	Ac-PTTT$^{\Psi}$PLKNH$_2$	$C_{50}H_{82}N_{10}O_{19}$	1126.6	1127.8	1	2	24.3	—
O-Acetylated **8**	H-PT$^{\Psi}$TTPLKNH$_2$	$C_{48}H_{80}N_{10}O_{18}$	1084.6	1085.6	1	2	23.9	—
Deprotected **1**	Ac-PT$^{\Delta}$T$^{\Delta}$T$^{\Delta}$PLKNH$_2$	$C_{60}H_{102}N_{12}O_{26}$	1406.7	1407.7	3	9	14.0	7
Deprotected **2**	Ac-PT$^{\Delta}$T$^{\Delta}$TPLKNH$_2$	$C_{52}H_{89}N_{11}O_{21}$	1203.6	1204.7	2	7	15.8	25
Deprotected **3**	Ac-PT$^{\Delta}$TT$^{\Delta}$PLKNH$_2$	$C_{52}H_{89}N_{11}O_{21}$	1203.6	1204.6	2	7	16.5	19
Deprotected **4**	Ac-PTT$^{\Delta}$T$^{\Delta}$PLKNH$_2$	$C_{52}H_{89}N_{11}O_{21}$	1203.6	1204.7	2	7	15.4	32
Deprotected **5**	Ac-PT$^{\Delta}$TTPLKNH$_2$	$C_{44}H_{76}N_{10}O_{16}$	1000.6	1001.6	1	5	17.0	30
Deprotected **6**	Ac-PTT$^{\Delta}$TPLKNH$_2$	$C_{44}H_{76}N_{10}O_{16}$	1000.6	1001.6	1	5	18.4	25
Deprotected **7**	Ac-PTTT$^{\Delta}$PLKNH$_2$	$C_{44}H_{76}N_{10}O_{16}$	1000.6	1001.6	1	5	17.8	29
Deprotected **8**	H-PT$^{\Delta}$TTPLKNH$_2$	$C_{42}H_{74}N_{10}O_{15}$	958.5	959.6	1	5	14.4	36
9, with free NH_2	H-PTTTPLKNH$_2$	$C_{34}H_{61}N_9O_{10}$	755.5	756.5	0	3	15.6	37
10, N-acetylated	Ac-PTTTPLKNH$_2$	$C_{36}H_{63}N_9O_{11}$	797.5	798.7	0	3	19.5	46

[a] T^{Ψ} = Thr(Ac$_3$-α-D-GalNAc); T^{Δ} = Thr(α-D-GalNAc).
[b] ESIMS: glycopeptides were dissolved in 1:1 CH_3CN–H_2O, and detected as $[glycopeptide+H]^+$ ions.
[c] RPHPLC conditions are in Figure 2 legend.
[d] Overall isolated yields, based on initial substitution of resin. Yields were not determined on the O-acetylated intermediates.

and ESIMS. The mass spectrometric data were in complete accord with theoretical predictions. Trends in the RPHPLC retention time are shown in Figure 3. Due to their decreased hydrophilicities, retention times were longer for O-acetylated glycopeptides **1**–**8**, with the longest for O-acetylated glycopeptide **1**, which has the greatest number of protected sugar moieties present. Once the glycopeptides were deprotected, an inverse correlation held, due to the increased hydrophilicities imparted by the free OH groups. The presence of a free N^{α}-amino group, as in **8**, led to shorter retention with respect to the usual N-acetyl compounds **5**–**7**.

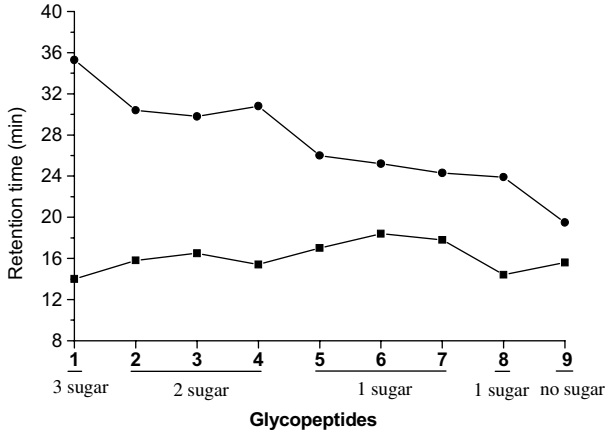


Figure 3. Relationship between RPHPLC retention time of free glycopeptides **1**–**8** (■) and their O-acetylated derivatives (●). For comparison, the last point is peptide **9** (free NH_2 terminal) and peptide **10** (N-acetylated). RPHPLC conditions are in Figure 2 legend.

While retention time is a function of the number of glycosylated residues, the dependence on this property is weaker than for the protected precursors. Comparing acetylated and fully deprotected glycopeptides, it appears that each free OH group contributes to a reduction of about 2 min in retention time under standardized conditions. Within the series of glycopeptides with permutations of one or two sites of glycosylation, respectively, retention times vary as a function of the specific locus of glycosylation for the same total number of pendant sugars, suggesting some conformational consequences of the specific pattern of glycosylation. Consistent with this, RPHPLC data published by Takeuchi et al.,[2] who accessed several N-terminal fluorescently labeled partially glycosylated peptides by enzymatic actions on PTTTPLK, show some variation in retention based on locus of glycosylation, even when the number of sugars was constant.

2.2. On-resin conversion of azido groups to acetamido groups

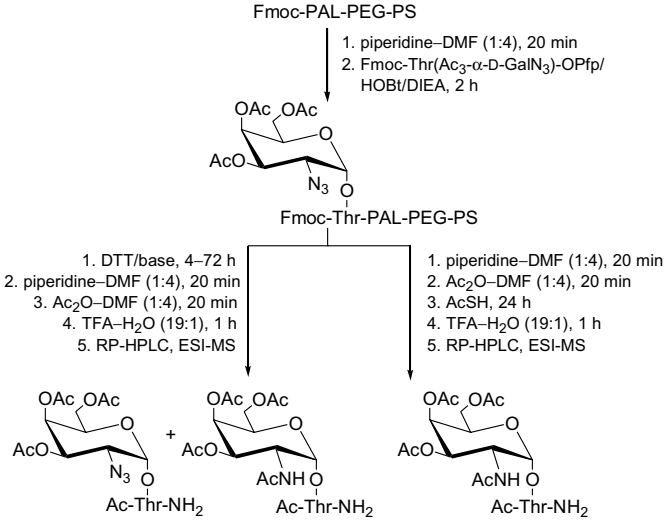
The synthetic method (Scheme 1) described in the previous section features a building block that has an azido function, rather than the finally desired N-acetyl group. While it would seem optimal to use directly a building block with N-acetyl, such compounds are difficult to obtain in good yield, since there are yield-diminishing side reactions along the way, and active ester moieties are unstable to intermediate reduction steps.[17,18] In the approach favored here, Fmoc-Thr(Ac$_3$-α-D-GalN$_3$)-OPfp[12] (or the corresponding Ser derivative) is used

to incorporate the azidosugar into the growing chain, and subsequently, reduction/acetylation is achieved by treatment of the resin-bound protected glycopeptide with neat thioacetic acid,[19] as first done in this context by Paulsen and co-workers[20] following a solution precedent of Rosen et al.[21] However, the thioacetic acid procedure is not without problems. First, the reaction is quite slow, taking on the order of days to reach completion at 25 °C. Second, the thioacetic acid must be of exquisite purity, achieved by multiple distillations;[22,23] otherwise, byproducts form in which a thioacetyl group replaces the desired acetyl [C═S in place of C═O], as first reported by Paulsen et al.[23] and confirmed in the present study. These facts provided impetus, first by others[18] and then in our laboratories (see below), to investigate alternative reducing agents, such as dithiothreitol (DTT), followed by a separate acetylation step. Our experiments were carried out primarily using Fmoc-Thr(Ac_3-α-D-$GalN_3$)-PAL-PEG-PS as a model, but additional experiments used a full glycoheptapeptide resin (Scheme 2).

With DTT as the reducing agent, we were not even able to convert half of the 2-azido groups to the corresponding amine under 'reasonable' conditions. Nevertheless, these experiments illustrate several interesting points: (i) reduction is base-catalyzed, with reaction in the presence of DIEA (Table 2, entries 2–5) or 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (entry 6), but with no reaction in the presence of the weak base, collidine[24] (entry 1); (ii) the catalysis by base is limited, insofar as there was no benefit from increasing the DIEA concentration from 0.1 mM to 0.1 M (entry 2 vs 4); (iii) there was negligible benefit in switching the solvent from CH_2Cl_2 to DMF (entry 4 vs 7); (iv) vigorous shaking was advantageous, by a factor of 5- to 8-fold, with respect to gentle mixing (entries 2, 4 vs 3, 5). The only way to achieve the desired conversion was to use the original AcSH reduction/acetylation protocol. Accentuating this conclusion, the complex protected glycoheptapeptide-resin Fmoc-Pro-Thr*-Thr*-Thr*-Pro-Leu-Lys-PAL-PEG-PS, which contains three consecutive Thr(Ac_3-α-D-$GalN_3$), was not at all converted with DTT (entry 9), but quantitatively converted with neat thioacetic acid (entry 10). [During the review process for this manuscript, an anonymous referee called our attention to several papers[25,26] which use 18–24 h treatment with AcSH–pyridine (2:1) to achieve similar conversions.]

2.3. Optimization of on-resin removal of O-acetyl groups

The carbohydrate portion of glycopeptides is most commonly protected for synthesis by easily introduced O-acetyl groups, which have the added benefit of conferring enhanced acid stability of glycosidic bonds to TFA (as used for resin cleavage), and also help with solubility of the glycosylated building blocks in organic solvents. According to the literature, O-acetyl groups can be removed with basic reagents either before or after cleavage from the resin. In the former case, hydrazine hydrate in methanol has been reported as the reagent of choice.[27] However, most researchers prefer to carry out deacetylation after glycopeptides have been released into solution, since the reaction can be more easily monitored. Both hydrazine–hydrate in methanol[28] and sodium methoxide in methanol[29–31] have been used successfully to effect solution deacetylation, with the latter more common. Catalytic sodium methoxide is believed to promote Zemplén transesterification, which occurs with neither



Scheme 2. Experimental design to evaluate methods for reduction of azido moiety in Ac-Thr*-PAL-PEG-PS. The same general design was used with glycoheptapeptide-resin Fmoc-Pro-Thr*-Thr*-Thr*-Pro-Leu-Lys-PAL-PEG-PS, with *attempted* reduction by treatment with DTT (0.2 M) and DIEA (0.1 mM) in CH_2Cl_2 for 2×4 h [see Table 2, entry 9]. Thr* = Thr(Ac_3-α-D-$GalN_3$).

Table 2. Conversion of azido moieties to N-acetylamines as a function of reducing agent and other experimental conditions[a]

Entry	Substrate[b]	Reducing agent (conc)	Base (conc)	Solvent	Time (h)	Shaker[c]	NHAc:N_3[d]
1	Fmoc-Thr*-PAL-PEG-PS	DTT (0.2 M)	Collidine (0.1 mM)	CH_2Cl_2	2 × 3	Multimixer	0:100
2	Fmoc-Thr*-PAL-PEG-PS	DTT (0.2 M)	DIEA (0.1 mM)	CH_2Cl_2	2 × 3	Multimixer	42:58
3	Fmoc-Thr*-PAL-PEG-PS	DTT (0.2 M)	DIEA (0.1 mM)	CH_2Cl_2	24	Labquake	2:98
4	Fmoc-Thr*-PAL-PEG-PS	DTT (0.2 M)	DIEA (0.1 M)	CH_2Cl_2	2 × 2	Multimixer	41:59
5	Fmoc-Thr*-PAL-PEG-PS	DTT (0.2 M)	DIEA (0.1 M)	CH_2Cl_2	72	Labquake	20:80
6	Fmoc-Thr*-PAL-PEG-PS	DTT (0.2 M)	DBU (0.1 M)	CH_2Cl_2	2 × 3	Multimixer	26:74
7	Fmoc-Thr*-PAL-PEG-PS	DTT (0.2 M)	DIEA (0.1 M)	DMF	2 × 3	Multimixer	45:55
8[e]	Fmoc-Thr*-PAL-PEG-PS	AcSH (Neat)	—	AcSH	24	Labquake	100:0
9	Glycoheptapeptide-resin	DTT (0.2 M)	DIEA (0.1 mM)	CH_2Cl_2	2 × 4	Multimixer	0:100
10[e]	Glycoheptapeptide-resin	AcSH (Neat)	—	AcSH	72	Labquake	100:0

[a] The overall outline of Scheme 2 was used, and reactions were carried out with 20 mg of substrate resin. The sequence of steps for use of DTT was (i) reduction with DTT; (ii) Fmoc removal; (iii) acetylation of peptidyl and sugar free amino groups; (iv) cleavage from support with 19:1 TFA–H_2O; (v) HPLC and ESIMS analysis. For use of AcSH: (i) Fmoc removal; (ii) acetylation of peptidyl free amino group; (iii) reduction/sugar N-acetylation with AcSH; (iv) and (v) the same as previous.

[b] Thr* = Thr(Ac$_3$-α-D-GalN_3); glycoheptapeptide-resin = Fmoc-Pro-Thr*-Thr*-Thr*-Pro-Leu-Lys-PAL-PEG-PS, the precursor to glycopeptide **1**.

[c] Mistral multimixer provided vigorous shaking; Labquake provided gentle agitation by rotation.

[d] Reduced/acetylated:unreacted azido groups, calculated from peak areas upon analytical RPHPLC.

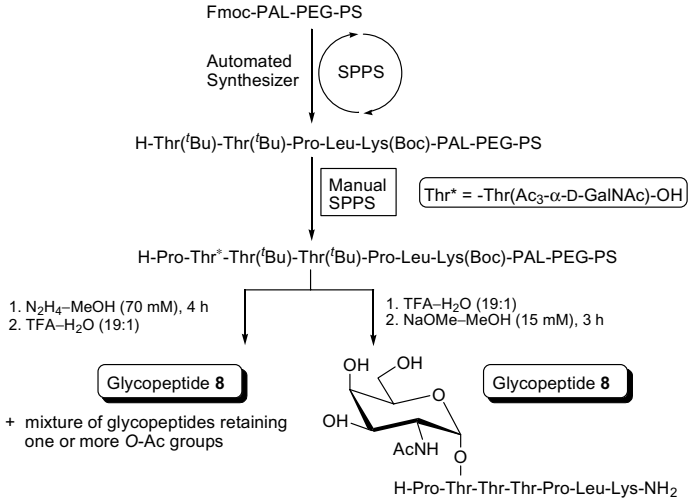
[e] As controls, samples that had not been treated with reducing reagents showed 100% unreacted N_3.

β-elimination nor epimerization of the stereocenters.[32] Despite outstanding results with a model glycopeptide,[32] the need for careful choice and control of reaction conditions in order to minimize undesirable base-catalyzed side reactions has been stressed.[33]

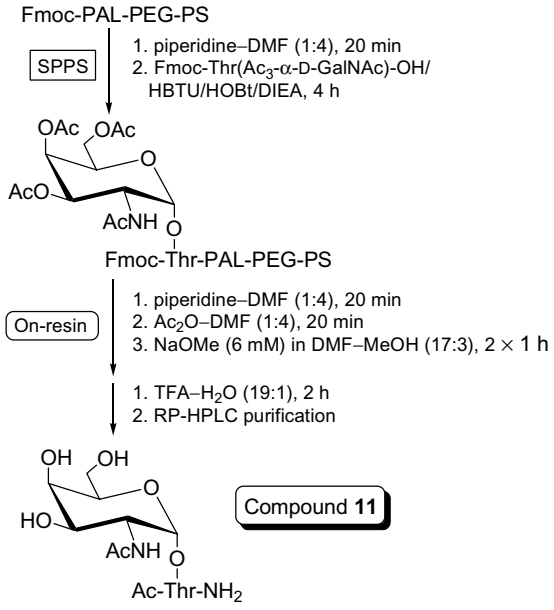
Leading to the synthesis of glycopeptide **8**, the resin-bound intermediate H-Pro-Thr-(Ac$_3$-α-D-GalNAc)-Thr(^{t}Bu)-Thr(^{t}Bu)-Pro-Leu-Lys(Boc)-PAL-PEG-PS was treated with hydrazine (70 mM) in methanol for 4 h (Scheme 3, left side). After acidolytic cleavage of the glycopeptide from the resin, the product mixture was monitored by RPHPLC and ESIMS. These results showed that only about half of the O-acetyl groups had been removed. In contrast, when the glycopeptide was first cleaved from the resin, and then treated with sodium methoxide in methanol (pH 11–12) for 3 h, O-acetyl groups were removed smoothly and completely.

For on-resin removal of O-acetyl groups, a solution of sodium methoxide (6 mM) in DMF–MeOH (17:3) was used.[34] The solvent milieu was chosen for optimal resin swelling. The model Ac-Thr(Ac$_3$-α-D-GalNAc)-PAL-PEG-PS was treated for 2 h, after which acid cleavage was carried out, and final RPHPLC (see Fig. S12 in Supplementary data) and ESIMS analysis of the product revealed that the desired deacetylation had proceeded quantitatively (Scheme 4).

The aforementioned on-resin deacetylation method was applied next to the synthesis of glycopeptide **4** (Scheme 5, right side). For comparison, solution deacetylation after cleavage from the support was also carried out (left side). While both reactions went smoothly, the on-resin procedure required slightly less time (<6 h vs 8 h) and made it possible to avoid an RPHPLC purification step.



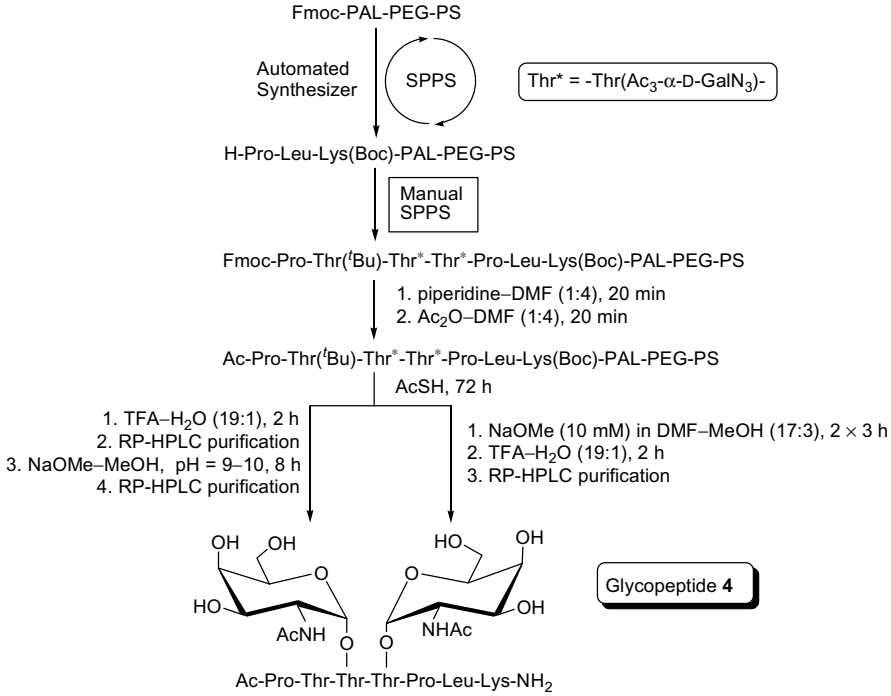
Scheme 3. Solid-phase synthesis of glycopeptide **8**, H-Pro-Thr-(α-D-GalNAc)-Thr-Thr-Pro-Leu-Lys-NH_2, emphasizing alternative deacetylation strategies.



Scheme 4. Solid-phase synthesis of Ac-Thr(α-D-GalNAc)-NH_2 (compound **11**).

2.4. Preliminary 1D ^{1}H NMR study of synthetic glycopeptides

This work has provided a series of glycopeptides, the conformations of which are being evaluated by NMR spectroscopy. From this, we can discern progressive conformational changes in the molecules as the density of glycosylation is increased, and thereby provide a window on their behavior as GalNAc transferase substrates. ^{1}H NMR shifts and dispersion of the peptide backbone amide protons provide indications of conformational change, with greater dispersion being associated with a more well-defined conformation. Amide ^{1}H NMR spectra, in H_2O–D_2O (9:1), of the N^{α}-acetylated peptide itself (**10**), and of N^{α}-acetylated glycopeptides with sequentially one, two, or three threonine residues glycosylated, starting from residue 2 (glycopeptides **5**, **2**, and **1**), are presented in Figure 4. There is a pronounced downfield shift of the respective amide protons upon glycosylation of the residue in question, relative to what is found for the peptide itself. With attachment of GalNAcs to residues 2 (glycopeptide **5**), or 2 and 3 (glycopeptide **2**), the effect seems more local to the glycosylated residue. However, when all three threonines (residues 2, 3, and 4) are glycosylated (glycopeptide **1**), a more long-range effect seems to emerge that propagates beyond the subsequent Pro5 residue to the backbone of the C-terminal Leu6 and Lys7 residues. The residue-dependent shift pattern for the amide protons of **1** is quite similar to that for the glycosylated residues in Ac-S*T*T*AV-OH (with asterisks denoting α-GalNAc) that we reported on previously,[35,36] suggesting that organization of the triplet mucin motif in **1** is homologous. In both instances, the N-terminal residue of the glycosylated triplet is the most upfield. We also showed a similar pattern for a sequence based on Ac-S*S*S*AVAV-OH.[36] This ordering is seen for a triplet of glycosylated Ser residues in a different context as well.[37]



Scheme 5. Solid-phase synthesis of glycopeptide **4**, Ac-Pro-Thr-Thr(α-D-GalNAc)-Thr(α-D-GalNAc)-Pro-Leu-Lys-NH_2.

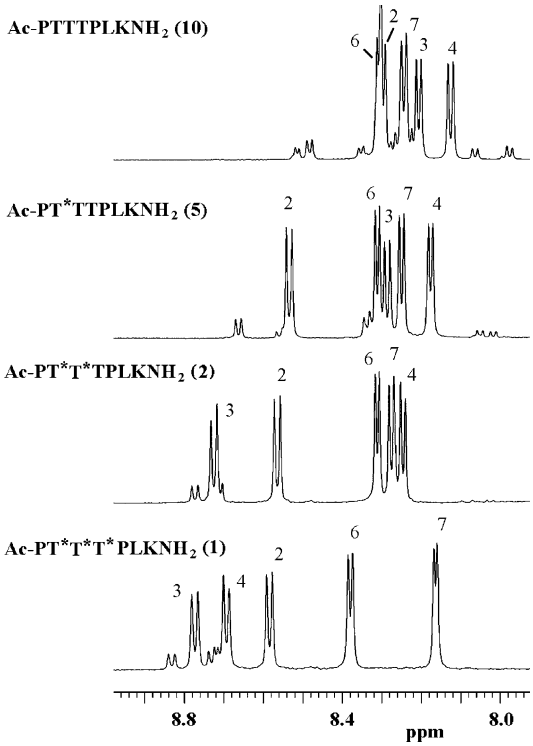


Figure 4. Amide proton signals as a function of glycosylation state. From top to bottom are shown heptapeptide **10** (Ac-PTTTPLKNH$_2$), monoglycosylated **5** (Ac-PT*TTPLKNH$_2$), diglycosylated **2** (Ac-PT*T*TPLKNH$_2$), and triglycosylated **1** (Ac-PT*T*T*PLKNH$_2$), where T* = Thr(α-D-GalNAc). Next to each doublet is the residue number in the peptide, starting with 1 for N-terminal Pro. Spectra were observed at 600 MHz, using solutions of 4 mg of each compound dissolved in 0.6 mL of 9:1 H_2O–D_2O, phosphate buffer (10 mM, pH ~3.7) at 25 °C. Shifts are relative to 2,3-dimethyl-2-silapentane-5-sulfonate (DSS, sodium salt). The water solvent signal was suppressed by presaturation. The downfield minor signals arise from the presence of a small proportion of *cis*-peptide bonds involving proline.

Together, these imply an underlying common conformational preference for triplet mucin motifs.

3. Experimental

3.1. General methods

Unless indicated otherwise, solvents and reagents were used directly as obtained from Aldrich Chemical Co. (Milwaukee, WI), in HPLC and/or the highest available grade. Methanol was maintained over 3 Å molecular sieves. AcSH (bp 87–88 °C) was distilled on a 500-g scale, overall five consecutive times, always at atmospheric pressure, using a 5-cm Vigreux column with an ice-cooled condenser, and stored at 4 °C whereupon it was usable for the next several months to effect azido reduction. NaOMe and DTT needed to be used within a month of opening the respective bottles. Protected N^{α}-Fmoc-amino acid derivatives and Fmoc-PAL-PEG-PS supports (initial loading 0.18–0.21 mmol/g) for peptide synthesis were obtained from PerSeptive Biosystems (now Applied Biosystems) (Framingham, MA) and Novabiochem (La Jolla, CA). TFA and DIEA were from Fisher (Pittsburgh, PA). HBTU was from Advanced ChemTech (Louisville, KY). The glycosylated amino acid building block, Fmoc-L-Thr(Ac_3-α-D-GalN_3)-OPfp, was prepared as described previously.[12] Analytical RPHPLC was performed using a Vydac analytical C_{18} reversed-phase column (218TP54; 5 μm, 300 Å, 4.6 × 250 mm) on a Beckman system configured with a Model 125 programmable solvent module pump and a Model 165 variable wavelength detector controlled from an IBM PC running Beckman System Gold software, with detection at 220 nm. Samples were chromatographed at 1.0 mL/min using linear gradients of 0.1% aqueous TFA (buffer A) and 0.1% TFA in CH_3CN (buffer B), from 0% to 40% buffer B over 40 min, except as indicated otherwise. Crude glycopeptides were purified by preparative RPHPLC on a Waters system configured with a 600E system controller, 625 pump, and a 484 detector controlled from a PC running Millennium chromatography software under Windows 3.1, using Vydac C_{18} (218TP1010; 10 μm; 300 Å; 10 × 250 mm) in a radial compression module (Waters, Milford, MA), all on a Waters Deltaprep system at 3–5 mL/min, using gradients which varied according to the properties of the particular sequence, and detection at 220 nm. Fractions with the desired peptide or glycopeptide were combined and lyophilized. Yields were calculated based on the loading of the resin used. ^{1}H NMR spectra were observed at 600 MHz on a Varian instrument, using solutions of 4 mg of each compound dissolved in 0.6 mL of 9:1 H_2O–D_2O, phosphate buffer (10 mM, pH ~3.7) at 25 °C. Shifts are relative to 2,3-dimethyl-2-silapentane-5-sulfonate (DSS, sodium salt). The water solvent signal was suppressed by presaturation.

3.2. Automatic solid-phase syntheses of H-Pro-Leu-Lys(Boc)-PAL-PEG-PS and Thr(^{t}Bu)-Thr(^{t}Bu)-Pro-Leu-Lys(Boc)-PAL-PEG-PS

Automated peptide chain assembly was carried out in the continuous-flow mode on a PerSeptive Biosystems Pioneer Peptide Synthesizer (Framingham, MA). Unless indicated otherwise, molar equivalents are given relative to resin-bound amine. Side-chain protection used was Boc for Lys and ^{t}Bu for Thr. Fmoc removal was achieved with 1:4 piperidine–*N*-methylpyrrolidone (NMP) for 20 min. Fmoc-amino acids (4 equiv) were coupled for 1 h in the presence of HBTU (4 equiv)/HOBt (4 equiv)/DIEA (8 equiv) in NMP at 25 °C. Washings between reactions were carried out with NMP, without capping. Double couplings were performed at all positions. The H-Pro-Leu-Lys(Boc)-PAL-PEG-PS resin, which is sub-

sequently referred to as 'tripeptide-resin', was assembled starting with Fmoc-PAL-PEG-PS (2.2 g, 0.18 mmol/g, 0.40 mmol). Acidolytic deprotection/cleavage of the peptide resin (~10 mg, carried out after an Fmoc removal step) with TFA–H_2O (19:1) gave the expected molecular weight of the tripeptide H-Pro-Leu-Lys-NH_2: ESIMS m/z 378.3 $[M+Na]^+$, 356.3 $[M+H]^+$. The H-Thr(^{t}Bu)-Thr(^{t}Bu)-Pro-Leu-Lys(Boc)-PAL-PEG-PS resin, which is subsequently referred to as 'pentapeptide resin', was assembled starting with Fmoc-PAL-PEG-PS (0.21 mmol/g) following the same procedures.

3.3. General procedure for solid-phase glycopeptide synthesis

The 'tripeptide resin' described in Section 3.2 was distributed, respectively, to six glass vessels (8.5 mL) containing a porous polypropylene frit and used for the manual completion of glycopeptides **1**–**4** and **6**–**7**. The 'pentapeptide resin', described in Section 3.2, was distributed to two glass vessels (8.5 mL), respectively, for the manual completion of glycopeptides **5** and **8**. A Multimixer was used to achieve vigorous shaking. Fmoc removal was achieved with piperidine–DMF (1:4) for 20 min, followed by 2-h couplings of Fmoc-amino acids (4 equiv), preactivated for 10 min and mediated by HBTU (4 equiv)/HOBt (4 equiv)/DIEA (5 equiv) in DMF at 25 °C. Coupling of Fmoc-O-(Ac_3-α-D-$GalN_3$)-L-Thr-OPfp (1.4–1.5 equiv) in DMF was carried out in the presence of stoichiometric amounts each of HOBt and DIEA for 2 h at 25 °C. Washings between reactions were carried out with DMF and CH_2Cl_2, and no intermediate capping steps were done. After complete chain assembly, N-acetylation (except for **8**) was achieved by treatment with Ac_2O–DMF (1:4) for 20 min. Completion of each coupling/deprotection/acetylation reaction was monitored either by the Kaiser ninhydrin test [reagents were from PerSeptive Biosystems (Framingham, MA) and used directly] or the isatin test [reagents were prepared and used according to the literature,[38] specifically for monitoring Fmoc deprotection and coupling of proline]. After chain assembly was completed, the glycopeptide resin was washed with DMF, CH_2Cl_2, and 2-PrOH, dried with a stream of nitrogen, and then treated with AcSH (again, except for **8**) for 72 h (fresh AcSH was added every 24 h) to achieve azido reduction on a Labquake shaker. Subsequently, the resin was washed thoroughly with DMF and CH_2Cl_2, and dried in a vacuum desiccator for the next reaction.

3.4. General procedure for resin cleavage and removal of acetyl groups with sodium methoxide

The glycopeptide resin (typically 250 mg) was transferred to a sterile plastic syringe with a porous polypropylene frit (10 mL). O-Acetylated glycopeptides were cleaved from the resin with concurrent removal of the ^{t}Bu and Boc side-chain protecting groups of Thr and Lys, respectively, by treatment with TFA–H_2O (19:1) for 2 h at 25 °C. The cleaved resin (~250 mg) was then washed extensively with TFA (3 × 0.5 mL), the combined filtrates were evaporated, and the residue was coevaporated with toluene (3 × 20 mL) to remove TFA. O-acetylated glycopeptides were purified by semi-preparative RPHPLC, and the correct fractions were pooled and lyophilized. The lyophilized material was then dissolved in methanol (~5 mg/mL) for removal of O-acetyl groups as applicable. Sodium methoxide (0.14 M) in methanol (0.4–0.5 mL) was added dropwise to the glycopeptide solution. Depending on the sample, the pH was between 9 and 11 (as detected by pH paper), and the final concentration of NaOMe was between 10 and 15 mM. The O-deacetylation reaction was monitored by analytical RPHPLC and ESIMS. Deprotection time varied among different glycopeptides. Neutralization was carried out by addition of dry ice until pH 6 was reached. Pure glycopeptides were obtained after semi-preparative RPHPLC and lyophilization.

3.5. Solid-phase synthesis of glycopeptides 1–8, aglycopeptides 9 and 10, and amino acid derivative 11

3.5.1. Preparation of glycopeptide 1: Ac-Pro-Thr(α-D-GalNAc)-Thr(α-D-GalNAc)-Thr(α-D-GalNAc)-Pro-Leu-Lys-NH_2. *Method A*: Starting with 'tripeptide resin' (450 mg), and following Sections 3.3 and 3.4 to complete assembly of the protected glycopeptide sequence, achieve on-resin conversion of azido to acetamide groups, and cleave material from the support, O-protected Ac-Pro-Thr(Ac_3-α-D-GalNAc)-Thr(Ac_3-α-D-GalNAc)-Thr(Ac_3-α-D-GalNAc)-Pro-Leu-Lys-NH_2 was obtained. ESIMS: m/z 1786.1 $[M+H]^+$, t_R 35.3 min. Removal of O-acetyl groups was performed, following Section 3.4, at pH 8–9 for 5 h and monitored by ESIMS: m/z 1407.9 $[M+H]^+$ for fully deacetylated; 1450.0 $[M+H]^+$ for monoacetylated, 1492.0 $[M+H]^+$ for diacetylated; 1556.0 $[M+Na]^+$ for triacetylated; 1576.0 $[M+H]^+$; 1598.0 $[M+Na]^+$ for tetraacetylated; 1619.1 $[M+H]^+$ for pentaacetylated; 1660.1 $[M+H]^+$ for hexaacetylated; and 1702.1 $[M+H]^+$ for heptaacetylated. A second O-deacetylation step was then performed at pH 9–10 for 5 h and provided the title product (8 mg, 7%), after purification by preparative HPLC [buffer B: 0–5% over 100 min at 3 mL/min; Analytical RPHPLC, t_R 14.0 min]. ESIMS: m/z 1430.0 $[M+Na]^+$, 1407.7 $[M+H]^+$. *Method B*: Following Section 3.3, but with no reduction of azido groups, the glycopeptide resin (50 mg) was treated with DTT (0.2 M) and DIEA (0.1 mM) in CH_2Cl_2 on a Multimixer (2 × 4 h). After removal of Fmoc, acetylation, and cleavage, the residue was analyzed by RPHPLC [t_R 17.1 min, from 30% to 70% buffer B over 40 min] and ESIMS.

Ac-Pro-Thr(Ac_3-α-D-$GalN_3$)-Thr(Ac_3-α-D-$GalN_3$)-Thr-(Ac_3-α-D-$GalN_3$)-Pro-Leu-Lys-NH_2: *m/z* 1738.0 $[M+H]^+$, indicating that the DTT did not reduce the azido group.

3.5.2. Preparation of glycopeptide 2: Ac-Pro-Thr(α-D-GalNAc)-Thr(α-D-GalNAc)-Thr-Pro-Leu-Lys-NH_2. Starting with 'tripeptide resin' (250 mg), and following Sections 3.3 and 3.4 to complete the assembly of the glycopeptide and achieve on-resin conversion of azido to acetamido groups, O-protected Ac-Pro-Thr(Ac_3-α-D-GalNAc)-Thr(Ac_3-α-D-GalNAc)-Thr-Pro-Leu-Lys-NH_2 was obtained. ESIMS *m/z* 1457.0 $[M+H]^+$, t_R 30.4 min. Removal of O-acetyl groups was performed, following Section 3.4, at pH 10–11 for 2 h, and provided the title product (13 mg, 25%) after purification by preparative HPLC [buffer B: 0–25% over 100 min at 4 mL/min; analytical HPLC, t_R 15.8 min] and lyophilization. ESIMS: *m/z* 1204.7 $[M+H]^+$.

3.5.3. Preparation of glycopeptide 3: Ac-Pro-Thr(α-D-GalNAc)-Thr-Thr(α-D-GalNAc)-Pro-Leu-Lys-NH_2. Starting with 'tripeptide resin' (250 mg), and following Sections 3.3 and 3.4 to complete the assembly of the glycopeptide and achieve on-resin conversion of azido to acetamido groups, O-protected Ac-Pro-Thr(Ac_3-α-D-GalNAc)-Thr-Thr(Ac_3-α-D-GalNAc)-Pro-Leu-Lys-NH_2 was obtained. ESIMS *m/z* 1457.2 $[M+H]^+$, t_R 29.8 min. Removal of O-acetyl groups was performed, following Section 3.4, at pH 10–11 for 2 h, and provided the title product (10 mg, 19%) after purification by preparative HPLC [buffer B: 0–15% over 90 min at 4 mL/min; Analytical RPHPLC, t_R 16.5 min] and lyophilization. ESIMS: *m/z* 1204.6 $[M+H]^+$.

3.5.4. Preparation of glycopeptide 4: Ac-Pro-Thr-Thr(α-D-GalNAc)-Thr(α-D-GalNAc)-Pro-Leu-Lys-NH_2. Starting with 'tripeptide resin' (250 mg), and following Section 3.3 to complete the assembly of the glycopeptide, a portion of the glycopeptide resin (50 mg) was treated with NaOMe (10 mM) in DMF–MeOH (17:3), agitated on a Multimixer (2 × 3 h). Glycopeptide-resin cleavage followed Section 3.4 and provided 'filtrate A'. Following Section 3.4, the remaining glycopeptide resin (~200 mg) provided O-protected Ac-Pro-Thr-Thr(Ac_3-α-D-GalNAc)-Thr(Ac_3-α-D-GalNAc)-Pro-Leu-Lys-NH_2. ESIMS: *m/z* 1457.0 $[M+H]^+$, t_R 30.8 min. Again following Section 3.4, removal of O-acetyl groups was performed at pH 9–10 for 8 h. Combining with 'filtrate A', this provided the title product (17 mg, 32%) after purification by preparative HPLC [buffer B: 0–5% over 100 min at 4 mL/min; Analytical RPHPLC, t_R 15.4 min] and lyophilization. ESIMS: *m/z* 1204.7 $[M+H]^+$.

3.5.5. Preparation of glycopeptide 5: Ac-Pro-Thr(α-D-GalNAc)-Thr-Thr-Pro-Leu-Lys-NH_2. Starting with 'pentapeptide resin' (210 mg), and following Section 3.3 to complete the assembly of the glycopeptide and achieve on-resin conversion of azido to acetamido, O-protected Ac-Pro-Thr(Ac_3-α-D-GalNAc)Thr-Thr-Pro-Leu-Lys-NH_2 was obtained. ESIMS: *m/z* 1127.7 $[M+H]^+$, t_R 26.0 min. Additionally, a trace amount of byproduct, Ac-Pro-Thr(α-D-GalNH(C=S)CH_3)-Thr-Thr-Pro-Leu-Lys-NH_2 was also isolated and identified. Analytical RPHPLC, t_R 30.0 min. ESIMS: *m/z* 1143.6 $[M+H]^+$. Following Section 3.4, removal of O-acetyl groups from the main product was performed at pH 10–11 for 1 h, and provided the title product (13 mg, 30%) after purification by preparative HPLC [buffer B: 0–15% B over 60 min at 4 mL/min; Analytical RPHPLC, t_R 17.0 min] and lyophilization. ESIMS: *m/z* 1023.7 $[M+Na]^+$, 1001.6 $[M+H]^+$. A second component (6 mg, 14%), apparently the β-isomer, was also separated. t_R 18.5 min. ESIMS: *m/z* 1001.6 $[M+H]^+$.

3.5.6. Preparation of glycopeptide 6: Ac-Pro-Thr-Thr(α-D-GalNAc)-Thr-Pro-Leu-Lys-NH_2. Starting with 'tripeptide resin' (250 mg), and following Sections 3.3 and 3.4 to complete the assembly of the glycopeptide and achieve on-resin conversion of azido to acetamido groups, O-protected Ac-Pro-Thr-Thr(Ac_3-α-D-GalNAc)-Thr-Pro-Leu-Lys-NH_2 was obtained. ESIMS *m/z* 1127.8 $[M+H]^+$, t_R 25.2 min. Removal of O-acetyl groups was performed, following Section 3.4, at pH 10–11 for 2 h and provided the title product (11 mg, 25%) after purification by preparative HPLC [buffer B: 0–10% over 100 min at 4 mL/min; analytical RPHPLC, t_R 18.4 min] and lyophilization. ESIMS: *m/z* 1023.6 $[M+Na]^+$, 1001.6 $[M+H]^+$.

3.5.7. Preparation of glycopeptide 7: Ac-Pro-Thr-Thr-Thr(α-D-GalNAc)-Pro-Leu-Lys-NH_2. Starting with 'tripeptide resin' (250 mg), and following Sections 3.3 and 3.4 to complete the assembly of the glycopeptide and achieve on-resin conversion of azido to acetamido groups, O-protected Ac-Pro-Thr-Thr-Thr(Ac_3-α-D-GalNAc)-Pro-Leu-Lys-NH_2 was obtained. ESIMS *m/z* 1127.8 $[M+H]^+$, t_R 24.3 min. Removal of O-acetyl groups was performed, following Section 3.4, at pH 10–11 for 2.5 h and provided the title product (13 mg, 29%) after purification by preparative HPLC [buffer B: 0–5% over 10 min, 5–7% over 90 min; Analytical RPHPLC, t_R 17.8 min] and lyophilization. ESIMS: *m/z* 1001.6 $[M+H]^+$.

3.5.8. Preparation of glycopeptide 8: H-Pro-Thr(α-D-GalNAc)-Thr-Thr-Pro-Leu-Lys-NH_2. Starting with 'pentapeptide resin' (250 mg), and following Section 3.3, glycopeptide assembly was completed, using commercially available Fmoc-Thr(Ac_3-α-D-GalNAc)-OH as a building block. O-Acetyl groups were removed by each of two methods. *Method A*: The glycopeptide resin (58 mg) was shaken with a solution of $N_2H_4 \cdot H_2O$

(98%, 3.2 mg, 70 μmol) in MeOH (1 mL), for 4 h at 25 °C, followed by washing with MeOH (5 × 1 mL) and Et_2O (5 × 1 mL). After cleavage as described in Section 3.4, the filtrates were added to cold Et_2O (100 mL). Centrifugation, purification by preparative HPLC [buffer B: 0–50% over 100 min at 5 mL/min; Analytical RPHPLC, t_R 14.4 min] and lyophilization provided the title glycopeptide **8** (4 mg, 36%). ESIMS *m/z* 981.6 $[M+Na]^+$, 959.6 $[M+H]^+$. Partially deacetylated compound **8′** (~2 mg) was detected and separated, t_R 17.0 min, ESIMS *m/z* 1023.6 $[M+Na]^+$, 1001.6 $[M+H]^+$. Fully protected compound **8″** was also detected: t_R 23.9 min (identified by using standard sample as external reference). The ratios of **8**:**8′**:**8″** were 14:11:1, determined from the HPLC profiles. *Method B*: Following Section 3.4, cleavage of glycopeptide resin (42 mg) provided O-protected H-Pro-Thr(Ac_3-α-D-GalNAc)-Thr-Thr-Pro-Leu-Lys-NH_2. ESIMS: *m/z* 1107.6 $[M+Na]^+$, 1085.6 $[M+H]^+$; t_R 23.9 min. Removal of O-acetyl groups was performed, following Section 3.4, at pH 11–12 for 3 h and provided the title product (2 mg, 25%) after purification by preparative HPLC [buffer B: 0–30% over 60 min at 4 mL/min] and lyophilization. ESIMS *m/z* 981.6 $[M+Na]^+$, 959.6 $[M+H]^+$.

3.5.9. Preparation of heptapeptide 9: H-Pro-Thr-Thr-Thr-Pro-Leu-Lys-NH_2. Starting with Fmoc-PAL-PEG-PS (250 mg, 0.21 mmol/g), and following Section 3.2, but omitting the final N-acetylation step, the title compound (14 mg, 37%) was obtained. ESIMS: *m/z* 756.5 $[M+H]^+$, t_R 15.6 min.

3.5.10. Preparation of heptapeptide 10: Ac-Pro-Thr-Thr-Thr-Pro-Leu-Lys-NH_2. Starting with 'tripeptide resin' (250 mg), and following Section 3.2, the title product was obtained (16 mg, 46%); t_R 19.5 min, ESIMS: *m/z* 798.7 $[M+H]^+$.

3.5.11. Preparation of compound 11: Ac-Thr(α-D-GalNAc)-NH_2. Fmoc-PAL-PEG-PS (500 mg, 0.18 mmol/g, 0.09 mmol) was placed into a glass solid-phase reaction vessel (8.5 mL). The Fmoc group was removed by treatment with piperidine–DMF (1:4) for 20 min, following which Fmoc-Thr(Ac_3-α-D-GalNAc)-OH (90.5 mg, 0.135 mmol), HBTU (58.5 mg, 1.8 mmol), HOBt (27.2 mg, 1.8 mmol), and DIEA (34 μL, 1.8 mmol) in DMF (5 mL) were added. Coupling proceeded for 4 h, then the Fmoc group was removed, and then the free N-terminus was acetylated by treatment with Ac_2O–DMF (1:4) for 20 min. The resin was then treated with NaOMe (6 mM) DMF–MeOH in (17:3, 4 mL), agitated on a Multimixer for 2 h. After washing with DMF (5 × 1 mL × 1 min), CH_2Cl_2 (5 × 1 mL × 1 min) and 2-PrOH (5 × 1 mL × 1 min) continuously and drying in vacuo, the resin was cleaved with TFA–H_2O (19:1, 5 mL) for 1 h. Purification by preparative HPLC [buffer B: 0–1% over 30 min at 4 mL/min, analytical RPHPLC, t_R 4.4 min] and lyophilization provided the title product (23 mg, 71%). ESIMS: *m/z* 402.1 $[M+K]^+$, 386.2 $[M+Na]^+$, 364.2 $[M+H]^+$.

3.6. Comparative study of reduction of azido groups (Table 2)

Fmoc-PAL-PEG-PS (500 mg, 0.18 mmol/g) was placed into a glass solid-phase reaction vessel (8.5 mL). The Fmoc group was removed by treatment with piperidine–DMF (1:4) for 20 min, following which Fmoc-Thr(Ac_3-α-D-$GalN_3$)-OPfp (105 mg, 0.13 mmol), HOBt (20 mg, 0.13 mmol) and DIEA (25 μL, 0.13 mmol) in DMF (5 mL) were added. Coupling proceeded for 2 h. For each test, an aliquot of resin (20 mg) was distributed to a glass vessel and treated with: (Table 2, Line 1) 0.2 M DTT/0.1 mM collidine in CH_2Cl_2 for 2 × 3 h on a Multimixer; (Line 2) 0.2 M DTT/0.1 mM DIEA in CH_2Cl_2 for 2 × 3 h on a Multimixer; (Line 3) 0.2 M DTT/0.1 mM DIEA in CH_2Cl_2 for 24 h on a Labquake; (Line 4) 0.2 M DTT/0.1 M DIEA in CH_2Cl_2 for 2 × 2 h on a Multimixer; (Line 5) 0.2 M DTT/0.1 M DIEA in CH_2Cl_2 for 72 h on a Labquake; (Line 6) 0.2 M DTT/0.1 M DBU in CH_2Cl_2 for 2 × 3 h on a Multimixer; (Line 7) 0.2 M DTT/0.1 M DIEA in DMF for 2 × 3 h on a Multimixer; (Line 8) Neat AcSH for 24 h on a Labquake. As a control, glycosylated threonine derivative was directly cleaved from resin without treatment with DTT or AcSH. Then, after removal of Fmoc, acetylation of N-terminal (and concurrent acetylation of the free 2-amino group), cleavage from the resin provided Ac-Thr(Ac_3-α-D-GalNAc)-NH_2, t_R 20.4–20.6 min, ESIMS: *m/z* 512.3 $[M+Na]^+$, 490.3 $[M+H]^+$; and/or Ac-Thr(Ac_3-α-D-$GalN_3$)-NH_2, t_R 26.1–26.3 min; ESIMS: *m/z* 496.3 $[M+Na]^+$, 474.3 $[M+H]^+$.

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Supplementary data

Supplementary information of ^1H NMR spectra of glycopeptides **1**–**8**, heptapeptides **9**–**10**, together with HPLC profile of compound **11** after deprotection (total 13 pages), is available, in the online version, at doi:10.1016/j.carres.2005.05.023.

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