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Neoglycopeptides as Inhibitors of Oligosaccharyl Transferase: Insight into Negotiating Product Inhibition

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Summary

Linear hexapeptides featuring the asparagine mimetics alanine-β-hydrazide, alanine-β-hydroxylamine, and 1,3-diaminobutanoic acid have been synthesized as oligosaccharyl transferase (OT) substrate mimetics and chemoselectively *N*-glycosylated to obtain the corresponding neoglycopeptides as OT product mimetics. The effect of glycosylation on the binding of these asparagine surrogates is in stark contrast with the effect of modification of native asparagine. In native *N*-linked glycosylation, product inhibition is minimal and glycopeptides show very low affinity for OT. In contrast, glycosylation of the substrate mimetics maintains or even improves affinity of the corresponding product mimetic for OT. Conformational considerations suggest that the flexibility of the *N*-glycosyl linkage in these neoglycopeptides allows them to be accommodated in the OT binding site while the native *trans* glycosyl amide linkage is rejected. These results provide insight into how OT minimizes product inhibition, thereby ensuring effective substrate turnover.

Introduction

Asparagine-linked glycosylation is a prevalent and critical modification of eukaryotic secretory and membrane-bound proteins [1]. The key bond-forming step in this process involves the en bloc transfer of a preassembled triantennary tetradecasaccharide $Glc_3Man_9GlcNAc_2$ from a dolichyl-pyrophosphate-linked donor to selected Asn-Xaa-Thr/Ser acceptor sites in nascent polypeptide chains [2]. This reaction is catalyzed by the multimeric, membrane-associated enzyme oligosaccharyl transferase (OT). In this transformation, OT appears to simultaneously accommodate both polypeptide and oligosaccharide substrates in a ternary complex. Due to the challenges associated with overexpression and purification of the enzyme complex, little is known about the active site of OT and how the enzyme may negotiate the large substrates and yet still turn over efficiently at a rate that is consistent with the rate of protein synthesis [3, 4]. At the current time, substrate and product-based probes of OT represent the most valuable tools for providing insight into the mechanism of asparagine-linked glycosylation [5].

Research efforts have resulted in the development of a potent and specific inhibitor of OT, c[Hex-Dab-Cys]-Thr-Val-Thr-Nph-NH_2 (K_i = 0.037 μM; Dab = 1,3-diaminobutanoic acid), where Dab replaces Asn in the consensus sequence for glycosylation [6–8]. Recent research aimed at improving the potency of this inhibitor led to the discovery that *N*-δ-alkylation of the Dab side chain with a bulky, aromatic naphthyl group improves inhibitory activity [9]. This effect is in stark contrast with modification of the native asparagine side chain of OT substrates, where a simple *N*-δ-methylation is known to eliminate binding to the enzyme [10]. As aromatic amino acids are often implicated in carbohydrate/protein binding interactions, we reasoned that the naphthyl group might be involved in π-stacking interactions within the saccharide binding pocket of the active site of the enzyme [11, 12]. This hypothesis led us to investigate neoglycopeptides as potential product analog inhibitors of OT. Product analogs would be anticipated to exhibit better affinity and specificity for the enzyme than compounds that mimic either substrate alone. Furthermore, such constructs might provide insights into the geometry of the active site as well as the mechanism of the enzyme [13].

The synthesis of glycopeptide mimetics (or "neoglycopeptides") has received considerable attention recently with the development of C- and S-glycoside analogs as well as targets prepared by chemoselective ligation techniques [14]. The strategy for the assembly of product analog inhibitors of OT described herein is based on the use of alanine-β-hydroxylamine (Aβx) [15], alanine β-hydrazide (Aβz) [15], and Dab [9] as asparagine surrogates. These residues have been chosen because the side chain functionality reacts chemoselectively with reducing sugars, without the need for protecting groups or activating agents, to afford *N*-linked glycoconjugates in highly convergent synthetic routes.

In the present work, we have designed linear hexapeptides of the general sequence Bz-Xaa-Ala-Thr-Val-Thr-Nph-NH_2, featuring the natural substrate, Asn (**1**), as well as Aβz (**2**), Aβx (**3**), and Dab (**4**) at the Xaa position (Figure 1). Peptides of this general sequence show good affinity for OT [6–8]. Glycoconjugates of **2**, **3**, and **4** with *N*-acetyl glucosamine (**2a**, **3a**, **3b**, and **4a**) were also prepared as potential product analog inhibitors of OT. Glycopeptide **1a** was prepared as a reference compound. The four nonnatural glycopeptides exhibited binding affinities that are similar to those of their respective parent peptides, while the natural glycopeptide has significantly diminished binding compared to its parent peptide. The contrast between the binding affinity of the glycopeptide bearing a natural amide linkage and those with nonnatural linkages is discussed in the context of the conformational preferences of the polypeptide substrate and glycopeptide product in the active site of OT.

Results and Discussion

Synthesis of Peptides and Glycopeptides

Peptides **1–4** were assembled using a combination of solid-phase and solution-state methods. An Asn(Glc-

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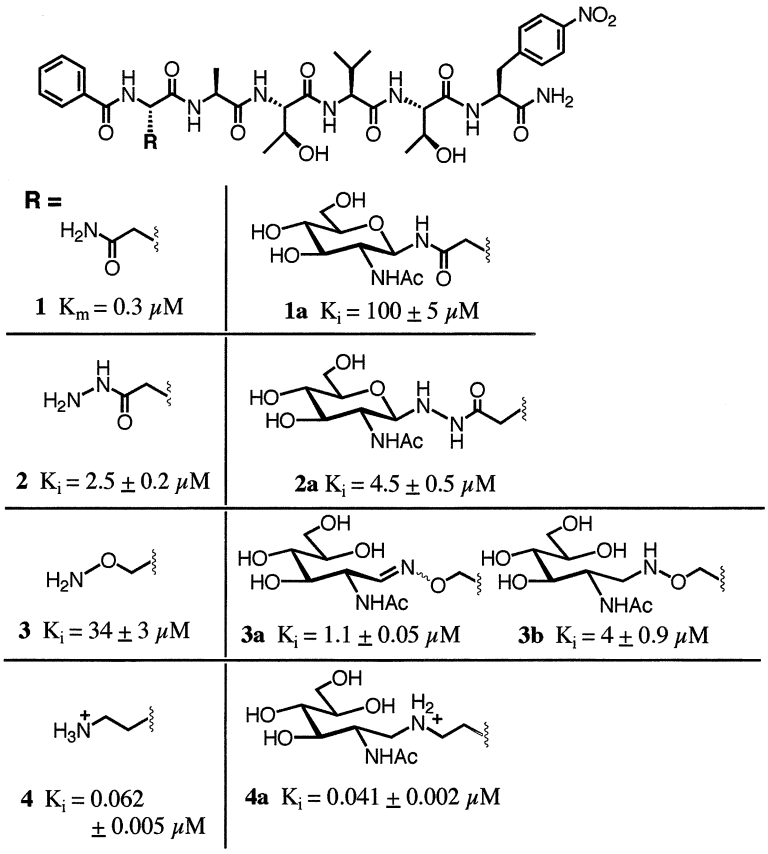

Figure 1. Peptides and Glycopeptide Conjugates Presented in This Study

NAc) building block was employed for the synthesis of **1a**. The hydrazide functionality in **2** was prepared from an orthogonally protected aspartic acid derivative, which enabled deprotection, and coupling of *t*-butylcarbazate onto the side chain prior to cleavage from the resin as described previously [15]. Formation of the glycosyl-hydrazide (**2a**) was carried out in solution by chemoselective ligation of the peptide (**2**) with unprotected *N*-acetyl glucosamine. For the preparation of the hydroxylamine analog (**3**), the corresponding phthalimide-protected derivative was prepared and integrated into polypeptide sequences via standard solid-phase synthesis methods [15]. After deprotection, reaction of **3** with *N*-acetyl glucosamine gave the glycosyl-oxime (**3a**) as a mixture of cyclic and acyclic saccharide derivatives. Subsequent reduction of **3a** with sodium cyanoborohydride resulted in the glycosyl-hydroxylamine (**3b**). Reduction fixes the sugar in the acyclic form. For the synthesis of **4**, commercially available *N*-α-Fmoc-Dab-(Boc)-OH was employed. Neoglycopeptide, **4a**, was prepared through treatment with *N*-acetyl glucosamine followed by reduction with sodium cyanoborohydride.

Kinetic Analysis of the Natural Substrate and Corresponding Glycoconjugate

Each of the compounds was evaluated for inhibition with the *S. cerevisiae* OT in competition assays with the disaccharide donor Dol-P-P-$(GlcNAc)_2$ and the tripeptide substrate Bz-Asn-Leu-Thr-NHMe [16]. The inhibition constants are summarized in Figure 1. The K_m of the asparagine-containing peptide (**1**) was determined by measuring the rate of reaction over a range of peptide concentrations. Kinetic parameters were determined using a Hanes plot. The glycopeptide featuring a secondary glycosyl amide at the glycosidic linkage, **1a**, is a very poor product inhibitor of OT ($K_i \sim 100\ \mu M$), reflecting a significant change in binding affinity from the peptide featuring asparagine in the same site ($K_m = 0.31\ \mu M$). This dramatic decrease in affinity may account for the lack of product inhibition that is necessary for the turnover of OT to keep up with the rate of protein translation and translocation. While these model systems are significantly smaller than the actual substrate and product that they represent, a direct comparison is being made between two peptides that are the same in length, only differing by the presence or absence of a single saccharide unit. It is postulated that the effect observed in the native system may be even more pronounced.

Kinetic Analysis of Nonnatural Peptides and Glycoconjugates

Each of the substrate mimetics (**2–4**) displays inhibition of OT (Figure 1). The estimated pK_a values of each nonnatural side chain functionality (based on model compounds in which the methylene adjacent to the α-carbon has been replaced by a methyl substituent) are 3.2, 4.6, and 10.6 for **2**, **3**, and **4**, respectively (pK_a values were determined with the software ACD/pKDB v. 4.59 [Advanced Chemistry Development Inc., Toronto, Canada]); so only **4** is protonated in the pH range (6–8) where

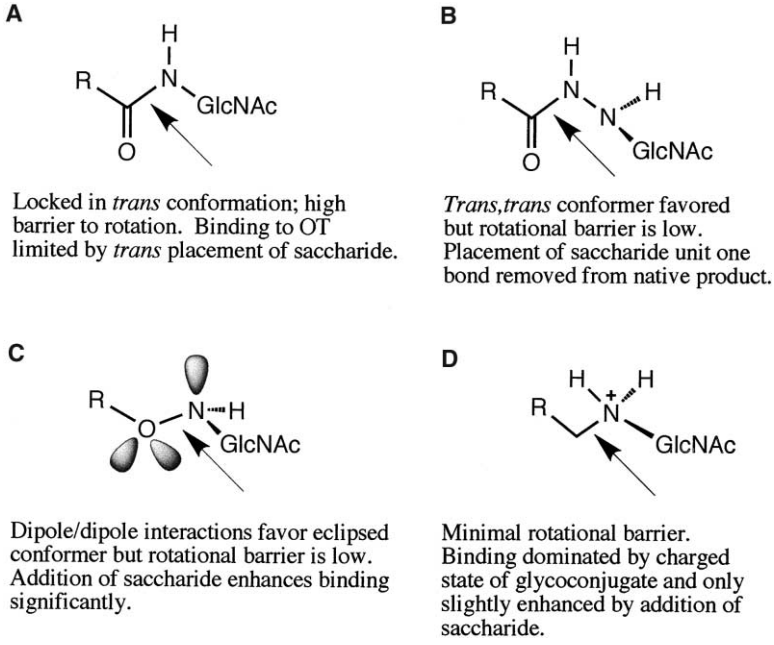

Figure 2. Conformational Preferences

Conformational preferences of (A) glycosyl amide, (B) glycosyl hydrazide, (C) glycosyl oxime, and (D) glycosylamine.

OT is active. As peptide **4** is notably more potent than peptides **2** and **3**, it is clear that the positive charge on the γ-nitrogen at neutral pH imparts additional binding affinity, potentially via interactions that may develop between high energy intermediates and the enzyme in the transition state of the reaction [17]. Due to limitations with the enzyme assay, a detailed study of the pH dependence of the inhibition constants is not feasible; therefore, the focus of the following analysis will be on the *relative* effects of saccharide modification within each peptide and neoglycopeptide family.

In contrast to the naturally occurring glycoside (**1a**), replacement of the glycosyl-amide bond with a glycosyl-hydrazide (**2a**), a glycosyl-oxime (**3a**), a glycosyl-hydroxylamine (**3b**), or a glycosyl amine (**4a**) affords low μM-nM inhibitors of OT. Therefore, replacing the native amide bond between the peptide and the *N*-acetyl glucosamine moiety with a more flexible linkage allows the neoglycopeptide to retain, and in some cases improve, binding affinity over that of the parent peptide.

A possible rationale for these observations lies in the conformational preferences of the various glycosidic linkages examined, together with previous proposals for the mechanism of OT [18]. A statistical survey of crystalline *N*-linked glycoproteins reveals that native glycosyl-amide bonds preferentially adopt a stable *trans* conformation (Figure 2), as would be expected for a secondary amide linkage [19]. However, as shown by studies on peptide **1a**, this *trans* conformation appears to be poorly accommodated by the enzyme (K_i 100 μM). This observation is further underscored by studies with a simple peptide featuring a δ-methylasparagine [10] which would also preferentially adopt a *trans* conformation and is neither a substrate nor an inhibitor. Thus, these studies indicate that even a minimal methyl group is not accommodated in the enzyme active site when the substituent favors a *trans* orientation relative to the asparagine side chain. Therefore, the conformation of the nascent glycopeptide product at the OT active site is likely to be represented by a relatively unstable species, such as a *cis* glycosyl amide or a product with a pyramidalized nitrogen, which would undergo a favorable equilibration to a more stable species such as the *trans* glycosyl amide. That ultimate species would no longer present the peptide and saccharide moieties in an appropriate orientation to bind to the enzyme. In contrast with the native glycosyl amide (**1a**), the Aβz(GlcNAc) featuring peptide, **2a**, shows a K_i of 4.5 μM. Modification of the parent peptide with the monosaccharide moiety effects a slight decrease in the affinity of the neoglycopeptide for the enzyme; however, compared with **1a** the effect is modest. The reaction of hydrazides at the anomeric center of saccharides is known to lead essentially to the β-pyranosidic form of the saccharide [20, 21]. Crystallographic studies show that substituted hydrazides feature a *trans* planar C-CO-NH-N fragment, the terminal nitrogen having a pyramidal sp^3 character with the substituents out of the hydrazide plane (Figure 2) [22, 23]. Additionally, a theoretical study of the conformational preferences of the hydrazide linkage in *N,N'*-diacetylhydrazide shows that the CO-NH bonds do not show resonance comparable with *N*-methyl acetamide, and therefore the barrier to rotation about these bonds would be low [24]. Therefore, the hydrazide neoglycopeptide may be able to make similar binding interactions with the peptidyl portion of the OT active site, but the length of the hydrazido side chain may preclude the saccharide moiety from engaging in additional binding. The freedom of rotation around the hydrazido linkage, however, is sufficiently flexible to enable the saccharide to move to a "neutral" position with respect to OT binding.

Replacing the native glycosyl-amide bond with a glycosyl-oxime bond in **3a** dramatically affects inhibitory potency of the hydroxylamine-peptide; the K_i is 1.1 μM (compared with 34 μM for the unmodified peptide). Glycosyl-oxime derivatives have been shown to exist in an

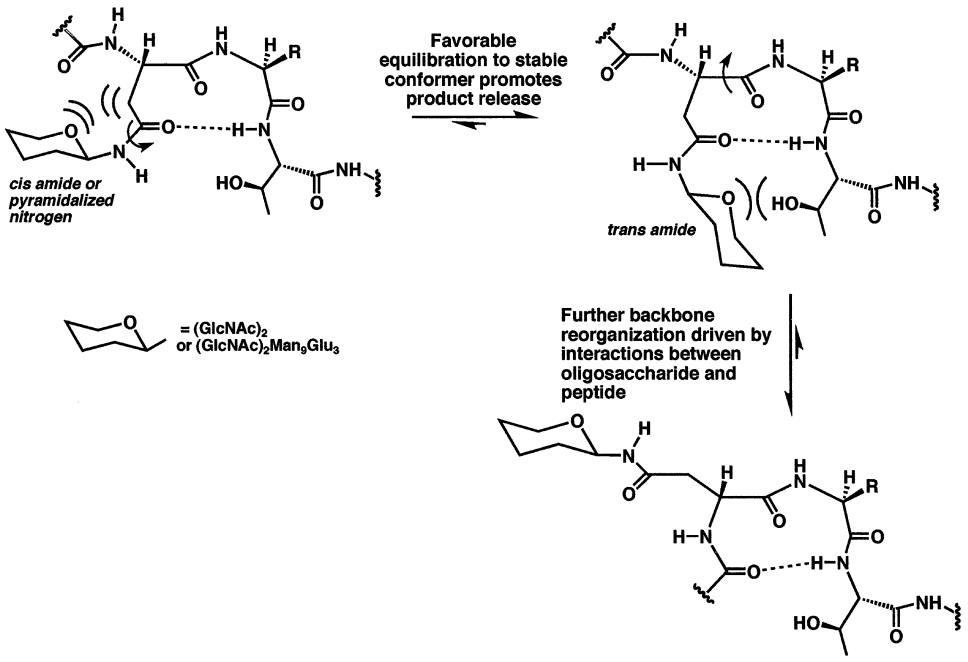

Figure 3. Proposal for the Role of Isomerization in Promoting Product Release

equilibrium mixture involving E and Z acyclic isomers as well as the cyclic β-pyranosidic glycosyl-hydroxylamine [25]. Reducing the glycosyl-oxime **3a** to the linear glycosyl-hydroxylamine **3b** resulted in a 4-fold weaker OT inhibition. This observation suggests that the most active species of **3a** is the β-pyranosidic form. The hydroxylamine bond is known to adopt unusual conformational preferences due to the lone pair electrons on both oxygen and nitrogen. Thus *N,O*-disubstituted hydroxylamines like **3a** and **3b** preferentially adopt a conformation where $\omega_{C\text{-}N\text{-}O\text{-}C} = 240°$ (Figure 2); however, the barrier to rotation in these species is low [26]. Thus, in the case of the glycosyl oxime derivatives a 30-fold improvement in binding is observed with the neoglycopeptide derivative.

Finally, the conjugate with Dab, **4a**, shows the most potent inhibition ($K_i = 0.041$ μM). In this case, it should be noted that the parent peptide without the glycosyl modification is also a potent inhibitor ($K_i = 0.061$ μM); thus, inhibitory effects are dominated by the charged state of the analog and only slightly effected by the glycosylation.

These conformational considerations suggest that neoglycopeptides can be accommodated in the OT binding site only when the nitrogen carrying the glycosyl moiety is not "locked" into a *trans* orientation relative to the asparagine side chain. In this context, we propose a mechanistic model in which the initial glycoconjugate formed in the OT active site may be represented by a species with *cis* or twisted amide geometry. Thus, an energetically favorable equilibration to the *trans* amide species may participate in the release of the *N*-glycosylated peptide from the OT active site and effectively eliminate product inhibition due to the greatly reduced binding affinity of the *trans* amide isomer for the OT active site (Figure 3). It should be noted that a great deal more energy would be required for such a conformational change in the case of the natural tetradecasaccharide as compared to a monosaccharide; however, the strain in the native product would also be significantly greater in this scenario.

In summary, the results of these studies reveal that binding in the OT active site depends heavily on both the geometry and electronics of the linkage between the peptide and saccharide components of the inhibitor. These product analog inhibitors also suggest a model for understanding the lack of product inhibition in the enzyme-catalyzed transformation. Current work in our laboratory is focused on the further study of this proposal.

Significance

Asparagine-linked glycosylation is a ubiquitous modification of eukaryotic secretory and membrane-bound proteins. This reaction is catalyzed by the heteromeric membrane-associated complex oligosaccharyl transferase (OT) during translocation of nascent polypeptides into the endoplasmic reticulum. As this is a cotranslational process occurring between two large substrates, each with significant affinity for the enzyme, insight into understanding how an enzyme such as OT may negotiate product inhibition is of significant interest. Herein we have shown, in contrast to the native glycosyl amide linkage, that some neoglyopeptides retain or even gain affinity for OT relative to the unglycosylated counterparts. We propose that this effect may be due to the fact that the nascent glycopeptide is initially synthesized in a metastable conformation that equilibrates to a stable *trans* amide isomer that is no longer accommodated at the enzyme active site. Such an event may represent a useful mechanism

for prevention of product inhibition, as it would result in a "mismatch" between the binding determinants in the product with those at the active site. Furthermore, these studies have led to the development of neoglycopeptides with flexible linkages that are accepted into the binding site, thus introducing the first product inhibitors of OT.

Experimental Procedures

General Procedure for Fmoc-Based Solid-Phase Peptide Synthesis

All peptides were prepared manually using a glass reaction vessel fitted with a sintered glass frit. Resin was washed and swollen in CH_2Cl_2 (2 × 10 ml/g resin × 15 min) and dimethylformamide (DMF) (1 × 10 ml/g resin × 15 min). *N*-α-Fmoc was removed by treatment with piperidine: DMF 1:5 (3 × 10 ml/g resin × 5 min) followed by washing with DMF (5 × 10 ml/g resin × 1 min). The number of equivalents was determined by quantification of Fmoc release. This was accomplished by measuring the UV absorbance at 300 nm of the pooled deprotection rinses and DMF washes. Coupling reactions were performed using 1.5 equivalents of *N*-α-Fmoc-protected amino acids activated in situ with 1.5 equivalents of PyBOP and 3 equivalents of diisopropylethylamine (DIEA) in DMF (10 ml/g resin) for 1 hr. The success of each coupling and deprotection was verified by a trinitrobenzene sulfonic acid (TNBS) test, whereby approximately a milligram of resin was removed and added to a solution of TNBS in DMF. TNBS reacts with free amines to form a bright pink product. *N*-terminal capping was accomplished by adding 10 equivalents of benzoic anhydride and 10 equivalents of pyridine in DMF (2.5 ml/g resin) to resin-bound peptides and shaking at room temperature for 1 hr. This capping step was followed by washing with DMF (5 × 10 ml/g resin × 1 min) and CH_2Cl_2 (2 × 10 ml/g resin × 1 min). Cleavage of peptides from resin with concomitant removal of side-chain-protecting groups was performed using 10 ml/g resin of trifluoroacetic acid (TFA): CH_2Cl_2: triisopropylsilane: H_2O (90:5:2.5:2.5). The cleavage reaction was allowed to proceed for 1 hr, then the beads were filtered and rinsed with TFA (2 × 1 ml). Filtrate was concentrated to 1 ml under a stream of nitrogen, then triturated with 14 ml of diethyl ether. The white solid obtained was isolated by centrifugation, washed twice with diethyl ether, and dried. All peptides were purified by preparative HPLC with a gradient of increasing acetonitrile/0.1% TFA (B) in water/0.1% TFA.

BzAsnAlaThrValThrNphNH$_2$ (1)

For kinetic comparison, a natural OT substrate was synthesized by standard Fmoc-based solid-phase peptide synthesis on PAL-PEG-PS resin. K_m was determined by measuring the rate of reaction at 5 nM, 50 nM, 125 nM, 250 nM, 500 nM, 1 μM, 2 μM, 3 μM, and 4 μM peptide. These results were fit to a Hanes plot. The assay was performed as described below, using the hexapeptide, **1**, as the substrate.

$C_{36}H_{49}N_9O_{12}$; HPLC:t_R = 20 min (C_{18}, 7%–100% B in 28 min); exact mass, 799.35; mol. wt., 799.83; ESMS:$[M+H]^+$ = 800.26.

BzAsn(GlcNAc)AlaThrValThrNphNH$_2$ (1a)

BzAsn(Ac_3GlcNAc)AlaThrValThrNphNH$_2$ was prepared by standard methods on PAL-PEG-PS resin, with the exception that coupling of the building block, FmocAsn(Ac_3GlcNAc)OPfp, proceeded without activating agent or base. Following deprotection and cleavage, the peptide was taken up in methanol (20 ml). Removal of the *O*-acetyl groups was accomplished by adding sodium methoxide (180 μmol, 9.7 mg). The reaction was monitored by HPLC and was 92% complete after 22 hr. The product was purified by preparative HPLC for an overall yield of 56%. The K_i was determined as described below and is reported as an average from seven measurements. The standard deviation was less than 10%.

$C_{44}H_{62}N_{10}O_{17}$; HPLC:t_R = 20 min (C_{18}, 7%–100% B in 28 min); exact mass, 1002.43; mol. wt., 1003.2; ESMS:$[M+H]^+$ = 1003.42.

BzAβzAlaThrValThrNphNH$_2$ (2)

Preparation of **2** began with the synthesis of BzAsp(OAll) AlaThrValThrNphNH$_2$ by standard Fmoc-based solid-phase peptide synthesis on Fmoc-XAL-PS resin. Removal of the allyl ester protecting group from Asp was done on the solid support using catalytic $Pd(PPh_3)_4$ (0.2 equivalents) and the scavenger, phenylsilane (25 equivalents). Coupling of *t*-butylcarbazate (1.5 equivalents) onto the Asp side chain in the presence of PyAOP (3 equivalents) and collidine (15 equivalents) on the solid support led to **2** after standard cleavage, deprotection, and HPLC purification for an overall yield of 24%. The K_i was determined as described below and is reported as an average from three measurements. The standard deviation was less than 10%.

$C_{36}H_{50}N_{10}O_{12}$; HPLC:t_R = 18 min (C_{18}, 7%–100% B in 28 min); exact mass, 814.36; mol. wt., 814.84; ESMS:$[M+H]^+$ = 815.30.

BzAβz(GlcNAc)AlaThrValThrNphNH$_2$ (2a)

Chemoselective ligation was used to prepare **2a** in solution by adding 5 equivalents of GlcNAc (22.5 μmol) to one equivalent of **1** (4.5 μmol) in DMSO (100 μl). After 18 hr at room temperature followed by concentration under reduced pressure, the HPLC trace showed 80% conversion to **2a**. Preparative HPLC was used to purify the product. The K_i was determined as described below and is reported as an average from three measurements. The standard deviation was less than 10%.

$C_{44}H_{63}N_{11}O_{17}$; HPLC:t_R = 19 min (C_{18}, 20%–70% B in 25 min); exact mass, 1017.44; mol. wt., 1018.03; ESMS:$[M+H]^+$ = 1221.29 $[M-H_2O]^+$ = 1018.31.

BzAβxAlaThrValThrNphNH$_2$ (3)

Preparation of **3** began with the synthesis of a phthalimide-protected serine building block, FmocAβx(Pht)OH, as reported [15]. The peptide was synthesized by standard Fmoc-based solid-phase peptide synthesis on Fmoc-XAL-PS resin, with the exception that coupling of the building block was accomplished using 2 equivalents of PyAOP as the activating agent and 3.7 equivalents of 2,4,6-collidine as the base. Removal of the phthalimide-protecting group with hydrazine: allyl alcohol: trifluoroethanol (1:3:46) (10 ml) for 20 hr was realized on the solid phase with 90% conversion by HPLC. Standard cleavage, deprotection, and purification led to **2**.

$C_{35}H_{49}N_9O_{12}$; HPLC:t_R = 20 min (C_{18}, 20%–70% B in 25 min); exact mass, 787.35; mol. wt., 787.82; ESMS:$[M+H]^+$ = 788.13.

BzAβx(GlcNAc)AlaThrValThrNphNH$_2$ (3a)

To a solution of **3** (15 μmol) in DMSO (30 μl) was added 30 μmol of GlcNAc (1 M) in 0.1 M sodium acetate buffer (pH 5.6, 30 μl). Additional DMSO (60 μl) was added to obtain a clear solution, then the reaction mixture was concentrated overnight under reduced pressure to obtain **3a** in 80% conversion by HPLC. Preparative HPLC was used to purify the product. The K_i was determined as described below and is reported as an average from three measurements. The standard deviation was less than 5%.

$C_{43}H_{62}N_{10}O_{17}$; HPLC:t_R = 16 min (C_{18}, 20%–70% B in 25 min); exact mass, 990.43; mol. wt., 991.01; ESMS:$[M+H]^+$ = 991.41.

BzAβx(rGlcNAc)AlaThrValThrNphNH$_2$ (3b)

To a solution of **3a** (15 μmol) in acetic acid (700 μl) was added sodium cyanoborohydride (120 μmol, 7.5 mg), and the progress of the reduction was monitored by analytical HPLC. After 10 min, the reaction was complete and the product was purified by preparative HPLC. Overall yield was approximately 20%. The K_i was determined as described below and is reported as an average from four measurements. The standard deviation was less than 20%.

$C_{43}H_{64}N_{10}O_{17}$; HPLC:t_R = 21 min (C_{18}, 7%–100% B in 28 min); exact mass, 992.45; mol. wt., 993.03; ESMS:$[M+H]^+$ = 993.29.

BzDabAlaThrValThrNphNH$_2$ (4)

Standard Fmoc-based solid-phase peptide synthesis procedures were used to obtain **4** on Fmoc-XAL-PS resin. The K_i was determined as described below and is reported as an average from five measurements. The standard deviation was less than 10%.

$C_{36}H_{51}N_9O_{11}$; HPLC:t_R = 18 min (C_{18}, 7%–100% B in 28 min); exact mass, 785.37; mol. wt., 785.84; ESMS:$[M+H]^+$ = 786.3.

BzDab(GlcNAc)AlaThrValThrNphNH$_2$ (4a)

A 25 μmol portion of GlcNAc (1 M in phosphate buffer, pH 7.5) was added to **4** (25 μmol in 100 μl DMF) and heated to 65°C. Sodium

cyanoborohydride (250 μmol of a 0.25 mg/ml solution in DMF) was then added, and the mixture was stirred at 65°C for 2.5 hr. Finally, the reaction mixture was diluted in 1:1 water: acetonitrile (10 ml) and purified by preparative HPLC. Overall yield was 20%. The K_i was determined as described below and is reported as an average from three measurements. The standard deviation was less than 5%.

$C_{44}H_{68}N_{10}O_{16}$; HPLC:t_R = 19 min (C_{18}, 7%–100% B in 28 min); exact mass, 990.47; mol. wt., 991.05; ESMS:$[M+H]^+$ = 991.39.

Determination of IC_{50} and K_i

The radiolabeled carbohydrate substrate Dol-PP-GlcNAc-[^{3}H]-GlcNAc was dissolved in DMSO for the control measurements or DMSO containing the inhibitor for the inhibition studies. Assay buffer (50 mM HEPES [pH 7.5], 140 mM sucrose, 1.2% Triton X-100, 0.5 mg/ml PC, 10 mM $MnCl_2$) and OT-containing solubilized microsomes from *S. cerevisiae* [2] were added to the carbohydrate substrate. After incubation for 30 min, the assay was initiated by adding the Bz-Asn-Leu-Thr-NHMe peptide substrate. Reaction aliquots (4 × 40 μl) were removed at 2 min intervals and quenched into 3:2:1 chloroform: methanol: 4 mM $MgCl_2$. The tritiated glycopeptide in the upper aqueous layer was separated from the unreacted glycolipid through a series of extractions. The combined aqueous layers were quantitated for tritium content. The disintegrations per minute (dpm) were plotted as a function of time for the control and three different inhibitor concentrations. The percentage of inhibition was determined from this plot in order to estimate the IC_{50}. Three concentrations were then selected to give between 30% and 70% inhibition. All experiments were run a minimum of three times. In each case, the approximate K_i was determined using the following equation [27]:

$$K_i = \frac{[I] \times (1 - i)}{i + \left(\frac{[S]}{K_M} \times i\right)}.$$

K_m of Bz-Asn-Ala-Thr-Val-Thr-Nph-NH_2

OT assays were performed as described above, except that instead of Bz-NLT-NHMe, Bz-Asn-Ala-Thr-Val-Thr-Nph-NH_2 was used as the disaccharide acceptor. The K_m of the tripeptide substrate, 0.25 μM, is similar to that of the hexapeptide containing asparagine that is used for comparison in this study, which has a K_m of 0.31 μM. The following concentrations were assayed: 5 nM, 50 nM, 125 nM, 250 nM, 500 nM, 1 μM, 2 μM, 3 μM, and 4 μM. From the slope of the dpm versus time plots, velocity (nmol/s) was calculated. Velocity versus substrate concentration was plotted to confirm saturation, and a Hanes plot ([S]/V versus [S]) was used to determine the K_m, with a linear regression of 0.99447. From the Hanes plot, the x intercept is equal to $(-)K_m$.

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