Structural and Functional Analysis of Peptidyl Oligosaccharyl Transferase Inhibitors†

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ABSTRACT: The peptide *cyclo*(hex-Amb$_{(1)}$-Cys$_{(2)}$)-Thr$_{(3)}$-Val$_{(4)}$-Thr$_{(5)}$-Nph$_{(6)}$-NH$_2$ was previously shown to be a slow, tight-binding inhibitor (K_i = 37 nM) of the yeast oligosaccharyl transferase (OT) [Hendrickson et al. (1996) *J. Am. Chem. Soc. 118*, 7636−7637]. This enzyme catalyzes the transfer of a carbohydrate moiety to an asparagine residue in the consensus sequence Asn-Xaa-Thr/Ser. Herein we present a study of the contribution of the residues in positions 1, 3, 4, and 5 to OT binding. Replacement of the threonine (residue 3) by valine or (*S*)-2-aminobutyric acid dramatically reduced the potency of the inhibitor while, surprisingly, the incorporation of an additional methylene into the side chain of residue 1 [(*S*)-2,3-diaminobutyric acid changed to ornithine] had very little effect. Variants with acidic, basic, hydrophilic/polar, and hydrophobic side chains in positions 4 and 5 were also evaluated for both yeast and porcine liver OT inhibition. This aspect of the study reveals that basic (lysine) and acidic (glutamic acid) residues are detrimental to the binding, whereas hydrophobic (valine) and polar/hydrophilic (threonine) residues are both well tolerated. The kinetic behavior of substrate analogs [*cyclo*(hex-Asn$_{(1)}$-Cys$_{(2)}$)-Thr$_{(3)}$-Xaa$_{(4)}$-Yaa$_{(5)}$-Nph-NH$_2$] corresponding to inhibitors of weak, medium, and strong potency was also examined in order to provide insight into the nature of these inhibitors.

Small synthetic peptidyl inhibitors are invaluable tools for identifying the mechanism of action and the binding specificity of protein processing enzymes such as proteases and prenyl transferases. Herein we investigate the properties of a new class of peptidyl inhibitors that are targeted at the multimeric membrane-associated glycoprotein oligosaccharyl transferase (OT).[1] This enzyme catalyzes the co-translational transfer of a complex carbohydrate (-GlcNAc$_2$-Man$_9$-Glc$_3$) from a lipid-linked pyrophosphoryl donor to specific asparagine residues in the consensus sequence Asn-Xaa-Thr/Ser. Currently, the composition and specific functions of the enzyme subunits as well as the details of the active site remain to be elucidated [for a recent review, see Imperiali and Hendrickson (1995)]. Prior to these studies, the only known inhibitor of *N*-linked glycosylation was the microbial natural product tunicamycin (Tamura, 1982). However, the effect of this compound on *N*-linked glycosylation is not specific; tunicamycin is a bisubstrate analog inhibitor of the enzyme that catalyzes the synthesis of Dol-P-P-GlcNAc, which is the first precursor in the biosynthesis of Dol-P-P-GlcNAc$_2$-Man$_9$-Glc$_3$. Therefore, inhibition of OT is not targeted to the actual protein glycosylation event, but rather to an upstream metabolic process. The importance of asparagine-linked glycosylation in numerous biological processes [for reviews, see Dwek (1996) and Varki (1993)] has prompted us to design inhibitors that would control the enzyme activity and provide insight into the underlying mechanism of the process.

Peptides presenting the minimum recognition sequence, Asn-Xaa-Thr/Ser, with the amino and carboxyl termini masked as the simple amide derivatives have been employed as substrates for the *in vitro* assay of OT (Welply et al., 1983). The excision of the carbonyl function in the essential asparagine residue affords simple analogs that competitively inhibit the enzyme. For example, the peptide Bz-Amb-Leu-Thr-NHMe [Amb, (*S*)-2,4-diaminobutyric acid] is an inhibitor of porcine OT with a K_i of 1 mM, while the corresponding substrate (Bz-Asn-Leu-Thr-NHMe) has a K_m of 0.24 mM (Imperiali et al., 1992). In terms of conformational requirements, studies with constrained substrate analogs have revealed that the Asx-turn conformation (Abbadi et al., 1991) is important for Asn-Xaa-Thr/Ser substrate recognition (Imperiali et al., 1994). Following this lead, the inhibitory amine functionality was incorporated within a cyclic peptide analog, *cyclo*(hex-Amb-Add)-Thr-NHMe that effectively mimicked the backbone conformation of the Asx-turn. The introduction of this constraint resulted in a significant improvement in the inhibitor potency (K_i = 50 μM; yeast OT). Furthermore, increasing the length of the peptide at the *C*-terminus to include extended binding determinants resulted in the most potent OT inhibitor to date. The peptide *cyclo*(hex-Amb-Cys)-Thr-Val-Thr-Nph-NH$_2$ (Hendrickson et al., 1996) shows slow, tight binding inhibition of yeast OT with a K_i of 37 nM. The choice of the -Val-Thr- dipeptide beyond the consensus sequence was guided by statistical studies on glycosylation sites *in vivo* (Gavel & von Heijne,

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[1] Abbreviations: Add, aminodecanedioic acid; Abu, (*S*)-2-aminobutyric acid; Amb, (*S*)-2,4-diaminobutyric acid; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; Boc, butyloxycarbonyl; Bz, benzoyl; DIPCDI, diisopropylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; dpm, disintegrations per minute; DPPC, dolichol-P-P-GlcNAc-GlcNAc; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; GlcNAc, *N*-acetylglucosamine; Glu, glucose; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; Man, mannose; NP-40, Nonidet P-40; OT, oligosaccharyl transferase; PC, phosphatidylcholine; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, triphenylmethyl.

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1990). The third residue in the *C*-terminal extension sequence, *p*-nitrophenylalanine (Nph), was introduced to allow facile concentration determination.

In the present study, we modulated the activity of the prototype constrained inhibitor by varying two residues beyond the consensus sequence to evaluate the role of the extended binding determinants in defining the potency of the inhibitors. This study represents the first systematic exploration of the OT exosites. A detailed examination of the kinetic behavior of the corresponding substrates provides insight into the nature of these inhibitors. Finally, variations made to the key residues that comprise the consensus sequence provide further information on the structural requirements for specific inhibitors of *N*-linked glycosylation.

MATERIALS AND METHODS

Peptide Synthesis. All peptides were synthesized by manual solid phase methods starting from Fmoc-PAL PEG resin (0.25 g, 0.2 mmol/g) and using Fmoc (9-fluorenylmethoxycarbonyl) as the protecting group for the α-amino functionality. Amino acids were coupled either as activated pentafluorophenyl esters or using diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt). Amino acids were used in the following side chain-protected forms: Amb(Boc), Asn(Trt), Cys(*S*-*t*Bu), Glu(*t*Bu), Lys(Boc), Orn(Boc), and Thr(*t*Bu). (All amino acid derivatives were purchased from commercial sources.) At the conclusion of the peptide synthesis, the amino terminus was capped with a large excess of 6-bromohexanoic acid anhydride and triethylamine (TEA). The cysteine was deprotected under nitrogen in degassed dimethylformamide (DMF) using a large excess of tri-*n*-butyl phosphine (3-hour treatments). The resin was then washed repeatedly with DMF and cyclization, between the thiolate of cysteine and the 6-bromohexanoyl group, was achieved in degassed DMF using a large excess of tetramethylguanidine as base (24 h) (Virgilio & Ellman, 1994). The peptides were cleaved from the resin in a trifluoroacetic acid (TFA)/water/dimethyl sulfide (95/5/5) mixture (2 h). Peptides were triturated in diethyl ether (three times), redissolved in water/acetonitrile, and lyophilized to dryness.

The peptides were purified by preparative reverse phase high-pressure liquid chromatography (RP HPLC) with a gradient elution (15−20% acetonitrile/water/0.1% TFA to 25−30% acetonitrile/water/0.1% TFA over 20 min, flow rate 9.5 mL/min). The identity of the peptides was confirmed by electrospray mass spectrometry and amino acid analysis.

The chromophore of the *p*-nitrophenylalanine allowed facile assessment of inhibitor concentrations. The net peptide content, determined by amino acid analysis, was correlated with the absorbance, allowing the determination of a common absorption coefficient for all the peptides. The absorption coefficient at 280 nm was calculated to be 12 500 cm^{-1} M^{-1}. Thus, peptide concentrations were determined by dissolving each sample in dimethyl sulfoxide (DMSO) and 0.1 N HCl and measuring the absorbance at that wavelength. A good correlation between quantitative amino acid analysis values and the concentrations calculated from UV absorbance was found.

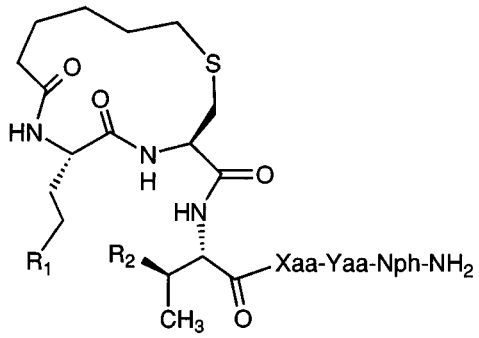
Preparation of Solubilized Membranes. Yeast (*Saccharomyces cerevisiae*) OT was purified to the solubilized membrane stage as described previously (Pathak et al., 1995). A similar method was used to purify the porcine liver enzyme. All steps were performed at 4 °C. Briefly, 12 mL of porcine microsome preparation was incubated with 30 mL of buffer (50 mM HEPES, pH 7, 40 mM sucrose, 2.5 mM $MgCl_2$, 2 mM DTT, 500 mM NaCl, 0.1 mM AEBSF, 0.5 mg/mL leupeptin, and 0.5 mg/mL pepstatin) on ice for 20 min and then centrifuged at 60 000 rpm (Ti60 rotor) for 60 min. The pellet was resuspended in 25 mL of buffer, and the concentration of Nonidet P-40 (NP-40) detergent was brought to 0.1% (v/v). The suspension was shaken for 20 min on ice, and then again centrifuged at 60 000 rpm for 60 min. The pellet was resuspended in the same buffer, and the concentration of NP-40 increased to 1% (v/v). The suspension was shaken for 20 min on ice and centrifuged at 45 000 rpm for 60 min. The supernatant was then aliquoted and stored at −80 °C. The preparations maintained constant OT activity for several weeks.

General Conditions for Binding Assays. Assays were performed at room temperature in a total volume of 200 μL (or 600 μL for slow binding analysis). The assay buffer consisted of 50 mM HEPES, pH 7.5, 140 mM sucrose, 15 mM $MnCl_2$, and 0.5 mg/mL phosphatidylcholine. The concentration of the peptide substrate, Bz-Asn-Leu-Thr-NHMe, was determined by amino acid analysis. The concentration and specific activity of lipid-linked glycosyl donor, dolichol-P-P-GlcNAc-[^{3}H]GlcNAc ([^{3}H]DPPC) (Imperiali & Zimmerman, 1990), is as indicated in the specific experiments. All measurements were carried out under the same conditions to allow a strict comparison of the constants. In particular, the same batch of enzyme was used, and the OT control rates were maintained in the 400−500 dpm/min range (for K_i determination).

Determination of Inhibitor Equilibrium Dissociation Constants (K_i). In a typical yeast OT assay, [^{3}H]DPPC (50 000 dpm, 60 Ci/mmol) was aliquoted from a chloroform/methanol stock solution into a microcentrifuge tube, and residual solvent was removed under a gentle stream of nitrogen. Mixtures of increasing amounts of peptidyl inhibitor (in 10 μL of DMSO) and a constant amount of enzyme in buffer were incubated on ice for 30 min. This incubation time was required to ensure pre-equilibration of the inhibitor with the enzyme. The reaction was initiated by adding 10 μL of a 2 mM solution of Bz-Asn-Leu-Thr-NHMe ($4K_m$) substrate in DMSO. Aliquots (40 μL) of the reaction mixture were quenched after 2, 4, 6, and 8 min, and the aqueous phase containing the radioactive *N*-glycosylated peptide was extracted and quantified as described previously (Sharma et al., 1981; Imperiali & Shannon, 1991). Identical conditions were used for porcine liver OT inhibition studies with the exception that the assay was initiated by adding 10 μL of a 10 mM Bz-Asn-Leu-Thr-NHMe ($2K_m$) solution. Preliminary experiments employed a broad range of inhibitor concentrations for each peptide to afford a rough estimate of the IC_{50}. Three concentrations were then selected to give between 30% and 70% inhibition. All experiments were run in duplicate. In each case, the approximate K_i was determined using the following equation (Segel, 1975):

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

where i represents the fraction inhibition, [I] is the concentra-



1 ($R_1 = NH_3^+$; R_2 = OH; Xaa = Val; Yaa = Thr)
2 ($R_1 = CH_2NH_3^+$; R_2 = OH; Xaa = Val; Yaa = Thr)
3 ($R_1 = NH_3^+$; $R_2 = CH_3$; Xaa = Val; Yaa = Thr)
4 ($R_1 = NH_3^+$; R_2 = H ; Xaa = Val; Yaa = Thr)
5 ($R_1 = NH_3^+$; R_2 = OH; Xaa = Val, Thr, Lys, or Glu; Yaa =Val, Thr, Lys, or Glu)
6a ($R_1 = CONH_2$; R_2 = OH; Xaa = Val; Yaa = Thr)
6b ($R_1 = CONH_2$; R_2 = OH; Xaa = Lys; Yaa = Lys)
6c ($R_1 = CONH_2$; R_2 = OH; Xaa = Glu; Yaa = Thr)

FIGURE 1: Structures of the variants of the parent peptide *cyclo*(hex-Amb-Cys)-Thr-Val-Thr-Nph-NH_2 presented in this study.

tion of inhibitor, and [S] is the concentration of Bz-Asn-Leu-Thr-NHMe. This assessment of K_i assumes competitive inhibition for all the peptides under investigation.

Determination of the Substrate Equilibrium Constants (K_m). [^{3}H]DPPC (100 000 dpm, 60 Ci/mmol) was dried in a microcentrifuge tube as described above. To this was added a mixture of 10 μL of DMSO, 150 μL of assay buffer, and 30 μL of enzyme (solubilized membranes). The reaction was initiated by the addition of 10 μL of peptide solution in DMSO (at increasing concentrations). Four aliquots (40 μL) of the reaction mixture were quenched at 2 min (or 4 min for low peptide concentrations) intervals. The kinetic constants (K_m and V_{max}) were determined using concentrations of peptides ranging between $0.5K_m$ and $5K_m$. The values of K_m and V_{max} were estimated using a Hanes plot (S vs S/V) (Cornish-Bowden, 1995).

Slow Binding. The method was adapted from Hendrickson et al. (1996). [^{3}H]DPPC (600 000 dpm, 60 Ci/mmol) was dried in a microcentrifuge tube. Unlabeled DPPC was added to give a final concentration of 100 nM. The reaction was initiated by addition of a mixture (50 μL) of inhibitor (increasing concentrations) and Bz-Asn-Leu-Thr-NHMe (600 μM) to the enzyme equilibrated in the buffer (550 μL). Time points were taken by quenching 40 μL aliquots at short time intervals (20−30 s) for the beginning of the kinetics and longer intervals as the reaction progressed.

RESULTS

The constrained peptide *cyclo*(hex-$Amb_{(1)}$-$Cys_{(2)}$)-$Thr_{(3)}$-$Val_{(4)}$-$Thr_{(5)}$-$Nph_{(6)}$-NH_2 (**1**, Figure 1) was shown to be a slow, tight-binding inhibitor of yeast oligosaccharyl transferase with a K_i of 37 nM (Hendrickson et al., 1996). The K_i value for **1** was previously determined using a progress curve analysis to afford k_{on} and k_{off} rates for enzyme/inhibitor complex formation and dissociation. In the present study, K_i values are calculated from the degree of inhibition in the presence of three concentrations of inhibitor (each run in duplicate), substrate concentration, and K_m (see Materials and Methods). The K_i value of 25 nM obtained for **1** by this method is in good agreement with the value of 37 nM obtained from the progress curve analysis. The following studies utilize inhibitor **1** as a starting point to explore both the requirements for the consensus sequence and the *C*-terminal extended binding determinants.

Exploration of the Consensus Sequence Requirements. The peptide *cyclo*(hex-Orn-Cys)-Thr-Val-Thr-Nph-NH_2 (**2**, Figure 1) incorporates an additional methylene unit within the side chain of the parent (*S*)-2,4-diaminobutyric acid residue that had been designed to mimic the length of the asparagine side chain in the corresponding substrate. Studies with yeast OT indicated that this peptide was approximately three times less potent than **1**, having a K_i of 75 ± 10 nM. The importance of the key hydroxy amino acid was probed with the peptides *cyclo*(hex-Amb-Cys)-Val-Val-Thr-Nph-NH_2 and *cyclo*(hex-Amb-Cys)-Abu-Val-Thr-Nph-NH_2 (**3** and **4**, Figure 1) in which the threonine residue in the consensus sequence was replaced by either valine or (*S*)-2-aminobutyric acid (Abu). Peptide **3** showed limited solubility in the assay buffer; at a concentration of 200 μM, approximately 10% inhibition was observed, which affords an estimate of 300 μM for the peptide K_i. Inhibition studies with peptide **4** revealed a K_i of 27 ± 6 μM. These results emphasize the importance of the residue 3 hydroxyl group for both inhibitor potency and substrate competence.

Design and Analysis of cyclo(hex-Amb-Cys)-Thr-Xaa-Yaa-Nph-NH_2 Variants. The importance of the extended binding determinants was assessed by varying the nature of the residues Xaa and Yaa in positions 4 and 5 of the inhibitor sequence. Hydrophobic, neutral/polar, acidic, and basic residues were employed. The hydrophobic (valine) and neutral/polar (threonine) residues were those of the parent inhibitor that had been selected based on the statistical survey of glycosylation sites (Gavel and von Heijne, 1990). The acidic and basic residues were represented by glutamic acid and lysine, respectively. All 16 combinations of these four amino acids in the Xaa and Yaa positions (general structure **5**) were prepared and evaluated (Figure 1). The potency of the peptidyl inhibitors was determined, and the K_i values for the 16 peptides with both yeast and porcine liver OT are shown in Table 1. Inhibition constants vary from low micromolar to low nanomolar with the most potent inhibition observed with the Val-Thr variant found in the parent peptide, **1** (K_i = 25 nM).

In studies with yeast OT, the first key observation is that all the peptide variants are less potent than **1**. The single replacement of valine at position 4 by threonine, glutamic acid, or lysine (Thr-Thr, Glu-Thr, and Lys-Thr variants) decreased inhibitor potency by 5−12-fold. Additionally, when valine is maintained at position 4 while threonine at position 5 is replaced with lysine or glutamic acid (Val-Lys and Val-Glu variants), more pronounced changes in the K_i values, corresponding to a loss of affinity (24−38-fold), are noted. However, when the threonine of the parent peptide is replaced with valine at position 5, as demonstrated with the double variants Thr-Val, Lys-Val, and Glu-Val, K_i values demonstrate much lower losses of affinity (*group c*). In general, all the other double replacements (Thr-Glu, Lys-Glu, Glu-Glu, Glu-Lys, and Lys-Lys variants), with the exception of the Thr-Lys variant, resulted in a more significant decrease in the inhibitory activity (64−340-fold,

Table 1: Equilibrium Dissociation Constants (K_i) for Yeast and Porcine Liver Oligosaccharyl Transferase Inhibition by *cyclo*(hex-Amb-Cys)-Thr-Xaa-Yaa-Nph-NH_2 Variants

group	Xaa-Yaa	K_i (μM) yeast OT	K_i (μM) porcine OT
a	Lys-Lys	8.5 ± 1.5	14 ± 4
a	Glu-Lys	4.3 ± 0.5	4.5 ± 1.2
a	Glu-Glu	4.3 ± 0.8	4.0 ± 0.8
a	Lys-Glu	3.2 ± 0.6	5.5 ± 1.5
a	Thr-Glu	1.6 ± 0.1	2.0 ± 0.2
a	Val-Glu	0.95 ± 0.15	1.1 ± 0.1
b	Thr-Lys	0.75 ± 0.07	2.7 ± 0.3
b	Val-Lys	0.60 ± 0.20	2.6 ± 0.4
c	Glu-Val	0.60 ± 0.08	0.68 ± 0.075
c	Lys-Val	0.55 ± 0.14	1.40 ± 0.10
c	Lys-Thr	0.43 ± 0.07	0.31 ± 0.06
c	Glu-Thr	0.31 ± 0.05	0.31 ± 0.06
c	Thr-Thr	0.14 ± 0.02	0.26 ± 0.02
c	Thr-Val	0.10 ± 0.03	0.41 ± 0.09
c	Val-Val	0.09 ± 0.015	0.22 ± 0.04
c	Val-Thr	0.025 ± 0.008	0.03 ± 0.006

[a] Variants with a $K_i \geq 1$ μM. [b] Variants that show the highest selectivity between yeast and porcine OT. [c] Variants with $K_i \leq 1$ μM.

K_i 1−10 μM range). Hydrophobic and polar/neutral residues (represented by valine and threonine, respectively) are well tolerated, the worst combination resulting in only a 5-fold decrease in potency (Thr-Thr variant) relative to **1**. In contrast, charged acidic and basic side chains are detrimental to binding; this is clearly demonstrated when the Xaa and Yaa positions each contain a charged residue. It is also noted that basic residues are less well tolerated than acidic residues.

Identical studies with porcine OT revealed the same general trends in inhibitory activity. As with yeast OT, all the variants are weaker inhibitors than the parent inhibitor **1**. The peptides Val-Val, Thr-Val, Thr-Thr, Glu-Thr, Lys-Thr, and Glu-Val were found to have sub-micromolar inhibitition constants whereas the variants Val-Glu, Lys-Val, Thr-Glu, Val-Lys, Thr-Lys, Lys-Glu, Glu-Glu, Glu-Lys, and Lys-Lys showed K_i values in the micromolar range (*group a*).

Comparison of Porcine and Yeast OT Inhibition. We examined the selectivity of these peptides toward yeast and porcine liver OT. The K_i values are very similar for the enzymes from the two species although inhibition of the yeast enzyme is generally slightly more effective. The highest selectivity is found for the variants that include the Thr-Lys and Val-Lys dipeptides (*group b*); the efficacy ratio [K_i (porcine OT)/K_i (yeast OT)] in these cases is approximately 4-fold (3.5 and 4.3).

Slow-Binding Kinetics. It has previously been reported that peptide **1** shows slow binding inhibition of yeast OT (Hendrickson et al., 1996). This kinetic phenomenon is also observed with **1** for porcine liver OT.

Study of the Potency of Corresponding Substrates. The variation of positions 4 and 5 in the constrained inhibitors leads to K_i constants in the nanomolar to micromolar range. In order to compare the inhibitor K_i values with the corresponding substrate K_m values, a series of peptides that included asparagine in place of (*S*)-2,4-diaminobutyric acid in the inhibitor sequence were prepared. Substrates corresponding to the best (Val-Thr; **6a**) and worst (Lys-Lys; **6b**) inhibitors and one of intermediate potency (Glu-Thr; **6c**) were evaluated. The K_m values for these peptides along with the corresponding K_i values for the analogous inhibitors with yeast OT are shown in Table 2. The relative V_{max} values for the three substrates were similar. A good correlation between substrate K_m and inhibitor K_i is observed.

Table 2: Comparison of Equilibrium Dissociation Constants (K_i) and Equilibrium Constants (K_m) of Three *cyclo*(hex-Amb-Cys)-Thr-Xaa-Yaa-Nph-NH_2 Inhibitors and Their Corresponding Substrates

Xaa-Yaa	peptide **4** K_i (μM)	peptide **5** K_m (μM)	**5**/**4** peptide K_m/K_i
Lys-Lys	8.5 ± 1.5	20.0 ± 1.5	2.35
Glu-Thr	0.31 ± 0.05	0.88 ± 0.20	2.84
Val-Thr	0.025 ± 0.008	0.13 ± 0.03	5.2

DISCUSSION

The peptide *cyclo*(hex-Amb-Cys)-Thr-Val-Thr-Nph-NH_2 (**1**) is the most potent oligosaccharyl transferase inhibitor obtained to date (Hendrickson et al., 1996). The high potency of this new class of glycosylation inhibitors prompted the further evaluation of the role of each individual residue in the inhibitor structure. Specifically, we have probed the importance of Amb (1), Thr (3), Val (4), and Thr (5) in the peptide sequence. Several studies have established the consensus sequence requirements for oligosaccharyl transferase activity. In particular, the absolute requirement for an asparagine residue together with a hydroxy amino acid (serine or threonine) in glycosylation substrates has been known for over 2 decades (Marshall, 1974). The synthetic accessibility of the peptidyl inhibitors described in this paper provides the first opportunity to carry out a systematic study of the structure/function relationships for OT inhibition.

The most potent inhibitor, **1**, presents an isosteric (*S*)-2,4-diaminobutyric acid analog in place of the asparagine in the consensus sequence (Hendrickson et al., 1996). The peptide *cyclo*(hex-Orn-Cys)-Thr-Val-Thr-Nph-NH_2, in which the (*S*)-2,4-diaminobutyric acid is replaced with ornithine, is still an inhibitor with only a 3-fold decrease in potency ($K_i = 75$ nM). This tolerance for the additional methylene unit in the inhibitor family is in sharp contrast to the stringent requirement for asparagine in the substrate peptides; it is well established that the OT shows no glycosylation of peptides that include the homologue glutamine. This result highlights the capacity of OT active site to accommodate the increased size of the flexible ornithine side chain, provided that it bears the requisite positive charge.

The peptides *cyclo*(hex-Amb-Cys)-Val-Val-Thr-Nph-NH_2 and *cyclo*(hex-Amb-Cys)-Abu-Val-Thr-Nph-NH_2 (**3** and **4**), in which the threonine residue in the consensus sequence was replaced by either valine or Abu, show significantly weaker inhibition of OT when compared with the parent peptide **1**. The literature has repeatedly highlighted the absolute requirement of a threonine or serine residue within the glycosylation consensus sequence. In general, peptides including threonine rather than serine show tighter binding and more efficient turnover. In inhibitor **3**, substitution of threonine with the β-branched amino acid valine would be predicted to maintain the conformational preferences of the peptide backbone, while abolishing the potential for hydrogen bonding by replacing the hydroxyl group with a methyl group. The studies presented herein demonstrate that this substitution dramatically compromises binding to the enzyme. The K_i has increased by approximately 4 orders of magnitude. Interestingly, inhibitor **4** shows slightly improved

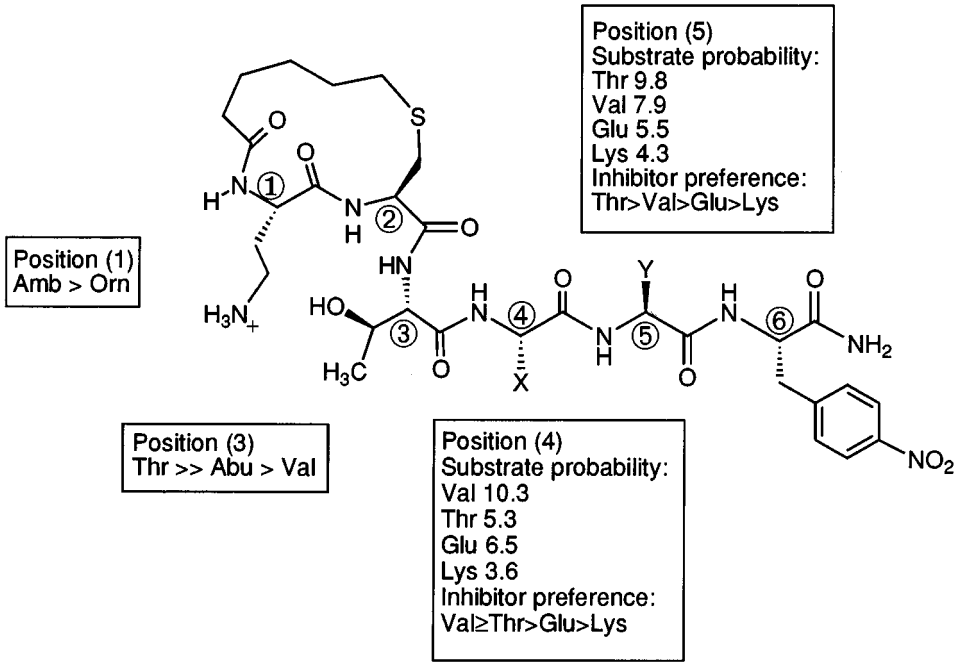


FIGURE 2: Summary of oligosaccharyl transferase amino acid preferences in position 1, 3, 4, and 5 using variants of the peptide *cyclo* (hex-Amb-Cys)-Thr-Val-Thr-Nph-NH_2.

inhibition relative to **3**. In this case, substitution with the Abu residue shows that the single hydroxyl group of the hydroxy amino acid contributes to about 3 orders of magnitide in inhibitor potency. This result underscores the importance of the hydroxy amino acid in both substrate turnover and inhibitor binding.

In contrast to the extensive exploration of the consensus sequence, there are currently no reports concerning the evaluation of the extended binding determinants of OT in synthetic substrates. In this study, we have systematically varied the nature of the two amino acid residues (Xaa and Yaa, peptide **5**) that flank the consensus sequence in the C-terminal direction. It is demonstrated that valine and threonine are tolerated well in positions 4 and 5. In contrast, charged and particularly basic amino acid residues are detrimental to enzyme binding. These results can be evaluated in terms of the statistical studies on native glycoproteins (Gavel & von Heijne, 1990). In these studies, information on the sequences flanking the glycosylation sites was assessed, and a probability ranking associated with finding each of the encoded amino acids at a specific position relative to the modified asparagine was done. The inhibition studies presented in this paper correlate very well with the statistical studies (see Figure 2). The highest frequency observed for valine at position 4 and threonine at position 5 is remarkably consistent with the structure of the most potent inhibitor, **1**, Val-Thr (Hendrickson et al., 1996). Furthermore, the Gavel and von Heijne study also reveals the low frequency of charged residues at positions 4 and 5, which is complemented by the inhibition studies with the Glu-Glu, Glu-Lys, Lys-Glu, and Lys-Lys variants. It should be noted that the presence of valine or threonine residues counterbalances the negative effect of lysine or glutamic acid residues (for example, in the Glu-Thr, Lys-Thr variants).

The inhibition studies were carried out on both yeast and porcine liver enzymes in order to assess whether subtle differences in binding at positions beyond the active site could be detected between the enzymes from the different species. It is now well documented that oligosaccharyl transferase is conserved throughout eukaryotic evolution. Since subunits from yeast and mammalian OT share significant sequence homology (Kelleher & Gilmore, 1994; Breuer & Bause, 1995; Kumar et al., 1995), it is likely that binding sites beyond those defining the interactions with the consensus triad may be less rigorously conserved and therefore distinguishable among species. Therefore the exploitation of extended binding determinants may present a means of generating species specific OT inhibitors.

The species selectivity (yeast/porcine) is rather weak; in general, inhibition of yeast OT is more effective. The highest species selectivity was found for variants having a lysine at position 5; the efficacy ratio in this case approached 4:1. Although these differences in affinity are not yet significant enough to be of practical utility, it is important to note that the variation of the inhibitor structure beyond the consensus sequence may open opportunities for enhanced species selectivities.

The parent inhibitor, **1**, was previously demonstrated to be a slow binding inhibitor of OT (Hendrickson et al., 1996). Since the slow binding kinetic phenomenon is frequently associated with transition state analog inhibition, this prompted us to evaluate the kinetic properties of selected substrates to assess whether the observed trend in K_i values correlated with the K_m for the corresponding substrates or with K_m/k_{cat}. This analysis is based on kinetic studies of thermolysin transition state analog inhibitors and their corresponding substrates (Bartlett & Marlowe, 1983). Therein it was shown that a good correlation between inhibitor K_i and substrate K_m/k_{cat} prevails and there was an absence of proportionality between K_i and K_m (Bartlett & Marlowe, 1983). In these studies with OT, three substrates were synthesized and evaluated. The sequences of these substrates was patterned after typical strong, medium, and weak OT inhibitors. The analysis revealed that both weak (μM K_i) and tight (nM K_i) binding inhibitors exhibited K_i constants 2−5-fold lower than the K_m values of their substrates and that the observed value

for K_i is proportional to the corresponding substrate K_m. Together, these results suggest that these inhibitors are acting as ground state analogs.

The study presented in this paper highlights the utility of this new class of peptidyl inhibitors in the examination of the active site and extended binding determinant of OT. Since the parent inhibitor, **1**, manifests such tight binding to the enzyme, it is possible to probe a wide range of structural variants while still obtaining measurable and reliable binding information that can in turn lead to a precise definition of the key characteristics that may be essential for peptide binding to OT. This study also emphasizes the importance of extended binding determinants in inhibitor potency. Future studies will aim to exploit additional interactions with the enzyme through the preparation of analogs with *N*-terminal sequence extensions. In this context, analysis of glycosylated peptide sequences *in vivo* (Gavel & von Heijne, 1990) suggests that incorporating bulky hydrophobic functionality may improve enzyme affinity. Finally, additional changes to the readily modifiable and modular peptide platform (such as increasing the lipophilicity and substituting the amides in the peptide backbone with isosteric replacements) may lead to bioavailable inhibitors of asparagine-linked glycosylation.

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