Design and Evaluation of Potent Inhibitors of Asparagine-Linked Protein Glycosylation

Tamara L. Hendrickson, Jeffrey R. Spencer, Mihoko Kato, and Barbara Imperiali*

Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125

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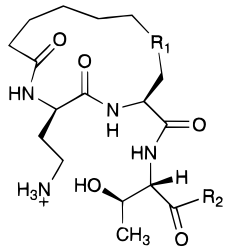
Specific and potent enzyme inhibitors have been invaluable as tools for elucidating the mechanisms of key biochemical transformations, providing insight into the biological implications of specific metabolic processes and inspiring new therapeutic strategies.[1] Recently, the biological importance of protein glycosylation[2] has gained attention as a critical modification with diverse ramifications including effects on protein stability and folding,[3] cellular targeting,[4] and intercellular recognition.[5] Asparagine-linked glycosylation[6] is the predominant protein-carbohydrate modification in eukaryotic cells.[7] Currently, the only inhibitor of *N*-linked protein glycosylation that demonstrates activity at a practical concentration is the microbial product tunicamycin:[8−10] However, the effect of tunicamycin on protein glycosylation is neither specific nor immediate because it functions indirectly by inhibiting the first step in the assembly of the oligosaccharide donor (Dol-P-P-$(GlcNAc)_2$-$(Man)_9$-$(Glc)_3$) essential in the formation of all asparagine-linked glycoproteins.[11] Furthermore, use of tunicamycin requires several cell cycles before the supply of the donor is sufficiently depleted to arrest glycosylation. Therefore, despite the centrality of asparagine-linked glycosylation, no potent inhibitors for oligosaccharyl transferase (OT), the enzyme that actually catalyzes the first committed step in this process, have been reported.

Herein, we describe a new class of slow, tight binding inhibitors for oligosaccharyl transferase. These constrained peptidyl compounds exhibit nanomolar inhibition constants. The modular nature of these inhibitors provides immediate opportunities for structural diversification through combinatorial synthesis.[12−14] These compounds present a readily modifiable platform for the further development of specific glycosylation inhibitors as diagnostic tools to evaluate the role of glycoproteins in biological systems, as potential therapeutic agents[15] and for the preparation of carbohydrate depleted glycoproteins for structural studies.



1. R_1 = CH_2, R_2 = NHMe
2. R_1 = S, R_2 = Val-Thr-Nph-NH_2
3. R_1 = S=O, R_2 = Val-Thr-Nph-NH_2

Figure 1. Chemical structures of **1**−**3** (Nph = *p*-nitrophenylalanine).

OT catalyzes the cotranslational glycosylation of asparagine residues in proteins in which the appropriate consensus sequence requirements are satisfied (-Asn-Xaa-Thr/Ser-; Xaa ≠ Pro).[16] Building from this primary sequence, we have incorporated structural features from three additional experimental observations into our inhibitor design. First, a study of peptide substrates for OT has revealed that binding to the enzyme is enhanced when the substrate is constrained to a specific conformation, namely the "Asx-turn".[17] Second, statistical studies of *N*-linked glycoproteins suggest that glycosylation is modulated by the identity of the residues beyond the consensus sequence, suggesting that interactions between OT and extended binding substrate determinants can be exploited.[18] Finally, mechanistic studies with the nonencoded amino acid γ-aminobutyrine (Amb) in the tripeptide Bz-Amb-Leu-Thr-NHMe afforded a weak competitive inhibitor (K_i = 1 mM) for porcine OT.[19] These features are incorporated into compounds **1**−**3** (Figure 1).

The preparation of compounds **2** and **3** followed standard Fmoc-based solid phase peptide synthesis procedures. In order to introduce the required conformational constraint, an orthogonally protected cysteine residue [Fmoc-Cys(S-S-*tert*-butyl)] was incorporated into the peptide.[20] Following deprotection, cyclization was effected *via* alkylation of an *N*-terminal 6-bromohexanoyl moiety. Resin cleavage at this stage afforded peptide **2**. Since it has previously been demonstrated that constrained thioethers and sulfoxides can favor different conformations and may therefore exhibit different inhibitory properties,[21] the related product **3** was also prepared and investigated. For comparison, the unconstrained analog **4**, with the peptide sequence N^{α}-hexanoyl-Amb-Cys(S-S-*tert*-butyl)-Thr-Val-Thr-Nph-NH_2, was synthesized.

Each of the compounds (**1**−**4**) was evaluated with *S. cerevisiae* oligosaccharyl transferase in competitive assays with the tripeptide substrate Bz-Asn-Leu-Thr-NHMe (Figure 2).[22] The inhibition constants are summarized in Table 1. The tripeptide **1** exhibited a modest K_i of 100 μM; however, extension to the hexapeptides (**2**−**4**) dramatically improved binding. The calculated K_i for **2** (37 nM) shows that this constrained peptide is at least three orders of magnitude more potent than any previously reported inhibitor of OT.[19,23] This

* To whom correspondence should be addressed.

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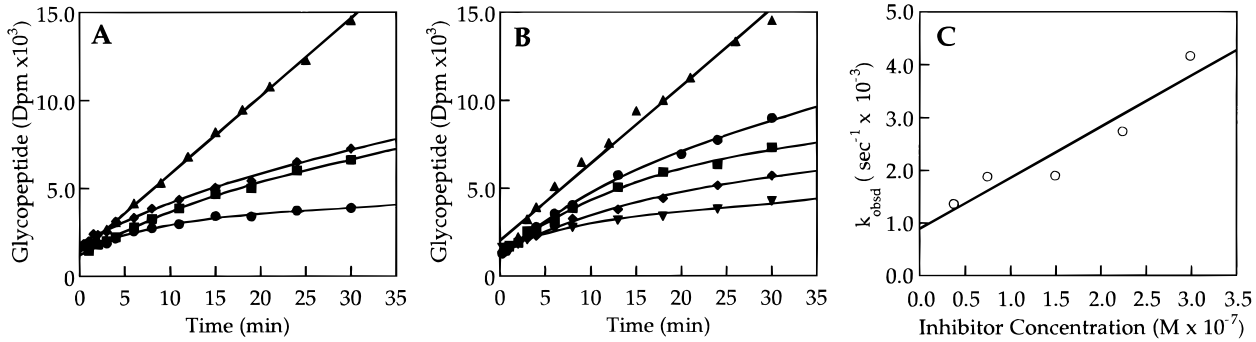

Figure 2. Slow binding inhibition of oligosaccharyl transferase. A. Inhibition by compounds **2**−**4** (▲ - no inhibitor; ◆ - 800 nM linear inhibitor **4**; ■ - 200 nM cyclic sulfoxide inhibitor **3**; ● - 200 nM cyclic thioether inhibitor **2**). B. Compound **2** (▲ - no inhibitor; ● - 37.5 nM inhibitor; ■ - 75 nM inhibitor; ◆ - 150 nM inhibitor; ▼ - 300 nM inhibitor; 225 nM data omitted for clarity). C. Plot of k_{obsd} (calculated from Figure 2B) *vs* inhibitor concentration.

Table 1. Kinetic Constants for Inhibitors **1**−**4**

peptide	K_M (μM)	K_i (nM)
Bz-NLT-NHMe	25	
1		100000[a]
2		37[b]
3		130[c]
4		360[c]

[a] Not slow binding; the K_i for peptide **1** was determined using standard procedures, by competition against several concentrations of Bz-NLT-NHMe. [b] $k_{on} = 2.4 \times 10^4$ s^{-1} M^{-1}; $k_{off} = 8.9 \times 10^{-4}$ s^{-1}. [c] Kinetic constants for compounds **3** and **4** were evaluated at a concentration which produced approximately 50% inhibition in the presence of 50 μM Bz-NLT-NHMe ($2 \times K_M$). The values for K_i were calculated as described by Segel[29] using the following equation: $K_i = ([I]-i[I])/(i + [S]i/K_M)$; i = percent observed inhibition.

enhancement in potency is due to the collective exploitation of the specific structural features incorporated into our design. The 3.5-fold difference in affinity between **2** and the corresponding sulfoxide **3** may result from slightly different conformational preferences. Since **2** exhibited slow tight binding inhibition and because it was the most potent of the inhibitors under investigation (see Figure 2A), a detailed kinetic evaluation, using a progress curve analysis (see Figure 2B,C) for the determination of k_{on} and k_{off}, was carried out.[24,25] The slow binding kinetic phenomenon is often associated with a slow, structural reorganization of the enzyme/inhibitor complex to a species that more closely resembles the transition state in the reaction coordinate.[26] Therefore, these compounds and related analogs may assist in elucidating the mechanism of action of OT.

The enzyme oligosaccharyl transferase has been characterized from several different species and shows significant structural homology throughout eukaryotic evolution.[27] It was therefore of interest to examine whether inhibitor **2** demonstrated any species selectivity. Thioether **2** was examined as an inhibitor against *S. cerevisiae* and porcine liver OT. Notably, inhibition of yeast OT was approximately three-fold more effective. Since extended binding interactions from the C-terminal residues contribute significantly to enzyme binding,[18] it may be possible to manipulate and enhance this species selectivity by simple changes in the inhibitor primary sequence. To verify that **2** was specific for *N*-linked rather that *O*-linked glycosylation, its activity against the common *O*-linked glycosylation enzyme, polypeptide *N*-acetylgalactosaminyl transferase,[28] was also assessed. The inhibitor showed no measurable activity against this enzyme even at elevated concentrations. The relative efficacy of inhibitor **2** against these enzymes is shown in Figure 3.

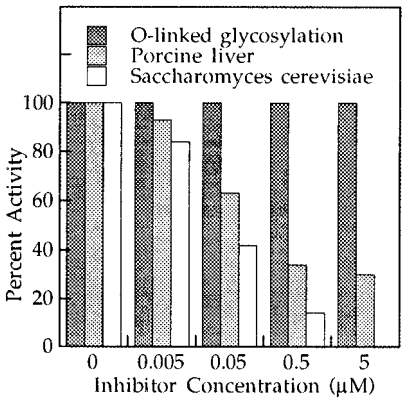

Figure 3. Cyclic inhibitor **2** shows species selective properties and specifically targets *N*-linked glycosylation. Polypeptide *N*-acetylgalactosaminyl transferase[28] was used to assess **2** as an inhibitor of the *O*-linked glycosylation process. Species selectivity was evaluated with porcine liver and *S. cerevisiae* OT.

Herein, we have reported a new class of slow, tight binding inhibitors that exhibit nanomolar inhibition constants for the enzyme oligosaccharyl transferase. This class of cyclic peptides provides the first example of a readily available and adaptable family of potent protein glycosylation inhibitors. These compounds will be valuable tools for future studies designed to elucidate the roles of glycosylation in complex cellular processes.

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Supporting Information Available: Synthetic details for the preparation of **1**, **2**, **3**, and **4** and assay details and kinetic data for studies reported herein (7 pages). See any current masthead page for ordering and Internet access instructions.

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