Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 281–284

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Probing the Extended Binding Determinants of Oligosaccharyl Transferase with Synthetic Inhibitors of Asparagine-Linked Glycosylation

Maria de L. Ufret and Barbara Imperiali*

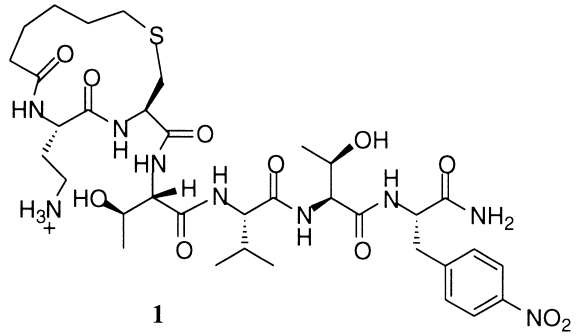
Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

Received 15 October 1999; accepted 3 December 1999

Abstract—Protein glycosylation is associated with many critical biological processes. In connection with studies on the mechanism of asparagine-linked glycosylation by the enzyme oligosaccharyl transferase, we have prepared peptide inhibitors that interact with the enzyme at nanomolar concentrations. Herein we describe efforts directed toward improving the binding properties of these inhibitors.

Introduction

Asparagine-linked glycosylation is one of the most complex enzyme-catalyzed protein modifications.[1] This process is catalyzed by the multimeric membrane-associated glycoprotein, oligosaccharyl transferase (OT), and involves the transfer of a tetradecasaccharide from a dolichol pyrophosphate-linked donor to the side chain of an asparagine residue in an Asn-Xaa-Thr/Ser (NXT/S) consensus sequence in a nascent polypeptide. The function of the individual enzyme subunits and the details of the active site of this enzyme remain to be elucidated.[2–4]



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Previously, peptide inhibitors were prepared in order to study the structural requirements for catalysis. In these studies, a prototype inhibitor, with the asparagine in the consensus sequence substituted with 2,4-diaminobutanoic acid (Dab) was obtained.[3,4] Additionally, peptides structurally constrained in an Asx-turn conformation have greatly enhanced enzyme/ligand affinity.[5] A constrained Asx-turn-type motif is achieved through cyclization of the inhibitor from the side chain of cysteine in the Xaa position of the consensus sequence to a 6-bromohexanoyl moiety used to cap the peptide at the N-terminus following the Dab.[3,4] The tripeptide inhibitor *c*[Dab-Add]-Thr-NHMe, which contains the amino acid (*S*)-2-aminodecanedioic acid (Add), showed a K_i of 100 μM.[3,4] Also, previous studies showed that the use of a valine-threonine dipeptide at the C-terminus of the consensus sequence results in greatly enhanced binding to OT relative to a variety of other dipeptide combinations.[3,4,6] For example, peptide **1** has a K_i of 37 nM.[3,4]

The current study explores the effect of modifications in the structure of a peptide on enzyme–inhibitor interactions. The modifications presented herein include C-terminus and Dab variations, as well as a comparison of linear and cyclized peptides. The compounds studied are presented in Figure 1.

Peptides **1**–**8**[7] were synthesized using standard Fmoc-based solid phase synthesis methods on Fmoc-PAL-PEG-PS resin. In order to prepare peptides **3**–**8**, an allyloxycarbonyl protected (*N*-alloc) Dab was employed.

Keywords: enzyme inhibitors; glycosylation; glycopeptides/glycoproteins; solid phase synthesis.

*Corresponding author. Tel.: +1-617-253-1838; fax: +1-617-452-2419; e-mail: imper@mit.edu

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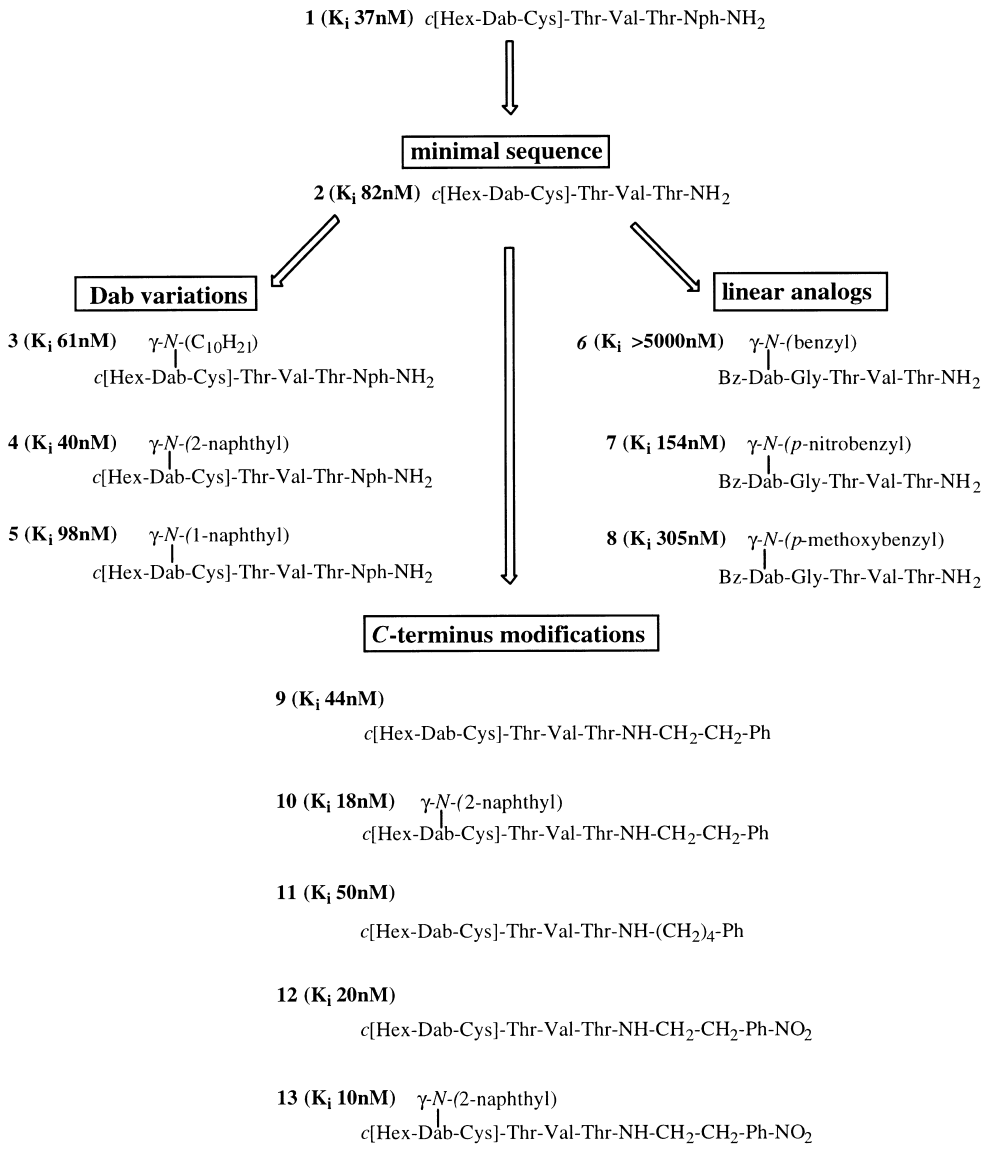
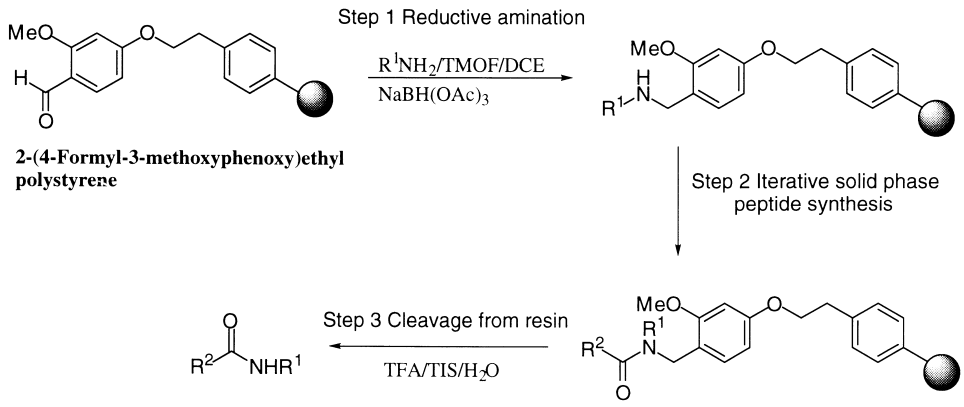


Figure 1. Summary of synthetic asparagine-linked glycosylation inhibitors presented in this report.

The *N*-alloc group can be deprotected on the resin without affecting other protecting groups; deprotection was achieved using tetrakis(triphenylphosphine) palladium in $CHCl_3$:morpholine:AcOH (37:1:2). Reductive amination was carried out by the addition of the desired aldehyde, followed by reduction with $NaBH_3CN$, as described by Devraj and Cushman.[8] It is important to wash away any excess aldehyde before adding the sodium cyanoborohydride in order to avoid reductive alkylation of the secondary amine product. To obtain inhibitors **1**–**5** and **9**–**13** the peptides were capped with 6-bromohexanoic acid. Following this, the Cys(S-*t*Bu) was orthogonally deprotected under nitrogen using tributylphosphine. Cyclization between the thiolate of the cysteine and the 6-bromohexanoyl group was achieved in degassed DMF, using an excess of 1,1,3,3-tetramethylguanidine as a base.[9]

Peptides **9**–**13** were synthesized on a 2-(4-formyl-3-methoxyphenoxy)ethyl polystyrene resin, as shown in Scheme 1. The desired amine and sodium triacetoxyborohydride were added to the resin in a 10-fold excess. Double addition to the resin is not observed due to steric hindrance. The first amino acid was coupled to the secondary amine using 1-hydroxy-7-azabenzotriazole/diisopropylcarbodiimide (HOAt/DIPCDI) activation. Further elongation of the peptide by iterative solid-phase peptide synthesis was performed using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate: *N*-hydroxybenzotriazole (HBTU: HOBt). The peptide was cleaved from the resin using TFA:triisopropylsilane:H_2O (95:2.5:2.5) to obtain the desired inhibitor.

The *p*-nitrophenylalanine (Nph) residue in peptide **1** was originally introduced to allow facile concentration determination.[3,4] Removal of this residue (peptide **2**) resulted in decreased enzyme–inhibitor affinity (K_i 82 nM versus 37 nM).[10] However, as illustrated in Figure 1, the replacement of the terminal amino acid in **1** by



Scheme 1. Synthesis of C-terminal-modified peptides. R^1 = C-terminal amide introduced via reductive amination with R^1NH_2 in Step 1; R^2 = polypeptide chain constructed in N-terminal direction in Step 2.

appropriately spaced and substituted aryl derivatives compensates for the loss of the binding determinants in **1**. For example, the *p*-nitrophenethyl-modified derivative, **12**, shows a 20 nM K_i.

Modification of the Dab side chain, by reductive amination with either *n*-decyl or 2-naphthyl aldehyde, also resulted in a lower K_i and increased affinity of peptides **3** and **4** to OT (K_i 61 and 40 nM, respectively). These derivatives were prepared to probe whether it was possible to exploit hydrophobic interactions that the enzyme might make with the glycosyl donor. Interestingly, the related 1-naphthyl analogue (**5**) shows a higher K_i which may be the result of unfavorable interactions, since the orientation of the naphthyl group is different in the two peptides. The choice of these highly hydrophobic groups was based on the observation that aromatic amino acids have been implicated in carbohydrate binding sites.[11,12]

Linear analogues **6–8** were prepared in order to study the importance of groups attached to the Dab in unconstrained analogues. The diminished binding of these analogues emphasize the importance of the constrained motif provided by the cyclization. Inhibitors **6–8** are all significantly less potent than the corresponding cyclic analogues.

Since modifications to the C-terminus led to increased binding of certain inhibitors to the enzyme and because the reductive amination of the Dab residue also enhanced binding, peptide **13** was prepared to examine whether additive effects could be achieved. This is indeed the case, peptide **13**, with both a *p*-nitrophenethyl at the C-terminus and a 2-naphthyl at the Dab side chain yields the most potent inhibitor of OT to date, having a K_i of 10 nM.

Conclusion

A family of peptide inhibitors for OT have been prepared, with binding constants ranging from 5000 to 10 nM. Appropriately substituted hydrophobic groups in the Dab side chain interact favorably with the enzyme, which suggests the presence of hydrophobic amino acids, most likely aromatic residues, in the peptide binding site of OT. Also, modifications with an aromatic functionality at the C-terminus greatly enhances the enzyme/inhibitor affinity. A combination of these two modifications produces the most potent inhibitor for OT. Further experiments are being carried out to study the membrane permeability of these compounds, which would allow the study of in vivo inhibition of oligosaccharyl transferase.

Acknowledgements

This research was supported by the National Institutes of Health (GM39334). The award of a Ford Foundation predoctoral fellowship to M. U. is also gratefully acknowledged.

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7. All peptides were characterized by ES–MS. Peptide **2**: calcd for $C_{26}H_{47}N_7O_8S$ 617, obsd 618 (MH^+); **3**: calcd for $C_{36}H_{67}N_7O_8S$ 757, obsd 758 (MH^+); **4**: calcd for $C_{37}H_{55}N_7O_8S$ 757, obsd 758 (MH^+); **5**: calcd for $C_{37}H_{55}N_7O_8S$ 757, obsd 758 (MH^+); **6**: calcd for $C_{33}H_{47}N_7O_8$ 669, obsd 670 (MH^+); **7**: calcd for $C_{33}H_{46}N_8O_{10}$ 714, obsd 715 (MH^+); **8**: calcd for $C_{34}H_{49}N_7O_9$ 699, obsd 700 (MH^+); **9**: calcd for $C_{34}H_{55}N_7O_8S$ 721, obsd 722 (MH^+); **10**: calcd for $C_{45}H_{63}N_7O_8S$ 861, obsd 862 (MH^+); **11**: calcd for $C_{36}H_{59}N_7O_8S$ 749, obsd 750 (MH^+); **12**: calcd for $C_{34}H_{54}N_8O_{10}S$ 766, obsd 767 (MH^+); **13**: calcd for $C_{45}H_{62}N_8O_{10}S$ 906, obsd 907 (MH^+).
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10. Determination of IC_{50} and K_i: The radiolabelled carbohydrate substrate Dol-P-P-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 crude OT-containing microsomes from *S. cerevisiae* 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 min (dpm) were plotted as a function of time for the control and different inhibitor concentrations (three inhibitor concentrations were selected to give between 30 and 70% inhibition). The IC_{50} was then derived from a plot of percent inhibition against inhibitor concentration. All assays were run in duplicate. The K_i was determined from the IC_{50} using the following equation (Segel, I. H. In *Enzyme Kinetics*; Wiley, J. and Sons: New York, 1975):

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

The K_i values provided are mean values; the observed deviation is maximally 15% of the mean value.

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