Synthesis and Evaluation of Tripeptides Containing Asparagine Analogues as Potential Substrates or Inhibitors of Oligosaccharyltransferase

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The amino acids 5-diazo-4-oxo-L-norvaline, 4-oxo-L-norvaline, and (methanesulfinyl)-L-alanine have been incorporated into three separate tripeptides wherein these nonnatural amino acids replace Asn in a known tripeptide substrate of oligosaccharyltransferase. Synthesis of both the diazoketone- and sulfoxide-containing peptides involved functionalization of the appropriate side chain after peptide assembly, whereas synthesis of the methyl ketone-containing peptide was effected by synthesis of the protected amino acid followed by its incorporation into the desired tripeptide. None of the three synthetic tripeptides showed activity as substrates, nor were they potent inhibitors of oligosaccharyltransferase at concentrations that were 10−35 times the K_m for the corresponding Asn-containing tripeptide substrate. NMR analysis in DMSO-d_6 showed that the diazoketone and methyl ketone peptides adopt the "Asx-turn" conformation, which has been postulated to be crucial for substrate binding. Furthermore, a nonsubstrate peptide, Ac-Asn-Pro-Thr-NH_2, was found to adopt the "Asx-turn" in both the solid state and in solution (DMSO-d_6). The collective data suggest that the ability to form an Asx-turn in the N-glycosylation consensus sequence (Asn-Xaa-Ser/Thr) may be a necessary but not sufficient condition for substrate binding and catalysis.

Introduction

Oligosaccharyltransferase (OST, EC 2.4.1.119), the enzyme responsible for formation of the peptide−carbohydrate bond in N-linked glycoprotein synthesis, catalyzes transfer of the oligosaccharide portion of $Glc_3Man_9GlcNAc_2$-P-P-dolichol (Dol-P-P-OS) to the Asn side chain carboxamide of the polypeptide.[1,2] Specifically, cotranslational N-glycosylation occurs in the consensus sequence Asn-Xaa-Ser/Thr, **1**, to give the β-linked glycopeptide **2** (Scheme 1).[3,4] This Asn-Xaa-Ser/Thr sequon is necessary, albeit not sufficient, for N-linked glycosylation. Peptides as short as the minimum tripeptide containing the Asn-Xaa-Ser/Thr consensus sequence are good in vitro substrates for OST.[5,6]

Amide nitrogens are poor nucleophiles,[7] and a reasonable N-glycosylation mechanism must explain the enhanced reactivity of the Asn β-carboxamide.[4,8−10] Various N-glycosylation mechanisms have been proposed, all focusing on an OST-mediated activation of the Asn β-carboxamide. While our previous research has ruled out several mechanisms based on analogy to glutamine-dependent amidotransferases[11] involving fully formed reactive intermediates,[4,9,12] other mechanisms involving more transient species are plausible.[8,10,13]

As depicted in Scheme 2, OST may activate the Asn carboxamide nitrogen in one of several ways. An active site nucleophile may add to Asn to form a tetrahedral intermediate **3** (Scheme 2, path a). The nitrogen atom in intermediate **3** is more nucleophilic than the carboxamide nitrogen in the parent amide **1**. Nucleophilic attack of tetrahedral intermediate **3** on the LOS glycosyl donor, followed by collapse of the glycosylated tetrahedral intermediate, would give the N-linked glycopeptide **2**. There is precedent for a similar mechanism operating for *E. coli* asparagine synthetase.[14,15] Alternatively, a "substrate-assistance" mechanism is possible for OST-catalyzed N-glycosylation (Scheme 2, path b). However, prior studies with isoAsn-containing[4] and deuterated Asn-containing[9] peptides argue against a variety of "substrate assistance" mechanisms, including that involving **4**. Finally, in path c (Scheme 2), deprotonation of the Asn β-carboxamide would generate a nucleophilic imidate **5**. N-Glycosylation of imidate **5** would then directly provide glycopeptide **2**. Variations of this amide deprotonation

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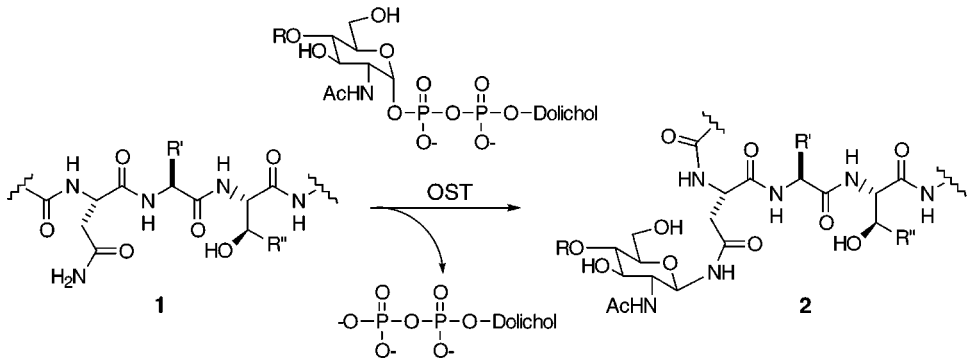
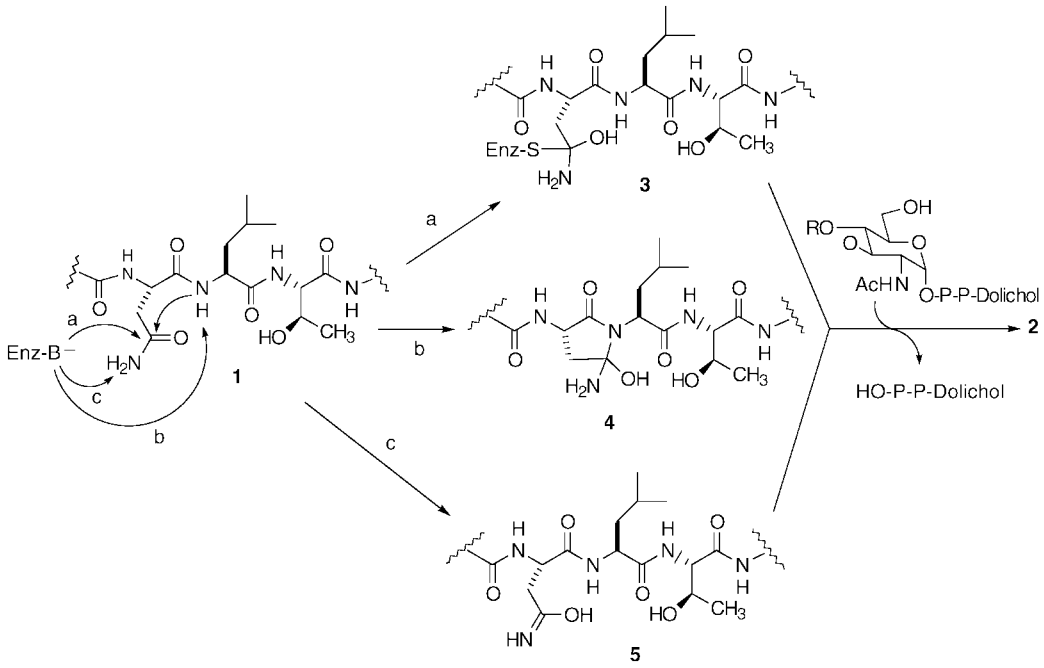
Scheme 1

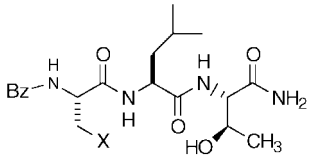


Scheme 2



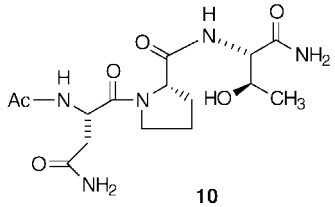
mechanism have been proposed by several groups to explain sequence specificity of the glycosylation process,[13,16] including a proposed role for an Asx-turn motif in facilitating such a mechanism.[17,18]

Peptides with the key Asn replaced by suitable amino acid analogues, such as β-fluoroasparagine[19] and 2,4-diaminobutyric acid (Amb),[10,17,20] have been shown to be OST substrates and/or inhibitors. In an attempt to gain more understanding about the OST mechanism, we have synthesized tripeptides **7**−**9**, wherein the carboxamide



6, X = $CONH_2$
7, X = $COCHN_2$
8, X = $COCH_3$
9, X = $SOCH_3$

moiety of the Asn targeted for glycosylation in substrate peptide **6** is replaced by a diazoketone (**7**), a methyl ketone (**8**), or a methyl sulfoxide (**9**). In addition, the synthesis of a known nonsubstrate peptide, Ac-Asn-Pro-Thr-NH_2 (**10**),[8] was carried out in order to provide comparative structural information on the role of proposed secondary structural motifs on ligand binding to OST.



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Tripeptides **7**−**9** were synthesized to address two general N-glycosylation mechanisms: (1) formation of an enzyme-bound tetrahedral intermediate (Scheme 2, path

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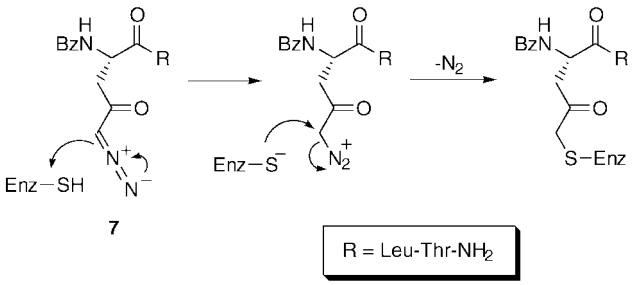
Scheme 3



a) and (2) Asn deprotonation (Scheme 2, path c). Thus, tripeptide **7** was proposed to target a nucleophilic residue such as cysteine in the enzyme active site (Scheme 3). Diazoketone-containing peptides have been widely used as irreversible inhibitors of cysteine and serine proteinases.[21] Since sulfhydryl-specific reagents such as *p*-(chloromercury)benzoate (pCMB) and methyl methanethiolsulfonate (MMTS) are time-dependent inhibitors of OST,[22,23] it is plausible that OST contains an essential cysteine that might act as either a nucleophile or a base to promote glycosylation. Thiol reagents such as pCMB and MMTS are not, however, substrate analogues, and it is unclear how OST inactivation by these compounds relates to the catalytic mechanism.

Tripeptides **8** and **9**, in which Asn was replaced by 4-oxo-L-norvaline (ONV) and methanesulfinyl-L-alanine, respectively, were designed as potential OST substrate analogues. If OST catalyzes N-glycosylation by amide deprotonation (Scheme 2, path c), the methyl ketone **8** and/or sulfoxide **9** may be substrates for N-glycosylation. Enzyme-catalyzed deprotonation of **8** or **9**, followed by glycosylation of the resulting carbanion, would give a C-linked glycopeptide (Scheme 4). Alternatively, ketone **8** with its electrophilic center located in the amino acid side chain may act as a reversible inhibitor of OST via formation of a hemiketal with an OST active site nucleophile.[24]

The solution conformations of the peptide analogues **7**−**9** in DMSO-d_6 were compared to the solution conformation of a substrate peptide, Bz-Asn-Leu-Thr-NH_2 (**6**), and a nonsubstrate peptide, Ac-Asn-Pro-Thr-NH_2 (**10**). Since it has been proposed that a specific secondary structure, the "Asx-turn," is required of OST substrates during catalysis,[8,17,18] these conformational studies in connection with the biochemical data presented herein provide a useful test of this hypothesis.

Results and Discussion

Synthesis of Peptide Analogues 7−9. Here, we present the synthesis and characterization of modified peptides **7**−**9** (Scheme 5). To our knowledge, this paper presents the first description of peptides containing these three amino acid analogues.[25]

Diazoketone 7. The amino acid 5-diazo-4-oxo-L-norvaline (DONV) is an irreversible inactivator of L-asparaginase and glycosylasparaginase.[28,29] In both cases, DONV reacts with an active-site Thr OH to give a covalent α-keto ether linkage. Peptidyl diazoketones are also irreversible inhibitors of many cysteine proteinases.[21] In these cases, however, the diazoketone functional group is always located at the C-terminus of the peptide.

Since the diazoketone moiety is labile to both acid and hydrogenolysis,[21] this functional group was introduced during the last step of peptide synthesis. We previously reported the use of Bz-Asp-Leu-Thr-NH_2 (**11**) as an intermediate in glycopeptide synthesis.[30] This protected tripeptide **11** was also used as the precursor for the diazoketone-containing peptide **7**. Mixed anhydride activation of the side-chain carboxylate of Bz-Asp-Leu-Thr-NH_2 (**11**) followed by the addition of diazomethane afforded the desired peptidyl diazoketone **7** as a crystalline solid in 31% yield (Scheme 5). An IR spectrum of peptide **7** clearly indicated the presence of the newly formed diazoketone, as evidenced by the characteristic diazo stretch at 2109 cm^{-1}. The ^1H (Table 1) and ^{13}C NMR spectra (see Supporting Information) were also consistent with the proposed structure for **7** with the diazomethyl proton at 6.09 ppm in DMSO-d_6 (5.92 ppm in CD_3OD) and the ketone carbonyl ^{13}C resonance at 194.8 ppm (in CD_3OD).

Methyl Ketone 8. Tripeptide **8** contains the rare amino acid 4-oxo-L-norvaline **12** (ONV) in place of the key Asn residue of peptide substrate **6**. To date, there is only a single report of a peptide or protein containing ONV, as Schultz used unnatural amino acid mutagenesis to incorporate ONV into T4 lysozyme.[31] Whereas synthesis of both the diazoketone- and sulfoxide-containing peptides **7** and **9** involved functionalization of the appropriate side chain after peptide assembly, the synthesis of Bz-ONV-Leu-Thr-NH_2 (**8**) was effected by the stepwise incorporation of ONV into the desired tripeptide (Scheme 5). ONV (**12**), prepared via diazomethane homologation of a suitably protected β-aspartyl-semialdehyde,[32] was first converted to its Boc carbamate in 86% yield.

1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC)-mediated coupling of Boc-ONV with Leu-Thr-NH_2 afforded the Boc tripeptide **13** in 47% yield after chromatography. The ^1H NMR spectra of both the crude reaction mixture and purified **12** revealed that no racemization had occurred at the ONV α-carbon during peptide coupling with Boc-ONV. Finally, acid-catalyzed removal of the Boc protecting group and reacylation of the resultant N-terminal amine with Bz_2O gave tripeptide **8** in 59% yield after purification.

The structure of peptide **8** was confirmed by NMR spectroscopy through the use of 2D $^1H-^1H$ COSY and $^1H-^1H$ ROESY experiments (see Table 1 for ^1H NMR assignments in DMSO-d_6). The ^1H and ^{13}C NMR data

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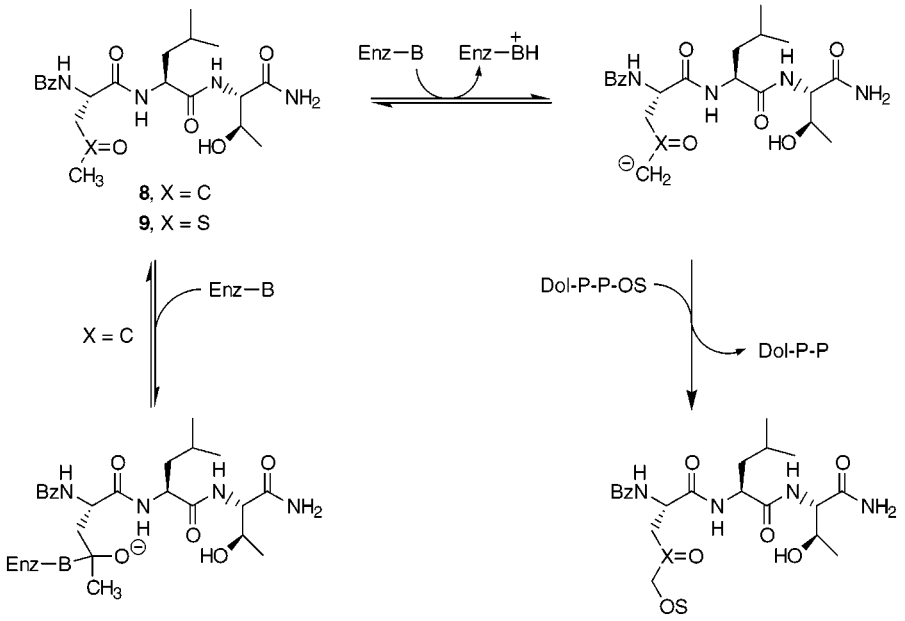
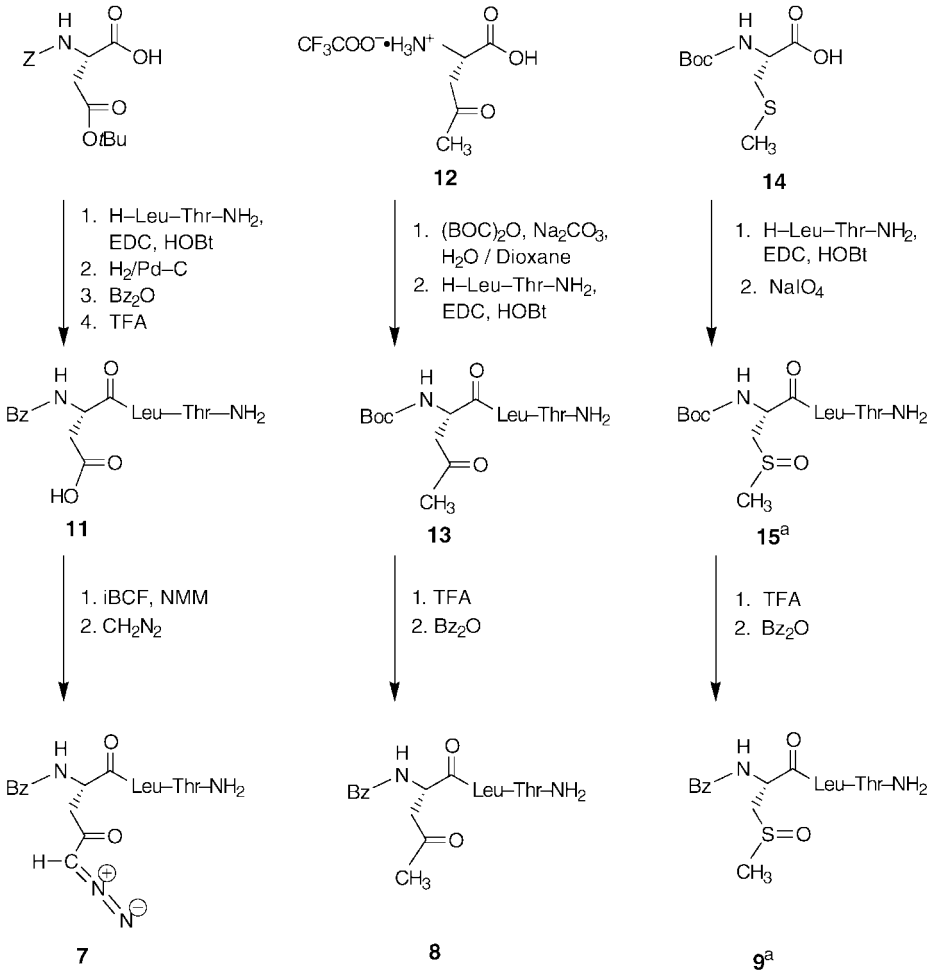
Scheme 4



Scheme 5



[a] Mixture of diasteromers (3:1) observed by HPLC and ^{1}H NMR.

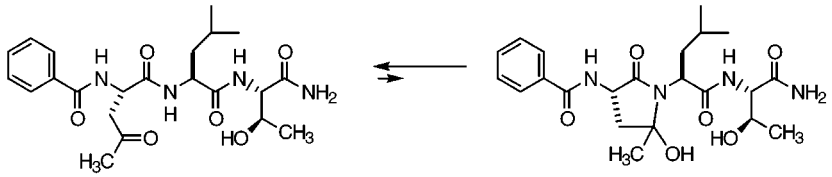
confirmed that the β-aspartyl methyl ketone in tripeptide **8** is the predominant tautomer in D_2O, DMSO-d_6, and CD_3CN. For example, the 2D ^{1}H−^{1}H COSY spectra of tripeptide **8** in DMSO-d_6 showed a strong Leu NH-αH cross-peak, with the Leu NH integrating for one proton, indicating that peptide **8** exists as an acyclic ketone and not as a cyclized aminal (Scheme 6). In addition, the ^{1}H resonance at 2.12 ppm and a ^{13}C resonance at 207.5 ppm

Table 1. Proton Chemical Shift Assignments for Tripeptides 6−9 (5.0 mM) in DMSO-d_6 at 298 K. Format: Splitting Pattern, ppm (J ^{1}H−^{1}H Listed in Hz)

resonance	6	7	8	9[a]
i-NH	d, 8.60 (7.8)	d, 8.64 (7.8)	d, 8.56 (6.8)	(minor) d, 8.89 (7.8) (major) d, 8.83 (7.8)
Leu NH	d, 8.16 (8.4)	d, 8.17 (7.8)	d, 8.12 (7.2)	(major) d, 8.45 (7.8) (minor) d, 8.29 (7.5)
Aromatic	d, 7.84 (7.2)	d, 7.84 (7.5)	d, 7.82 (6.9)	d, 7.86 (7.8)
Thr NH	d, 7.59 (8.7)	7.53[b]	7.51[b]	7.55[b]
Aromatic	m, 7.62−7.43	m, 7.68−7.52	m, 7.59−7.43	m, 7.60−7.48
term *cis*-NH_2	s, 7.06	s, 7.08	s, 7.10	s, 7.09
term *trans*-NH_2	s, 7.06	s, 7.06	s, 7.06	s, 7.09
trans-Asn δ-NH	s, 7.39			
cis-Asn δ-NH	s, 6.96			
Diazo-Ket		s, 6.09		
i-αH	m, 4.75	m, 4.84	m, 4.79	m, 4.85
Thr-OH	d, 4.81 (5.6)	4.84[b]	d, 4.86 (4.7)	4.85[b]
Leu-α	m, 4.24	m, 4.29	m, 4.28	m, 4.29
Thr-β	m, 4.00	m, 3.99	m, 4.01	m, 4.00
Thr-α	m, 4.05	m, 4.06	m, 4.06	m, 4.08
i-βH	dd, 2.65 (5.3, 15.2)	m, 2.83	m, 2.94(5.6, 17.0)	m, 3.18
i-β'H	dd, 2.58 (8.7, 15.2)	m, 2.83	m, 2.94(8.3, 17.0)	m, 3.18
i-CH_3			s, 2.12	(minor) s, 2.65 (major) s, 2.61
Leu γ	m, 1.62	m, 1.60	m, 1.60	m, 1.63
Leu β	m, 1.50	m, 1.49	m, 1.49	m, 1.51
Thr CH_3	d, 1.00 (6.2)	d, 0.99 (5.9)	d, 0.99 (4.7)	d, 1.00 (6.2)
Leu CH_3	d, 0.85 (6.5)	d, 0.85 (6.2)	d, 0.85 (6.5)	d, 0.87 (6.2)
Leu CH_3'	d, 0.80 (6.5)	d, 0.81 (6.2)	d, 0.81 (5.6)	d, 0.82 (6.5)

[a] Sulfoxide tripeptide **9** has doubling of peaks due to a 3:1 mixture of diastereomers. [b] Due to spectral overlap the ^3J values were not determined. The chemical shifts for the NH and OH resonances, which overlapped with other ^{1}H resonances in the 1D spectrum, were determined using a 2D ^{1}H−^{1}H COSY experiment.

Scheme 6



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were consistent with a methyl ketone. These NMR data ruled out the aminal or hydrate as major tautomers for tripeptide **8** in solution.[33] In addition, IR spectral data for **8** in CD_3CN indicated the presence of a ketone moiety as evidenced by a strong absorbance at 1734 cm^{-1}.

Sulfoxide 9. Synthesis of tripeptide analogue **9** started with the methylation of L-cysteine.[34] N-Protection of *S*-methyl-L-cysteine by Boc_2O gave **14** in 78% yield. Coupling of **14** with the Leu-Thr-NH_2 dipeptide followed by oxidation with $NaIO_4$[35] proceeded smoothly to give a mixture of two diastereomers **15** in 81% yield. The sulfoxide-containing peptide **9** was then obtained in 82% yield after Boc deprotection of **15** with TFA, followed by reacylation of the N-terminus with Bz_2O. After recrystallization, peptide **9** was found to be a mixture of diastereomers in a ratio of approximately 3:1 as determined by ^{1}H NMR spectroscopy (Table 1) and analytical reversed-phase HPLC. The 500 MHz ^{1}H NMR spectrum showed that the Leu NH ($\Delta\delta$ = 0.16 ppm), the (methanesulfinyl)-L-alanine NH ($\Delta\delta$ = 0.06 ppm), and the (methanesulfinyl)-L-alanine methyl group ($\Delta\delta$ = 0.04 ppm) had the largest chemical shift differences for the two diastereomers. With the exception of these three protons, all the other ^{1}H resonances showed no major chemical shift differences for the two peptide diastereomers in DMSO-d_6 over the temperature range from 298 to 316 K. These data are consistent with diastereomeric sulfoxides for peptide **9**.

Conformational Analysis of Tripeptides. Imperiali et al. have proposed that N-glycosylation substrates favor a "bioactive" conformation, wherein the Asn-Xaa-Ser/Thr consensus sequence forms a secondary structure known as the Asx-turn.[8,17,18] They suggest that OST recognizes the Asx-turn, and that once the peptide is bound to the enzyme's active site, the Asx-turn also activates the Asn β-carboxamide for subsequent N-glycosylation. An Asx-turn is formed by a hydrogen bond between an Asn/Asp side chain C $^\gamma$O and the i + 2 backbone NH. If the i + 2 residue is Thr or Ser, then the turn's 10-membered ring can be further stabilized by an additional Thr/Ser $^\gamma$OH to Asn C $^\gamma$O hydrogen bond. The Asx-turn is found in numerous protein crystal structures,[36] and it also occurs in the crystal structures of at least nine short peptides.[37] Marraud et al. have analyzed both the solid-state and solution structures of

(33) A related tripeptide with a β-aspartyl semialdehyde in position 1 predominates as the cyclic aminal; see: Hendrickson, T. L.; Imperiali, B. *Biochemistry* **1995**, *34*, 9444−9450.

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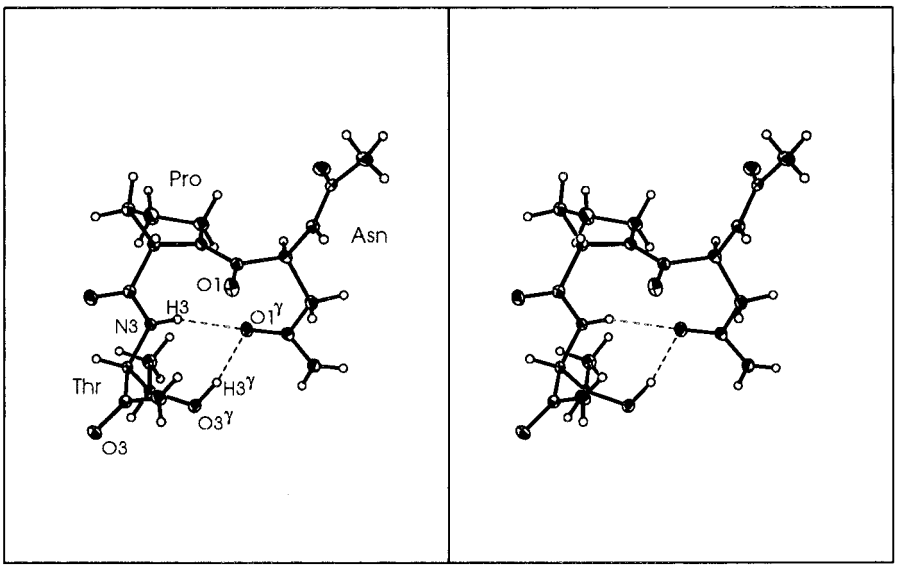

Figure 1. Stereoview of the crystal structure of Ac-Asn-Pro-Thr-NH_2 **10**. This nonsubstrate peptide adopts a Type I_t Asx-turn.

some short peptides that form the Asx-turn.[38] In the context of peptide N-glycosylation, Imperiali has provided evidence that the Asx-turn is a major solution conformer of substrate peptides in DMSO-d_6,[8] and we have also demonstrated that N-glycosylation of a substrate tripeptide stabilizes the Asx-turn of the resulting glycopeptide in a mixed organic solvent.[39] For the present work, we were concerned that the amino acid modifications in peptides **7**−**9**, relative to the standard N-glycosylation substrate Bz-Asn-Leu-Thr-NH_2 **6**, might result in conformational differences that would deter binding of peptide analogues **7**−**9** by OST.

Before evaluating the solution conformation of peptides **6**−**9**, we first studied the conformation of a nonsubstrate peptide, Ac-Asn-Pro-Thr-NH_2 **10**, in both the solid state and in DMSO-d_6 solution. First, we felt that such a comparison would allow us to better evaluate the solution NMR data for the noncrystalline peptides **6**−**9**. Second, the conformation of Ac-Asn-Pro-Thr-NH_2 **10** is particularly relevant to the N-glycosylation problem since Pro is the only amino acid that never occupies the Xaa position of the N-glycosylation consensus sequence.[40] Indeed, Imperiali et al. have shown that Ac-Asn-Pro-Thr-NH_2 **10** is neither an N-glycosylation substrate nor an inhibitor, and they further proposed that this nonsubstrate peptide does not adopt the Asx-turn in solution.[8]

Solid State Structure of the Nonsubstrate Ac-Asn-Pro-Thr-NH_2 10. Figure 1 shows a stereoview of the molecular conformation of the tripeptide Ac-Asn-Pro-Thr-NH_2 **10** after crystallization from 1:1 water−methanol. This tripeptide clearly adopts a type-I_t Asx-turn in the solid state, with a hydrogen bond between the Thr NH and the Asn β-carbonyl (N3···O1$^\gamma$, 3.06 Å). This backbone−side chain interaction, which defines the Asx-turn, is further strengthened by another hydrogen bond between the Thr $^\gamma$OH and the Asn β-carbonyl (O3$^\gamma$···O1$^\gamma$, 2.74 Å). In the crystal, the Asn side chain adopts a trans orientation about the C_α−C_β bond (χ_1 = 178°). With its doubly hydrogen-bonded Asn side chain, the crystal structure for Ac-Asn-Pro-Thr-NH_2 **10** is similar to the solid-state structure of the peptide Boc-Asn(Me)Ala-Ser-OMe reported by Marraud.[41] Finally, the crystal structure of Ac-Asn-Pro-Thr-NH_2 **10** also shows that the C-terminal trans amide NH and the Asn backbone carbonyl are close to each other, although the distance (N4···O1, 3.68 Å) is somewhat longer than the standard hydrogen-bond distance. This potential N4−O1 interaction makes the crystal structure of **10** reminiscent of other peptide crystal structures wherein an Asx-turn is overlapped by a β-turn.[42,43] Moreover, the solution NMR data for peptide **10**, presented below, are consistent with the C-terminal trans amide NH being involved in an intramolecular hydrogen bond.

Solution Conformation of the Nonsubstrate Ac-Asn-Pro-Thr-NH_2 10. A phase-sensitive ^{1}H−^{1}H NOESY experiment in DMSO-d_6 at room temperature revealed that Ac-Asn-Pro-Thr-NH_2 **10** can form the Asx-turn in solution.[44] Thus, in addition to the usual intraresidue NOEs, we observed some key interresidue NOEs that were consistent with the peptide's crystal structure. As shown in Figure 2, the most important NOE for constraining the peptide's solution conformation was a side chain−side chain interaction between the Thr $^\gamma$OH and the Asn cis amide NH. The Thr $^\gamma$OH−Asn$^\gamma$NH$_{cis}$ distance is 2.64 Å in the Asx-turn that defines the crystal structure of **10**. This Thr $^\gamma$OH−Asn$^\gamma$NH$_{cis}$ NOE clearly indicates that **10** can also form the Asx-turn in solution. In addition to this diagnostic side chain−side chain NOE, a strong $(i, i + 1)_{NN}$ NOE between the Thr NH and the C-terminal trans amide NH is also consistent with the

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(44) The ^{1}H NMR chemical shifts for **10** in DMSO-d_6 at 298 K were invariant at 1, 5, and 10 mM peptide concentration, indicating that tripeptide **10** does not aggregate in DMSO-d_6 over this concentration range. Moreover, the observed NOEs, measured at both 5 and 10 mM for **10**, were not concentration dependent. These data indicate that the NOEs were due to intramolecular interactions and not due to intermolecular peptide aggregation.

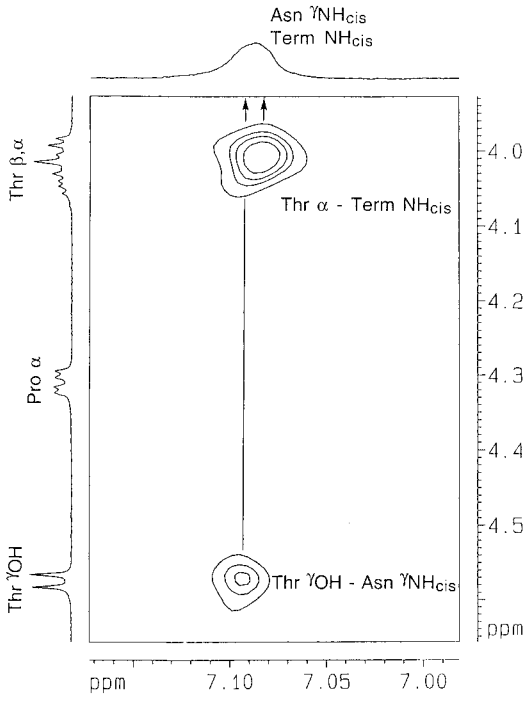

Figure 2. Contour plot of a region of a 500 MHz phase-sensitive NOESY spectrum of Ac-Asn-Pro-Thr-NH_2 **10** (5.0 mM) recorded at 298 K in DMSO-d_6. The mixing time was 200 ms. The NOE cross-peaks are labeled.

peptide's crystal structure. Finally, an intraresidue NOE between the backbone Thr NH and the side-chain Thr $^{\gamma}$OH is consistent with the Thr residue's double hydrogen bond to the Asn side chain.

The Asx-turn for Ac-Asn-Pro-Thr-NH_2 **10** is, of course, not static in DMSO-d_6 solution. The NOE data only indicate that such a structure is one of a family of low-energy conformations available to the peptide. For example, the magnitude of the Asn $^3J_{\alpha,\beta}$ coupling constants reveals that this peptide is conformationally flexible in DMSO-d_6 at room temperature. The crystal structure of **10** shows that the Asn $C_\alpha-C_\beta$ bond is in a trans orientation with $\chi_1 = 178°$. On the basis of Pachler's equations,[45,46] a trans $C_\alpha-C_\beta$ conformation predicts that the Asn $^3J_{\alpha,\beta}$ coupling constants should be close to 13.6 Hz for C_α-pro-*R* C_β and 2.6 Hz for C_α-pro-*S* C_β. That peptide **10** has observed $^3J_{\alpha,\beta}$ values of 7.8 and 6.6 Hz indicates that the peptide is not constrained to a type-I_t Asx-turn in DMSO-d_6. These Asn $^3J_{\alpha,\beta}$ values are, however, consistent with the trans orientation being a predominant rotamer in solution, while the g^- ($\chi_1 = -60°$) and g^+ ($\chi_1 = 60°$) rotamers make up the rest of the population (see Table 2).

Temperature coefficients for the exchangeable NH and OH protons in DMSO-d_6 are also consistent with the Asx-turn being a major solution conformation for the non-substrate peptide **10**. The magnitude of NH temperature coefficients ($-\Delta\delta/\Delta T$) in competitive solvents is a measure of an amide NH proton's solvent accessibility and potential involvement in intramolecular hydrogen bonding. In a polar solvent like DMSO-d_6, NH protons that are shielded from solvent and involved in intramolecular hydrogen bonds typically have temperature coefficients between 0 and -3.5 ppb/K, while NH protons with $\Delta\delta/\Delta T$ values less than -4 ppb/K are considered to be solvent-exposed.[47−50] We determined temperature coefficients in DMSO-d_6 for the nonsubstrate Ac-Asn-Pro-Thr-NH_2 **10**, for the N-glycosylation substrate Bz-Asn-Leu-Thr-NH_2 **6**, and for our three synthetic peptide analogues **7**−**9** (see Table 3). First, both the Thr NH and the Thr $^{\gamma}$OH in peptide **10** have relatively low-temperature coefficients that are in the hydrogen-bonded range (-3.4 and -3.1 ppb/K, respectively). These temperature-dependence data are compatible with the $^1H-^1H$ NOE and $^3J_{\alpha,\beta}$ data, which also support the contention that Thr NH and Thr OH can hydrogen bond in solution with the Asn side-chain carboxamide to form an Asx-turn. The C-terminal trans NH also has a reduced NH temperature coefficient of $\Delta\delta/\Delta T = -2.0$ ppb/K, consistent with this amide NH being involved in a hydrogen bond with the Asn backbone carbonyl to form a β-turn.[51] Again, such a β-turn structure overlapping an Asx-turn has been observed in both solid-state and solution structures for related Asn-Pro-Yaa tripeptides.[42,43] Finally, in addition to determining the temperature dependence of NH chemical shifts in DMSO-d_6, the effect of varying amounts of nonpolar organic solvent on the NH chemical shifts for **10** (5 mM) was also examined (data not shown). Thus, ^1H NMR spectra were recorded at room temperature in solvent mixtures ranging from 100% DMSO-d_6/0% CD_2Cl_2 to 10% DMSO-d_6/90% CD_2Cl_2. These experiments showed that the Thr NH and the C-terminal trans NH chemical shifts were relatively insensitive to the solvent composition ($\Delta\delta < 0.1$ ppm over this solvent range), as compared to the peptide's other four NH protons ($\Delta\delta$ ranged from 0.5 to 1.6 ppm). This minimal solvent effect on chemical shift supports the idea that Thr NH and the C-terminal trans NH are involved in intramolecular hydrogen bonds.[38]

Table 2. Homonuclear Coupling Constants (Hz) and Rotamer Populations for the Asn Side Chain of 6 and 10 and the Methyl Ketone Side Chain of 8 in DMSO-d_6 at 298 K[a]

peptide	$^3J_{\alpha,\beta}$	P_t	P_{g-}	P_{g+}
6	8.7, 5.3	55 (25)	25 (55)	20
8	8.3, 5.6	52 (27)	27 (52)	21
10	7.8, 6.6	47 (36)	36 (47)	17

[a] The $^3J_{\alpha,\beta}$ values were obtained from 500 MHz 1D ^1H NMR spectra. To unequivocally determine the rotamer population the *pro-S* and *pro-R* β-protons must be assigned. We did not make the diastereotopic assignments. The larger $^3J_{\alpha,\beta}$ value is assumed to be for the side chain's *pro-R* β-proton. The rotamer populations were then calculated from Pachler's equations.[45,46] The values in parentheses indicate rotamer populations if the larger $^3J_{\alpha,\beta}$ value actually corresponds to the side chain's *pro-S* β-proton.

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(51) Previously, it was concluded that the nonsubstrate Ac-AsnPro-Thr-NH_2 **10** adopts a significantly different conformation than that populated by N-glycosylation substrate peptides in solution.[8] However, two NH temperature coefficients for **10**, the C-terminal trans NH and a side chain Asn $^{\gamma}$NH, in Table 2 of ref 8 were inadvertently transposed. This neccesarily alters the conclusions regarding the solution conformation of the nonsubstrate peptide **10**. The NMR data for the other peptides in ref 8 appear correct, and the overall conclusion that substrate peptides can adopt the Asx-turn in solution remains the same. We thank Professor Imperiali for helpful correspondence.

Table 3. Temperature Dependence of Exchangeable ^{1}H Chemical Shift ($\Delta\delta/\Delta T$)[a] for Tripeptides 6−10 (5.0 mM) in DMSO-d_6

	peptide					
^{1}H resonance	BzNLT-NH_2 (**6**)	Diazo (**7**)	Ket (**8**)	Sulf (**9**) (major)	Sulf (**9**) (minor)	AcNPT-NH_2 (**10**)
Asn or Ketone NH	−3.9	−4.3	−3.3	−4.9	−4.1	−5.7
Leu NH	−4.5	−5.2	−5.0	−5.7	−4.7	
Thr NH	−3.2	−2.3	−1.7	−5.3	−5.3	−3.4
trans-Asn δ NH	−3.3					−4.8
cis-Asn δ NH	−5.0					−6.2
Term *cis*-NH_2	−3.9	−5.1	−5.5	−4.6	−4.6	−6.1
Term *trans*-NH_2	−3.9	−4.5	−3.9	−4.6	−4.6	−2.0
Thr OH	−4.3	−4.9	−4.6	−4.4	−4.4	−3.1

[a] In parts per billion per degree K (ppb/K). Measured at 500 MHz in 3° increments over the temperature range 291 to 312 K. In cases where NH resonances overlapped with benzoyl aromatic protons, the NH temperature coefficients were determined from a series of 2D ^{1}H−^{1}H COSY NMR spectra at different temperatures. The uncertainty is ±0.3 ppb/K.

In summary, the nonsubstrate peptide Ac-Asn-Pro-Thr-NH_2 **10** can adopt a type I_t Asx-turn in the solid-state. Using ^{1}H−^{1}H NOEs, $^3J_{\alpha,\beta}$ coupling constants, and NH temperature coefficients, we confirmed that a major solution conformer for this peptide was similar to its low-energy crystal structure. As detailed below, this comparison of crystal and solution structure for **10** has allowed us to better interpret NMR data for the synthetic peptides **6**−**9**.

Solution NMR Studies on Peptides 6−9. First, ROESY spectra of the substrate peptide Bz-Asn-Leu-Thr-NH_2 **6**, the ketone-containing peptide **7**, and the diazoketone-containing peptide **8** all showed similar NOE patterns in DMSO-d_6 at room temperature. Unlike for the Pro-containing tripeptide **10**, long-range Thr $^\gamma$OH-Asn $^\gamma NH_{cis}$ NOEs were not observed for peptides **6**−**8**. However, sequential $(i, i + 1)_{NN}$ NOEs between the Thr NH and the C-terminal trans amide NH, consistent with a turn, were apparent for peptides **6**−**8**. Second, the $^3J_{\alpha,\beta}$ coupling constants for the Asn side chain in Bz-Asn-Leu-Thr-NH_2 **6** and for the 4-oxonorvaline residue in ketone tripeptide **8** were similar (see Table 2). Thus, changing the Asn β-carboxamide in Bz-Asn-Leu-Thr-NH_2 **6** to a methyl ketone in peptide **8** seems not to perturb the rotamer population for the side chain.

Finally, we measured the NH temperature coefficients in DMSO-d_6 for peptides **6**−**9** (Table 3). For the substrate peptide **6**, Thr NH had the lowest temperature coefficient (−3.2 ppb/K). The temperature coefficients for Bz-Asn-Leu-Thr-NH_2 **6** are similar to those reported earlier by Imperiali and Shannon for the related substrate tripeptide Ac-Asn-Leu-Thr-NH_2 in DMSO-d_6 (−2.7 ppb/K).[8] This low-temperature coefficient for Thr NH in peptide **6** is consistent with the Asx-turn model, wherein Thr NH forms an intramolecular hydrogen bond with the Asn β-carboxamide. It was then of interest to determine if the three synthetic peptide analogues **7**−**9** had any major differences in their NH temperature coefficients, relative to peptide substrate **6**. Large differences in $\Delta\delta/\Delta T$ values, particularly increases in the value for Thr NH, might indicate deviations away from the Asx-turn conformation.

The diazoketone-containing peptide **7** has similar NH temperature coefficients when compared to the standard N-glycosylation substrate **6**. In particular, Thr NH has a temperature coefficient that is lower (−2.3 ppb/K) for the diazoketone-containing peptide **7** when compared to the Thr NH for the Asn-containing peptide **6** (−3.2 ppb/K). The methyl ketone tripeptide **8** has an even lower temperature coefficient for Thr NH (−1.7 ppb/K) than does peptides **6** and **7**. These reduced Thr NH $\Delta\delta/\Delta T$ values suggest that a significant population of the diazoketone **7** and ketone-containing peptide **8** adopt an Asx-turn in solution. Compared to peptide analogues **7** and **8**, there were significant differences in the NH temperature coefficient for the sulfoxide-containing tripeptide **9**. Thus, Thr NH in sulfoxide **9** has a relatively large temperature coefficient (−5.3 ppb/K) when compared to the other peptides. This increased $\Delta\delta/\Delta T$ value for Thr NH indicates that the sulfoxide peptide **9**, because of differences in geometry, electronics, or solvation, has a different average solution conformation than does the parent peptide **6** and the other peptide analogues **7** and **8**.

These NMR data indicate that the average solution conformation of the substrate tripeptide **6** and two of the peptide analogues, namely diazoketone **7** and methyl ketone **8**, are similar. The NMR evidence suggests that an Asx-turn is a significant conformer for peptides **6**−**8**. Changing the Asn β-carboxamide in substrate peptide **6** to either a diazoketone (peptide **7**) or to a methyl ketone (peptide **8**) does not apparently result in any major changes in Thr NH hydrogen bonding. If an Asx-turn in the N-glycosylation consensus sequence is indeed crucial for OST recognition, then the ketone peptide **8** and diazoketone peptide **7** might be expected to bind with some affinity to OST. However, as indicated below, studies with analogues **7** and **8** show that they are poor OST substrates and inhibitors. Taken together with the demonstration that the nonsubstrate Ac-Asn-Pro-Thr-NH_2 **10** forms an Asx-turn in the solid state and in solution, the collective data suggest that either an Asx-turn may not be that critical for binding and catalysis or that this secondary structure may be necessary but not sufficient for peptide N-glycosylation.[52]

Biochemical Results

OST Substrate Assays. OST assay results showed that ketone-containing peptide **8** and sulfoxide-containing peptide **9** are not N-glycosylation substrates. One possible explanation for the failure of the ketone-containing peptide **8** to act as an OST substrate is that its methyl protons have a higher pK_a value than do the Asn carboxamide protons. Such a pK_a difference may make

(52) A cyclic peptide containing substitution of 2,4-aminobutyric acid for Asn in its N-glycosylation site is a potent inhibitor (K_i = 37 nm) of OST.[20] Because of this particular amino acid substitution, this peptide is unlikely to form an Asx-turn structure, yet it still has a strong affinity for binding to OST.

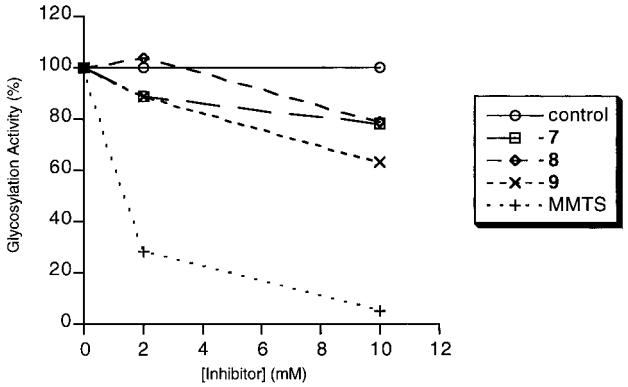

Figure 3. Inhibition of oligosaccharyltransferase by peptide substrate analogues.

it difficult for an OST active-site base to deprotonate the methyl ketone. The pK_a of a methyl ketone is higher (pK_a = 19.2 for acetone) than that of a carboxamide (pK_a = 15.1 for acetamide) in water. This pK_a difference is, however, much less in a nonaqueous environment, as may exist in the active site of the membrane-bound OST protein complex. For example, Bordwell found that acetone (pK_a = 25.5) and acetamide (pK_a = 26.5) have similar pK_a values in DMSO.[53,54] Such a pK_a variation corresponds to a difference of less than 1 kcal/mol for deprotonation of a ketone and a carboxamide. That ketone peptide **8** is not an N-glycosylation substrate may also suggest that the deprotonation mechanism (path c in Scheme 2) may not operate for OST. Of course, interpretation of negative results should be done with caution. Specifically, we still need to consider the following issues: (1) there may be steric problems in binding analogue **8**, as a methyl group still may not fit into an active site designed for an sp^2-hybridized amide; (2) the methyl ketone peptide **8** may not be acidic enough to be deprotonated by OST; and (3) the methyl ketone peptide **8** may be deprotonated, but not glycosylated, by OST. Since a methyl sulfoxide has an even higher pK_a (pK_a = 33 in DMSO)[55] than a methyl ketone, a similar argument may be made to explain the lack of substrate activity exhibited by **9**. Furthermore, possible electrostatic interactions between the putative OST active-site base (Scheme 2) and the positively charged sulfur atom of the sulfoxide moiety could make enzyme-catalyzed glycosylation of **9** even more difficult.[56]

OST Inhibitor Assays. Figure 3 shows the results of OST competition assays wherein potential inhibitors (2 or 10 mM) were incubated with the N-glycosylation substrate Bz-Asn-Leu-Thr-NH_2 (**6**) (360 μM) under standard assay conditions using ^{3}H-labeled Dol-P-P-DS.[30] As previously reported by Pathak et al.,[23] MMTS is an effective inactivator of OST. Thus, at 2 mM concentration, MMTS inhibits N-glycosylation of Bz-Asn-Leu-Thr-NH_2 (**6**) by approximately 75%, while at 10 mM this thiol-specific reagent almost totally inhibits peptide N-glycosylation. These results clearly indicate that a cysteine residue is important for OST activity. Unlike MMTS, peptides **7**−**9** showed little inhibition of N-glycosylation at 2 mM concentration. Even at 10 mM, a concentration that is well above the K_m of the substrate peptide Bz-Asn-Leu-Thr-NH_2 (**6**) (K_m = 282 μM),[57] only the sulfoxide-containing peptide **9** showed any significant inhibition (ca. 30−35%). Interestingly, peptide **9** is the only peptide of those examined in this work that does not form the Asx-turn (Table 3). At 10 mM concentration, the diazoketone-containing peptide **7** and the methyl ketone-containing peptide **8** showed less inhibition (<25%) of peptide N-glycosylation. Since **7** is proposed as an affinity label to covalently bond the OST active site, it was also preincubated with OST at 2 mM for up to 30 min prior to the assay of OST activity. However, **7** did not show any time-dependent inhibition of the OST-catalyzed glycosylation of Bz-Asn-Leu-Thr-NH_2 (**6**), while MMTS showed almost total inhibition after 30 min at the same concentration (data not shown).[58]

Recent mechanistic studies of PRPP amidotransferase by Krahn et al.[59] showed that enzyme conformation changes after PRPP binding are required for the binding of glutamine and glutamine analogues, e.g., 6-diazo-5-oxo-L-norleucine (DON). Since we proposed a possible mechanism by analogy to glutamine-dependent amidotransferases such as PRPP amidotransferase,[12] OST may also need glycolipid binding to effect a conformation change for associated peptide binding. This hypothesis is reasonable since N-glycosylation, involving OST and a glycolipid substrate, occurs at the lumenal side of endoplasmic reticulum (ER) following transit of the growing polypeptide through the ER membrane. Since the concentration of ^{3}H-labeled Dol-P-P-DS used above is much lower than its K_m value of 200 μM,[57] a higher concentration of Dol-P-P-DS may be needed for the proposed enzyme conformation change. Synthetic Dol-P-P-DS[30] was used to study the potential time-dependent inactivation of OST by diazoketone **7** at 2 mM. However, these experiments with [Dol-P-P-DS] = 200 μM also failed to show OST inactivation (data not shown). Of interest is the work of Bause and colleagues,[60] in which a hexapeptide containing α-(epoxyethyl)glycine in place of Thr in the Asn-Xaa-Thr sequon was found to be an effective active site-directed inhibitor (affinity label) of OST when incubated in the presence of Dol-P-P-DS. However, it is not known what amino acid residues are involved in labeling the OST subunits.

Although both the ketone-containing peptide **8** and sulfoxide-containing peptide **9** are structurally similar to the Bz-Asn-Leu-Thr-NH_2 tripeptide substrate **6**, neither peptide analogue is a good competitive inhibitor of OST. The lack of significant inhibition, even at 10 mM, indicates that the structural modifications in **8** and **9** dramatically hamper recognition by the enzyme. Any one

(53) Bordwell, F. G. *Acc. Chem. Res.* **1988**, *21*, 456−463.
(54) Bordwell, F. G.; Zhang, S.; Zhang, X.-M.; Liu, W.-Z. *J. Am. Chem. Soc.* **1995**, *117*, 7092−7096.
(55) Stewart, R.; Jones, J. R. *J. Am. Chem. Soc.* **1967**, *89*, 5069−5071.
(56) It was repeatedly observed that the diazoketone-containing peptide **7** showed slight activity as an OST acceptor substrate. The apparent ability of the diazoketone-containing peptide **7** to serve as a weak OST substrate is an intriguing but, as yet, unexplained observation.
(57) Gibbs, B. S.; Coward, J. K. Unpublished results.
(58) Boc-DONV-OBn was also synthesized and tested as an inactivator of OST. Interestingly, it also shows slight inhibition toward OST-catalyzed glycosylation. At 10 mM, it shows even more inhibition than the diazoketone **7** (data not shown), suggesting that both **7** and Boc-DONV-OBn may be similar to MMTS and act as nonspecific inactivators of OST. Possibly, they all react with a cysteine that is not in the OST active site, and this cysteine has a structural role rather than a catalytic function.
(59) Krahn, J. M.; Kim, J. H.; Burns, M. R.; Parry, R. J.; Zalkin, H.; Smith, J. L. *Biochemistry* **1997**, *36*, 11061−11068.
(60) Bause, E.; Wesemann, M.; Bartoschek, A.; Breuer, W. *Biochem J.* **1997**, *322*, 95−102.

of several factors, including steric bulk, conformation, hydrogen-bonding pattern, and relative electrophilicity of the side-chain carbonyl, may be responsible for tripeptides **7**−**9** not being effective OST inhibitors. Since Bause et al. found that an epoxide-containing peptide and the oligosaccharide moiety derived from Dol-P-P-OS form a covalently bound ternary complex with OST,[60] it is likely that the diazoketone, the methyl ketone, and the methyl sulfoxide groups are unable to form a similar complex due to steric and/or conformational constraints. Alternatively, the primary amide NH protons of the Asn β-carboxamide may make essential contacts with the enzyme that are needed for effective binding. It is also possible that there is no acidic residue in the enzyme active site that allows for protonation and subsequent activation of the diazoketone **7** for alkylation of an active-site nucleophile. Without protonation of the diazomethyl group, peptide **7** cannot serve as an affinity label. The third possibility is that the active-site cysteine residue may be more important for Dol-P-P-OS binding and that this cysteine may not be within a suitable distance for alkylation of the peptidyl diazoketone. The observation that $GlcNAc_2$-P-P-dolichol (Dol-P-P-DS) partially protects OST from being inactivated by MMTS further supports this possibility.[23,61]

While the importance of a cysteine residue in OST activity is evident, its actual role during binding and catalysis is still not clear. Furthermore, the proposed active-site base (Schemes 2 and 3) has not been identified, and its role in catalysis definitely needs further investigation. Incorporation of three functional groups into peptide analogues **7**−**9** has allowed us to test some of the proposed OST N-glycosylation mechanisms outlined in Scheme 2. While the results of these modifications on peptide binding and glycosylation by OST do not identify a specific active-site nucleophile (e.g., Cys), the chemistry used to synthesize peptides **7**−**9** is useful in allowing access to peptide analogues that, to date, have not been described. In addition, detailed structural studies indicate that the putative inhibitor peptides **7** and **8**, in addition to a known nonsubstrate, **10**, adopt the Asx-turn motif in solution (**7**, **8**, and **10**) and in the solid state (**10**), thus calling into question the importance of the Asx-turn in ligand binding and/or catalysis by OST.

Experimental Section

General Methods.[30,32] H-Leu-Thr-NH_2·TFA and *N*-Bz-Asn-Leu-Thr-NH_2 **6** were synthesized as previously described.[4] *N*-Ac-Asn-Pro-Thr-NH_2, **10**,[8] was synthesized by standard solid-phase methods. *N*-Bz-Asp-Leu-Thr-NH_2 (**11**) was synthesized according to the procedure of Lee and Coward.[30] *S*-Methyl-L-cysteine was prepared as described by Hwang et al.[34] 4-Oxo-L-norvaline (**12**) was synthesized as previously described.[32] The synthesis of Boc-DONV-OBn from Boc-Asp-OBn and CH_2N_2 in the presence of isobutyl chloroformate (*i*BCF) followed standard procedures; details are available from the authors. P_{40} yeast microsomes and [^{3}H]Dol-P-P-DS were prepared as described by Clark et al.[4] and Lee and Coward,[30] respectively. Dol-P-P-DS was synthesized as described by Lee and Coward,[30] and its concentration was determined by high pH anion-exchange chromatography (HPAEC)[62] following hydrolysis to glucosamine. HPLC analyses were performed on a Waters liquid chromatography system (6100A and 510 pumps), Rainin Microsorb-MV 5 μm C_{18}, 300 Å, 4.6 × 250 mm column, and monitored using a Waters 996 diode array spectrometer equipped with Millennium software. Gradient profiles at 10−100% MeOH in 90 min (condition A) or 10−25% MeOH in 20 min followed with 25% MeOH isocratic (condition B) were employed. Radioactivity was determined using a Packard 1600 TR liquid scintillation analyzer.

Spectral Characterization and X-ray Crystallography of Ac-Asn-Pro-Thr-NH_2 (10). Synthetic compound **10** was purified by RP-HPLC, and 50 mg of **10** was recrystallized from 1 mL of water/methanol (1:1) by slow evaporation to give long thin plates (0.50 × 0.25 × 0.05 mm). The crystals, when dissolved in DMSO-d_6 at 298 K, gave the following spectral data: ^{1}H NMR (400 MHz, 2D ^{1}H−^{1}H COSY) (DMSO-d_6) δ 8.26 (d, 1H, Asn-NH, J = 7.7 Hz), 7.60 (s, 1H, Asn trans $^\gamma$NH), 7.58 (d, 1H, Thr-NH, J = 8.9 Hz), 7.08 (s, 2H, Asn cis $^\gamma$NH and C-terminal cis NH), 6.85 (s, 1H, C-terminal trans NH), 4.81 (m, 1H, Asn-a, J = 7.7, 7.2 Hz), 4.58 (d, 1H, Thr-OH, J = 6.8 Hz), 4.31 (m, 1H, Pro-α, J = 8.1, 2.8 Hz), 4.04−3.98 (m, 2H, Thr α and β), 3.76−3.67 (m, 2H, Pro δ), 2.57 (dd, 1H, Asn-β, J = 15.3, 8.0 Hz), 2.37 (dd, 1H, Asn-β', J = 15.3, 6.4 Hz), 2.05 (m, 1H, Pro β), 1.88 (m, 3H, Pro β',γ), 1.79 (s, 3H, Ac), 1.03 (d, 3H, Thr-CH_3, J = 6.2 Hz); ^{13}C NMR (100 MHz) (DMSO-d_6) δ 172.2, 172.1, 171.3, 170.9, 168.9, 66.1, 60.6, 58.6, 47.4, 47.0, 37.3, 29.1, 24.2, 22.2, 20.2. FABMS m/z (M^+ + 1) 372 (16), 216 (24), 100 (100), 70 (55); HRMS (M^+ + 1) m/z calcd for $C_{15}H_{26}N_5O_6$, 372.1883, found 372.1872. For the details of X-ray diffraction measurements and for single-crystal data and refinement protocols, see the Supporting Information. All the crystal data for **10**, including atomic coordinates, have been deposited in the Cambridge Crystallographic Data Centre.

NMR Experiments. 2D NMR experiments were performed on a Bruker AMX-500 NMR spectrometer using TPPI quadrature detection.[63] The spectrometer ^{1}H frequency was 500.13 MHz, and its ^{13}C frequency was 125.77 MHz. The temperature was controlled to ±0.1 °C. The spectral window was 12 ppm for ^{1}H and 200 ppm for ^{13}C. Typical 90° pulse widths were 11 μs for ^{1}H and 8 μs for ^{13}C. Data processing was done with Triad (Tripos) software on a SUN sparc station. The 1D spectra were processed by zero-filling to 32 K and multiplied by a Lorentzian (0.3−1 Hz) prior to Fourier transformation. Both zero- and first-order phase corrections were applied. The 2D NMR spectra were recorded as 1 K complex points for 512 increments. The spectra were zero filled to 2K × 1K matrices. Shifted sinebell filter functions were applied for both dimensions before Fourier transformations. Both zero- and first-order phase corrections were applied.

^{1}H−^{1}H Correlation Experiments. The DQF-COSY, PS-NOESY, and ROESY experiments were recorded in the TPPI phase-sensitive mode. Four dummy scans were applied before each experiment. Each experiment used a 2 s relaxation delay. The 2D ROESY experiments were done using a 200 ms mixing pulse with a 2.5 kHz field strength.[64] The R_f carrier frequency was placed at 4.9 ppm in order to avoid problems from Hartmann−Hahn correlations.

NH Temperature Coefficients. Peptides **6**−**10** were made 5.0 mM in DMSO-d_6 for amide NH temperature coefficient analysis (see Table 3 for values). Changes in amide NH chemical shift were monitored over a temperature range of 291 to 312 K in 3° increments. All changes in NH chemical shift ($-\Delta\delta/\Delta T$) for peptides **6**−**10** were linear over the above temperature range. A relaxation delay of 1.5 s was used for each sample. See the Supporting Information for graphical presentation of the data.

***N*-Bz-DONV-Leu-Thr-NH_2 (7).** *N*-Bz-Asp-Leu-Thr-NH_2 (**11**) (450 mg, 1.0 mmol) was dissolved in 40 mL of THF/CH_3-CN (v/v, 9:1), and the solution was cooled to −10 °C. NMM (110 μL, 1.0 mmol) was added followed by iBCF (145 μL, 1.1 mmol). The mixture was stirred for 10 min, CH_2N_2/Et_2O (freshly prepared from 3.0 mmol Diazald) was added, and the reaction was stirred overnight. The reaction mixture was

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concentrated in vacuo, and then 40 mL EtOAc was added to redissolve the brown residue. This solution was washed with 5% aqueous citric acid (10 mL), saturated $NaHCO_3$ (10 mL), and brine (10 mL), dried by anhydrous $MgSO_4$, filtered, and rotary evaporated under reduced pressure. The crude product was crystallized from MeOH/Et_2O, and the slightly yellowish solid obtained was further recrystallized carefully from MeOH/Et_2O to give 146 mg (31%) of **7** as a slightly yellowish powder: mp 160−162 °C; R_f 0.70 (MeOH/EtOAc, 1:1, 4-(4-nitrobenzyl)-pyridine[65] positive); IR (KBr) 3286, 3080, 2956, 2108, 1674, 1633 cm^{-1}; ^1H NMR (500 MHz) (DMSO-d_6) assignments were aided through the use of 2D $^1H-^1H$ COSY NMR spectra; see Table 1 for chemical shifts, ^3J coupling, and assignments; ^{13}C NMR (CD_3OD) δ 20.4, 21.8, 23.7, 26.0, 41.4, 42.2, 52.1, 54.2, 60.1, 68.4, 128.7, 129.7, 133.2, 135.1, 170.4, 173.9, 175.1, 175.2, 194.8; MS (FAB^+) *m/e* (rel intensity) 475 (MH^+, 3.3), 447 ($-N_2$, 4.7), 105 (25), 79 (100); HRMS calcd for $C_{22}H_{31}N_6O_6$ (MH^+) 475.2305, found 475.2281.

***N*-Boc-4-oxo-norvaline-OH.** A solution of the TFA salt of 4-oxo-L-norvaline **12**, (309 mg, 1.26 mmol) in 3.78 mL of 0.33 M Na_2CO_3 was cooled to 0 °C, and a solution of $(Boc)_2O$ (303 mg, 1.39 mmol) in 3.48 mL of dioxane was added dropwise. The reaction mixture was stirred at 0 °C for 1 h, allowed to warm to room temperature, and then stirred at room temperature for 17 h. The reaction mixture was adjusted to pH 10 with Na_2CO_3 solution and then washed with Et_2O (3 × 20 mL). The aqueous layer was cooled to 0 °C, and cold 6 N HCl was added dropwise to adjust the solution to pH 2. The aqueous solution was extracted with CH_2Cl_2 (4 × 20 mL), and the combined organic layer was dried over Na_2SO_4 and concentrated in vacuo to give a colorless oil. Trituration of the oil with hexane gave 252 mg (86% yield) of *N*-Boc-4-oxo-norvaline-OH as a white gummy solid: ^1H NMR ($CDCl_3$) (400 MHz) δ 5.57 (d, 1H, J = 7.4 Hz), 4.44 (m, 1H), 3.11 (dd, 1H, J = 7.5, 17.9 Hz), 2.91 (dd, 1H, J = 7.3, 17.9 Hz), 2.12 (s, 3H), 1.37 (s, 9H); ^{13}C NMR ($CDCl_3$) (100 MHz) δ 206.9, 175.6, 155.7, 80.3, 49.2, 45.1, 29.7, 28.2; FABMS *m/z* (rel intensity) 232 ($M^+ + 1$) (8), 176 (44), 133 (31), 86 (22), 57 (100); HRMS ($M^+ + 1$) *m/z* calcd for $C_{10}H_{18}NO_5$ 232.1185, found 232.1184.

***N*-Boc-4-oxo-norvaline-Leu-Thr-NH_2 (13).** To a solution of *N*-Boc-4-oxo-norvaline-OH (219 mg, 0.95 mmol) in 4 mL of THF at 0 °C were added HOBt (192 mg, 1.42 mmol), EDC·HCl (199 mg, 1.04 mmol) and NMM (114 μL, 1.04 mmol). The mixture was stirred for 5 min at 0 °C, and a precooled solution of H-Leu-Thr-NH_2·TFA (0.95 mmol) and NMM (114 μL, 1.04 mmol) in 4 mL of DMF was added. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. The brown reaction mixture was concentrated in vacuo, and the resulting residue was partitioned between EtOAc (50 mL) and 5% aqueous citric acid solution (6 mL). The organic layer was washed with saturated $NaHCO_3$ (2 × 10 mL) and water (10 mL), dried over Na_2SO_4, and concentrated in vacuo to give an oil. Flash chromatography on silica gel (15:1 CH_2Cl_2/MeOH) afforded 168 mg (47%) of the methyl ketone tripeptide **13** as a white solid: IR (CD_3CN) 1733, 1684, 1652, 1649, 1635 cm^{-1}; ^1H NMR ($CDCl_3$) (400 MHz) δ 7.14 (d, 1H, J = 6.1 Hz), 6.98 (d, 1H, J = 6.5 Hz), 6.45 (s, 1H), 5.82 (s, 1H), 5.77 (d, 1H, J = 6.3 Hz), 4.34 (m, 1H, J = 6.2, 6.5, 7.1 Hz), 4.16 (m, 2H, J = 6.1 Hz), 4.13 (m, 2H, J = 6.3 Hz), 3.38 (dd, 1H, J = 7.1, 17.5 Hz), 2.95 (dd, 1H, J = 6.2, 17.5), 2.11 (s, 3H), 1.72−1.56 (m, 3H), 1.40 (s, 9H), 1.08 (d, 3H, J = 6.4), 0.92 (d, 3H, J = 6.5), 0.86 (d, 3H, J = 6.4); ^{13}C NMR (CD_3CN) (100 MHz) δ 207.9, 173.8, 173.4, 156.7, 80.4, 67.6, 58.9, 53.6, 51.5, 44.8, 40.8, 30.3, 28.4, 25.3, 23.3, 21.6, 19.9; FABMS *m/z* (rel intensity) 445 ($M^+ + 1$) (7), 271 (22), 227 (15), 119 (17), 86 (100), 57 (86); HRMS ($M^+ + 1$) *m/z* calcd for $C_{20}H_{37}N_4O_7$ 445.2662, found 445.2652.

Bz-4-oxo-L-norvaline-Leu-Thr-NH_2 (8). The tripeptide **13** (168 mg, 0.378 mmol) was dissolved in 6 mL of a TFA/CH_2Cl_2 (1:1) solution and stirred at room temperature for 30 min. The reaction mixture was concentrated in vacuo and azeotroped with benzene, and the resulting colorless oil was triturated with Et_2O to give H-ONV-Leu-Thr-NH_2 as a white precipitate. After being washed with Et_2O, this crude tripeptide was dissolved in 5 mL of H_2O, and the solution was cooled to 0 °C. To this solution of tripeptide was added Et_3N (52.7 μL, 0.378 mmol) followed by 5 mL of a solution of benzoic anhydride (94 mg, 0.416 mmol) in dioxane. The reaction mixture was stirred for 5 min at 0 °C, and a further aliquot of Et_3N (26.5 μL, 0.189 mmol) was added. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. The reaction mixture was concentrated in vacuo, and the resulting residue was triturated with Et_2O to give a white solid. Flash chromatography on silica gel (CH_2Cl_2/MeOH 10:1) afforded 99 mg (59%) of **8** as a white solid. An analytical sample of **8** was obtained by recrystallization from CH_3CN/Et_2O: mp 148−150 °C; IR (CH_3CN): 1734, 1675, 1669, 1662, 1653; ^1H NMR (500 MHz) (DMSO-d_6) assignments were aided through the use of 2D $^1H-^1H$ COSY NMR spectra; see Table 1 for chemical shifts, ^3J coupling, and assignments; ^{13}C NMR (125 MHz) (CD_3CN) δ 207.5, 173.5, 173.2, 172.9, 168.9, 134.4, 132.9, 129.5, 128.3, 67.7, 59.2, 53.9, 51.7, 44.4, 40.3, 30.2, 25.4, 23.3, 21.4, 19.9; FABMS *m/z* (rel intensity) 449 ($M^+ + 1$)(13), 431(7), 331(7), 218(23), 148(11), 119(12), 105-(100), 86(57); HRMS (M^+) *m/z* calcd for $C_{22}H_{33}N_4O_6$ 449.24002, found 449.24192. Anal. Calcd for $C_{22}H_{32}N_4O_6 \cdot 2.0H_2O$: C, 54.53; H, 7.49; N, 11.56. Found: C, 54.52; H, 7.21; N, 11.55.

***N*-Boc-*S*-methyl-L-cysteine (14).** *S*-Methyl-L-cysteine (216 mg, 1.60 mmol) was suspended in 3.2 mL of dioxane, 1.6 mL of water, and 1.6 mL of 1 M Na_2CO_3. The reaction mixture was cooled to 0 °C, $(Boc)_2O$ (384 mg, 1.76 mmol) was added, and the reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 15 h. The mixture was adjusted to pH 10 and then washed with Et_2O. The aqueous layer was then cooled to 0 °C, and cold 6 N HCl was added dropwise to adjust the solution to pH 2 during vigorous stirring. This mixture was washed with chloroform (3×), and the combined organic layer was dried by anhydrous $MgSO_4$ and then concentrated in vacuo. The clear oil obtained was triturated with hexane to give 292 mg of white powder (78% yield): R_f 0.48 (MeOH/EtOAc 1:1); mp 73−75 °C (lit.[66] mp 71−73 °C); ^1H NMR ($CDCl_3$) δ 1.33 (s, 9H), 2.02 (s, 3H), 2.85 (m, 2H), 4.43 (m, 1H), 5.51 (d, 1H); ^{13}C NMR ($CDCl_3$) δ 15.9, 28.0, 36.2, 52.7, 80.1, 155.3, 174.3. $[\alpha]^{20}_D = -24.6°$ (AcOH, 0.03 M) (lit.[66] $[\alpha]^{20}_D = -27.5$ (AcOH).

***N*-Boc-(*S*-methyl)Cys-Leu-Thr-NH_2.** Cysteine analogue **14** (306 mg, 1.30 mmol) was dissolved in 4 mL of dry THF at 0 °C, and HOBt (264 mg, 1.95 mmol), EDC·HCl (274 mg, 1.43 mmol), and NMM (157 μL, 1.43 mmol) were added. The mixture was stirred for 5 min at 0 °C under N_2, and a precooled solution of H-Leu-Thr-NH_2·TFA (1.30 mmol) and NMM (143 μL, 1.30 mmol) in 4 mL of dry DMF was added. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. The brown mixture was concentrated in vacuo, and the resulting residue was partitioned between EtOAc (50 mL) and 5% aqueous citric acid solution (6 mL). The organic layer was further washed with saturated $NaHCO_3$ (2×, 6 mL) and water (6 mL), dried by anhydrous $MgSO_4$, and then concentrated in vacuo. The solid residue obtained was crystallized from MeOH/Et_2O to give 390 mg of white solid (67% yield): mp 170−172 °C; R_f 0.74 (MeOH/EtOAc, 1:1); ^1H NMR (CD_3OD) δ 0.95 (dd, 6H), 1.15 (d, 3H), 1.45 (s, 9H), 1.66 (m, 2H), 1.76 (m, H), 2.17 (s, 3H), 2.80 (m, 2H), 4.24 (m, 1H), 4.29 (m, 2H), 4.45 (m, 1H); ^{13}C NMR (CD_3OD) δ 16.0, 20.4, 22.0, 23.7, 25.9, 28.9, 37.0, 41.7, 54.0, 55.3, 59.8, 68.2, 80.9, 157.8, 174.4, 174.9, 175.1; MS (CI) *m/e* (rel intensity) 449 (MH^+, 100); HRMS calcd for $C_{19}H_{38}N_4O_6S$ (MH^+) 449.2434, found 449.2421; HPLC t_R = 51.1 min (condition A).

***N*-Boc-(methanesulfinyl)Ala-Leu-Thr-NH_2 (15).** *N*-Boc-(*S*-methyl)Cys-Leu-Thr-NH_2 (368 mg, 0.82 mmol) was dissolved in 5 mL of MeOH and then cooled to 0 °C. A solution of $NaIO_4$ (202 mg, 0.94 mmol) in 5 mL of water was added, and the reaction mixture was stirred at 0 °C for 1 h and then

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at room temperature for 1 day. The reaction mixture was filtered, and the white solid collected was washed with MeOH. The filtrate was concentrated in vacuo to give a white solid. This solid was dissolved in 4 mL of MeOH, and a small amount of insoluble material was removed. The filtrate was then concentrated, and the crude product was further recrystallized from MeOH/Et_2O to give 308 mg of a white solid (81% yield): mp 173−176 °C; R_f 0.51 (MeOH/EtOAc 1:3); ^{1}H NMR (CD_3-OD) δ 0.96 (dd, 6H), 1.18 (d, 3H), 1.46 (s, 9H), 1.68 (m, 2H), 1.77 (m, H), 2.75 (m, 3H), 3.20 (m, 2H), 4.21 (m, 1H), 4.30 (d, 1H), 4.44 (m, 1H), 4.60 (m, 1H); ^{13}C NMR (DMSO-d_6/CD_3OD) δ 20.3 and 20.4, 21.9, 23.7, 25.9, 28.8, 38.6 and 39.0, 41.6, 51.1 and 51.3, 54.1, 56.1 and 57.0, 59.9 and 60.1, 68.4 and 68.5, 81.2, 157.5, 172.8 and 173.2, 174.9, 175.1 and 175.2; MS (CI) *m/e* (rel intensity) 465 (MH^+, 11), 401 (60), 345 (66), 328 (54), 111 (100); HRMS calcd for $C_{19}H_{38}N_4O_7S$ (MH^+) 465.2383, found 465.2398; HPLC (condition A) t_R = 44.1 min (major), 44.7 min (minor). Ratio of peak areas 3:1.

***N*-Bz-(Methanesulfinyl)Ala-Leu-Thr-NH$_2$ (9).** Tripeptide **15** (116 mg, 0.25 mmol) was dissolved in 6 mL of TFA/CH_2Cl_2 (v/v, 1:1) at room temperature and was allowed to sit for 30 min. The yellowish solution was concentrated in vacuo, and the resulting colorless oil was triturated with ether to give a white precipitate. The mixture was filtered, and the isolated white solid was rinsed with Et_2O. The solid collected was dissolved in 5 mL of water and then cooled to 0 °C. Et_3N (35 μL, 0.25 mmol) was added followed by 5 mL of dioxane and benzoic anhydride (62 mg, 0.28 mmol). The reaction mixture was stirred for 5 min, and Et_3N (18 μL, 0.13 mmol) was further added. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. The reaction mixture was concentrated, and the white residue obtained was dissolved in H_2O/THF (v/v, 1:1). A 6 mL bed volume of Dowex 50W×8 (H^+) resin was added, and the mixture was gently stirred. The mixture was filtered, and the filtrate was concentrated in vacuo. The resulting white solid was triturated with ether, and the white precipitate was collected by filtration and then crystallized from H_2O/THF (v/v, 1:2) to give 96 mg of white solid (82% yield): R_f 0.17 (EtOAc/MeOH 3:1); mp 194−197 °C; ^{1}H NMR (500 MHz) (DMSO-d_6) assignments were aided through the use of 2D ^{1}H−^{1}H COSY NMR spectra; see Table 1 for chemical shifts, ^3J coupling, and assignments; ^{13}C NMR (DMSO-d_6/CD_3OD) δ 20.0, 21.6, 23.1, 24.8, 38.7, 40.6, 49.6, 55.0, 55.7, 58.6, 67.0, 127.8, 128.7, 132.1, 134.1, 167.7, 170.7, 172.8, 172.9; MS (FAB$^+$) *m/e* (rel intensity) 469 (MH^+, 55), 174 (41), 105 (93), 86 (100); HRMS calcd for $C_{21}H_{34}N_4O_6S$ (MH^+) 469.2121, found 469.2107. Anal. Calcd for $C_{21}H_{33}N_4O_6S\cdot 0.5H_2O$: C, 52.81; H, 6.96; N, 11.73. Found C, 52.65; H, 6.78; N, 11.36. ^{1}H NMR showed **9** as a mixture of two diastereomers in a 3:1 ratio while HPLC (condition A) showed two peaks with t_R = 38.0 min (major) and t_R = 39.5 min (minor). Ratio of peak areas 3:1.

Biochemical Studies. Standard Assay To Measure Peptide as Potential OST Substrate. The synthetic peptides **7**, **8**, and **9** were tested as OST substrates using the method previously described by this laboratory[30] and others,[8] both of which are based on early work of Sharma et al.[67] The assay mixture contained ca. 6000 dpm [^{3}H]Dol-P-P-DS, 50 mM Tris, pH 7.5, 1% Triton X-100, 1 mM $MnCl_2$, 360 μM peptide substrate (**6**, **7**, **8**, or **9**) in DMSO (final [DMSO] = 5% v/v), and 875 μg of P_{40} yeast microsome in a total volume of 100 μL. The reaction mixture was shaken at 250 rpm for 2 h at room temperature and then quenched by the addition of 3 mL of cold $CHCl_3$/MeOH (v/v = 3:2). The mixture was then set on ice for 30 min, and the layers were separated by centrifugation at 1000*g* for 15 min. The supernatant was removed and extracted with 1 mL of 4 mM $MgCl_2$. The biphasic mixture was thoroughly agitated (Vortex) and then centrifuged at 1000*g* for another 15 min. The upper aqueous layer containing the water-soluble ^{3}H-labeled glycopeptide was carefully removed. A significant increase in ^{3}H radioactivity observed in the aqueous layer compared with that observed in the aq layer from a control assay not containing the peptide substrate provided evidence for the formation of ^{3}H-labeled glycopeptide. The relative activity of the tested peptide was determined by dividing the net % ^{3}H in the aqueous layer derived from an assay of each tested peptide by that derived from an assay of the standard peptide, Bz-Asn-Leu-Thr-NH$_2$ (**6**).

Standard Assay To Measure Peptide as Potential OST Inhibitor. The competition assay was performed by incubating standard peptide Bz-Asn-Leu-Thr-NH$_2$ (**6**) under standard assay conditions in the presence of a potential inhibitor **7**, **8**, **9**, or MMTS. The assay mixture contained ca. 6000 dpm [^{3}H]-Dol-P-P-DS, 50 mM Tris, pH 7.5, 1% Triton X-100, 1 mM $MnCl_2$, 360 μM peptide substrate Bz-Asn-Leu-Thr-NH$_2$ (**6**), 2 or 10 mM potential inhibitor **7**, **8**, **9**, or MMTS in DMSO (final [DMSO] = 10% v/v), and 875 μg of P_{40} yeast microsome in a total volume of 100 μL. The reaction mixture was shaken at 250 rpm for 2 h and then worked up as described above. OST activity in either the absence or presence of an inhibitor was obtained by determining the % ^{3}H in the aqueous phase due to formation of the glycopeptide product.[30] For each inhibitor evaluated, the % inhibition was calculated by dividing the activity in the presence of inhibitor by that observed in its absence. It is possible that the Tris buffer could react with **7** or **8**, and therefore, these inhibition assays were also carried out with 2 mM **7** or **8** in the presence of 50 mM HEPES, pH 7.5. The results obtained with HEPES were identical to those shown in Figure 3.

Inhibition assays were also performed in which 2 mM **7** (proposed alkylating reagent for OST) or MMTS (known alkylating reagent for OST) was preincubated for 30 min with microsomal enzyme under the conditions described above in the absence of [^{3}H]Dol-P-P-DS and Bz-Asn-Leu-Thr-NH$_2$ (**6**). The preincubated mixture was transferred to another tube that contained [^{3}H]Dol-P-P-DS and 360 μM Bz-Asn-Leu-Thr-NH$_2$ (**6**). The reaction mixtures were shaken for 2 h and then worked up as described above, and the glycosylation activities were determined. Each assay was run in duplicate, and the result is the average of the duplicates.

Time-Dependent OST Inhibition Studies Using Synthetic Dol-P-P-DS and Diazoketone 7. Each OST assay mixture contained 200 μM chemically synthesized Dol-P-P-DS, 50 mM HEPES, pH 7.5, 1% Triton X-100, 1 mM $MnCl_2$, 2 mM diazoketone **7** in DMSO, and 600 μg of P_{40} yeast microsome in a total volume of 95 μL. The assay was preincubated for 0, 5, 10, 15, or 30 min, and then 5 μL of [^{14}C]Bz-Asn-Leu-Thr-NH$_2$ in DMSO was added (final [[^{14}C]Bz-Asn-Leu-Thr-NH$_2$] = 360 μM; final [DMSO} = 10% v/v). The assay was shaken at 250 rpm for 2 h and then worked up as described above. The aqueous layer, which contained the newly formed [^{14}C] glycopeptide, was concentrated and then analyzed by reversed phase HPLC (condition B). The unreacted [^{14}C]Bz-Asn-Leu-Thr-NH$_2$ (t_R = 69 min) and the glycopeptide product [^{14}C]Bz-Asn(GlcNAc)$_2$-Leu-Thr-NH$_2$ (t_R = 76.5 min) were separated and quantitated using a liquid scintillation counter.

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Supporting Information Available: NMR (^{1}H, ^{13}C) spectra for compound **7**, crystal structure and data for **10**, ^{1}H−^{1}H PS-NOESY spectrum for **10**, and graphs of NH temperature vs chemical shift data (DMSO-d_6) for **6**−**10** (12 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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