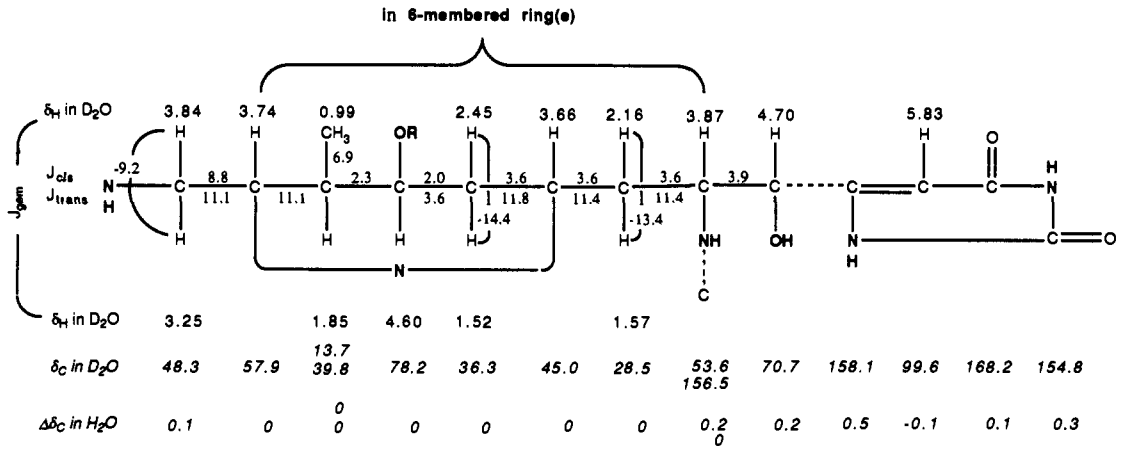
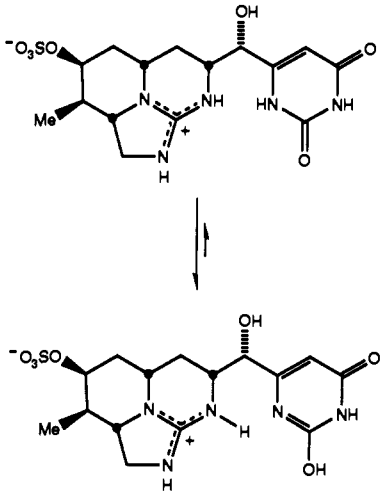
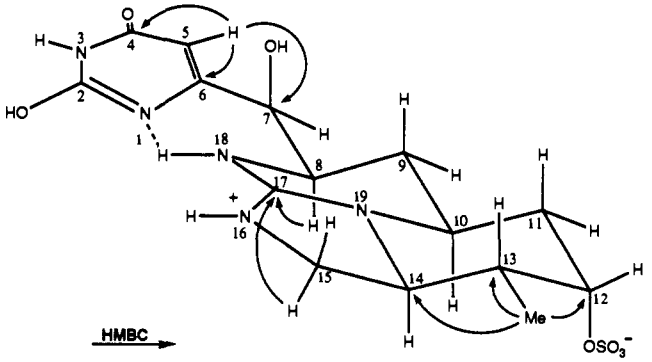



Figure 1. $^1H$ and $^{13}C$ chemical shift and $^1H$–$^1H$ coupling constant assignments for cylindrospermopsin.

tricyclic guanidine as depicted in **1**. The uracil ring had to be attached to C-7 since HMBC cross peaks were clearly seen between H-5 and C-4, C-6, and C-7 (**1a**). The $^{13}C$–$^{13}C$ COSY spectrum of uniformly 80+% $^{13}C$ enriched **1**, isolated from alga grown on $NaH^{13}CO_3$ (99%), confirmed the gross structure (see supplementary material).



**1**




**1a**

The $^{13}C$ signals for C-2 and C-6 were broad, and their chemical shifts, unlike the ones for uracil, were very sensitive to small changes in pH around 7, hinting that N-1/C-2 might have an enol rather than an amide structure. Favoring the enol tautomer could be a consequence of the uracil and guanidine units being coplanar with 18(N)-H hydrogen-bonded to N-1. If so, then the NOESY spectrum (NOE correlations: 5-H ↔ 7-H; 7-H ↔ 8-H; 9-$H_{eq}$ ↔ 11-$H_{eq}$; 10-H ↔ 14-H; 13-H ↔ 11-$H_{ax}$, 12-H, and 15-H (cis); and 13-$CH_3$ ↔ 12-H, 14-H, and 15-$H_2$) suggests that the toxin has the relative stereochemistry shown in **1a**.

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**Supplementary Material Available:** 500-MHz $^1H$ and 125-MHz $^{13}C$ NMR spectra of **1** in $D_2O$, $^1H$–$^{13}C$ HMQC, $^1H$–$^{13}C$ HMBC, and $^1H$–$^1H$ NOESY spectra of **1** in $D_2O$, $^{13}C$–$^{13}C$ COSY spectrum ($D_2O$) of [U-$^{13}C$]-**1**, and $^{13}C$ NMR spectrum of [U-$^{13}C$,$^{15}N$]-**1**[7] in $D_2O$ (7 pages). Ordering information is given on any current masthead page.

## Role of Peptide Conformation in Asparagine-Linked Glycosylation

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The first committed step in the biosynthesis of N-linked glycoproteins is catalyzed by the membrane-associated enzyme oligosaccharyltransferase (OT) and involves the cotranslational transfer of a complex carbohydrate from a lipid-linked pyrophosphate donor to the side-chain nitrogen of an asparagine (Scheme I).[1] The primary peptide sequence requirements for the process include a minimum -Asn-Xaa-Ser/Thr- tripeptide recognition motif.[2] This transformation is intriguing in that it formally involves nucleophilic attack by an asparagine primary amide nitrogen and displays remarkable selectivity considering the competing functionality in the peptidyl substrates in which the "reactive" asparagine is localized. Given these considerations and the recent proposal that the unique reactivity of asparagine may arise from a conformational bias resulting in the formation of the Asx turn,[3,4] we have synthesized and evaluated conformationally *constrained*[5] peptide substrates in order to probe the

(1) (a) Hubbard, S. C.; Ivatt, R. J. *Annu. Rev. Biochem.* **1981**, *50*, 555. (b) Kaplan, H. A.; Welply, J. K.; Lennarz, W. J. *Biochim. Biophys. Acta* **1987**, *906*, 161.
(2) Marshall, R. D. *Biochem. Soc. Symp.* **1974**, *40*, 17.
(3) Abbadi, A.; Mcharfi, M.; Aubry, A.; Premilat, S.; Boussard, G.; Marraud, M. *J. Am. Chem. Soc.* **1991**, *113*, 2729.
(4) Imperiali, B.; Shannon, K. L. *Biochemistry* **1991**, *30*, 4374.

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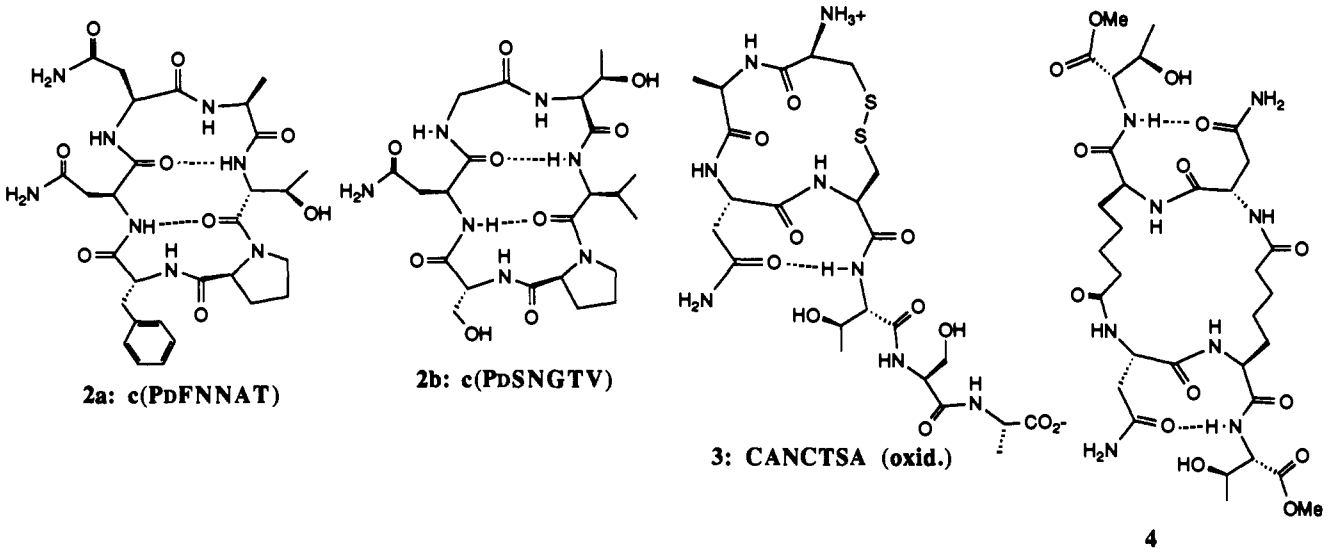
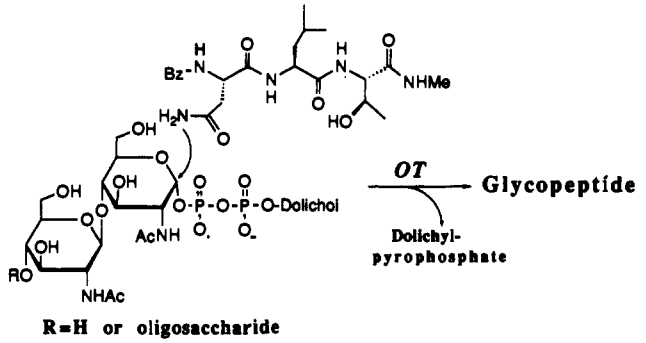



Figure 1.

**Scheme I**




importance of conformation in N-linked glycosylation.

OT may bind either an extended peptide conformer, in which no direct interaction exists between the asparagine and hydroxy amino acid side chains, or alternatively a motif such as a $\beta$-turn[6] or Asx-turn[3], in which the two side chains are brought into proximity. In a $\beta$-turn, the asparagine and hydroxy amino acid side chains can interact if the asparagine is in either the ($n$) or ($n$ + 1) position.[6] In the turn motifs the opportunity exists for one functional group to directly influence the other's reactivity. To distinguish between the extended and folded structures we have compared the solution-state conformational properties and substrate reactivity of the peptidyl substrates shown in Figure 1. The linear tripeptides **1a** and **1b** (Ac-Asn-Leu-Thr-OMe, **1a**; Ac-Asn-Leu-Thr-NHMe, **1b**) are both substrates. The cyclic hexapeptides **2a** and **2b** were designed to bias the conformation of the [-Asn-Xaa-Thr-] tripeptide into a type I $\beta$-turn, with the reactive asparagine at either the ($n$) or ($n$ + 1) position, through judicious incorporation of a prolyl-D-amino acid dipeptide.[7] The cyclic disulfide **3**[8] and peptide analog **4** are potential substrates in which the Asx-turn may be favored through a side chain to back-bone covalent constraint. NMR analysis of **2a**, **2b**, and **4** provides evidence that the major solution-state conformer is as shown, while for **3**, studies indicate that interactions within the large disulfide-containing ring are dominant.[9] While **3** does not represent a "fixed" Asx turn, the structure appears flexible enough to achieve this motif, but cannot adopt a $\beta$-turn motif.

**Table I.** Enzyme Kinetic Analysis of Conformationally Constrained Peptides[a]

| PEPTIDE | apparent $K_M$ (mM) | rel $V$ (%)[b] |
|---|---|---|
| Ac-Asn-Leu-Thr-OMe (**1a**) | 6.7 | 100 |
| Ac-Asn-Leu-Thr-NHMe (**1b**) | 0.3 | 150 |
| c(Pro-D-Ser-Asn-Gly-Thr-Val) (**2a**) | >20 | |
| c(Pro-D-Phe-Asn-Asn-Ala-Thr) (**2b**) | >20 | |
| oxid(Cys-Ala-Asn-Cys)-Thr-Ser-Ala (**3**) | 2.8 | 1600 |
| **4** | 0.6 | 75 |
| linear **4** | >20 | |

[a] Determined in the presence of 200000 dpm, 6.1 Ci/mmol $(GlcNAc)_2$-P-P-Dol ($4 \times 10^{-9}$ M).[4] [b] Peptide **1a** as standard.

Results of enzyme kinetic analysis[10] of **1–4** are documented in Table I. It is evident from the cyclic hexapeptides **2a** and **2b** that OT recognition of the tripeptide sequence in a $\beta$-turn structure is not favored. For **2a**, we investigated whether the corresponding linear peptide was a substrate; kinetic analysis of Pro-D-Ser-Asn-Gly-Thr-Val afforded kinetic parameters comparable to those of Ac-Asn-Leu-Thr-NHMe. Thus, the constraint imposed by cyclization severely impacted substrate properties. The Asx-turn analogs **3** and **4** are viable substrates with binding in the submillimolar range. The enhanced turnover of the disulfide-containing peptide **3** is attributed to the presence of additional binding determinants adjacent to the tripeptide recognition element.[11] It is noteworthy that a linear analog of **4**, Boc-Asn-$\alpha$-aminopimelic acid($\epsilon$-Asn-$\alpha$-aminopimelic acid-$\epsilon$-OBn-Thr-OMe)-Thr-OMe, is a very poor substrate with an *apparent* $K_M$ in excess of 20 mM. Therefore, in contrast to **2a**, the added constraint for **4** enhanced binding.

These studies indicate that glycosyl acceptor properties are indeed related to the ability of the potential substrates to adopt an "Asx-turn" conformation. This information will be invaluable for assessing the features of the local peptide environment which enhance asparagine primary amide reactivity and provide for the remarkable specificity observed in the glycosylation process.

**Acknowledgment.** This work was supported by the NIH (GM39334) and Sterling Drug Inc. In addition we gratefully

(5) Smith, P. E.; Al-Obeidi, F.; Pettitt, B. M. *Methods Enzymol.* **1991**, *202*, 411.

(6) Due to stereochemical considerations the $\beta$-turn is most likely to be a type I turn; for a review, see: Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1.

(7) This peptide enforces a type II $\beta$-turn[6] at the nonfunctional end of the substrate thus restricting the conformational freedom of the cyclic peptides.

(8) Bause et al. have considered the effect of incorporating two cysteine residues into glycosylation substrates; however, their studies did not reach a definitive conclusion regarding the role of peptide conformation. Bause, E.; Hettkamp, H.; Legler, G. *Biochem. J.* **1982**, *203*, 761.

(9) An evaluation of both NOEs and amide proton temperature coefficients provides evidence[4] to indicate that the conformation is as depicted.

(10) Kinetic results (apparent $K_M$ and $V_{max}$) are generated from a single crude microsomal preparation of the enzyme to circumvent inherent variations in the absolute activity.

(11) The groups adjacent to the tripeptide sequence exert a major effect on the kinetic parameters [apparent $K_M$ (mM), relative $V$ (%)]; for example: Bz-Asn-Leu-Thr-OMe (0.6, 370), Bz-Asn-Leu-Thr-NHMe (0.25, 1015).

acknowledge an NIH traineeship in biotechnology (GM08346) to K.L.S. and the award of a Fannie and John Hertz Foundation Fellowship to K.W.R.

**Supplementary Material Available:** Synthetic details and spectroscopic data for **2a**, **2b**, **3** and **4**, including ROESY spectra and variable temperature data for **2a** and **4**, and enzyme kinetic data for all substrates analyzed (9 pages). Ordering information is given on any current masthead page.

## A Mechanistic Proposal for Asparagine-Linked Glycosylation

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The origins of specificity and reactivity are presently unresolved issues in our understanding of co- and post-translational protein modification.[1] Central to unraveling the mechanism of asparagine-linked glycosylation[2] is ascertaining how the reactivity of a relatively poor nucleophile, the carboxamido group, might be enhanced. We have demonstrated[3] that glycosyl transfer acceptor properties may be related to the ability of the peptide to adopt an Asx-turn motif.[4] For glycosylation, the specific hydrogen-bonding interactions in this motif also bring the carboxamido oxygen of the asparagine side chain and the hydroxyl proton of the "essential" hydroxy amino acid into proximity. This added interaction may then provide additional stabilization for the Asx-turn and align the functional groups that comprise the specificity determinants for the process.

Mechanistic models to explain primary amide reactivity in the oligosaccharyltransferase-catalyzed (OT-catalyzed) process have previously been proposed by Marshall[5] and Bause et al.[6] While both models consider that the hydroxy amino acid functions by assisting ionization at nitrogen, neither model recognizes the potential importance of peptide backbone interactions nor implicates the Asx-turn in providing for a uniquely reactive side chain.

Toward deriving a mechanistic picture consistent with the observed conformational model, we have examined the glycosyl acceptor properties of peptides **1**–**5** in which the side chain functionality of the asparagine residue is replaced by groups with different ionization properties (Figure 1). The transfer kinetics of these peptides were determined[7] and compared against the standard substrate **1** (Table I). As a result of these studies, we propose that the enhanced asparagine reactivity may be attributed to a conformationally specific neighboring group assistance.

Asp-Xaa-Thr sites, such as **2**, are not glycosylated.[8,9] In addition, peptide **2** shows no competitive binding to OT, although the peptide adopts an Asx-turn conformation analogous to that observed in the substrates.[4] This suggests that a negative charge is not tolerated at the active site. Accordingly, those mechanisms which propose involvement of a nitrogen anion are unlikely, since **2** mimics that charge distribution and thus should inhibit OT as a transition-state analog.[10] Likewise, **3** is not recognized by OT. This modification does not affect either the conformation of the peptide or the hydrogen-bonding interaction to the hydroxyl proton. However, the side chain no longer carries a proton donor, and this appears to impact binding. Peptide **4** competitively inhibits OT and has a $K_i$ comparable to the $K_M$ of the standard substrate **1**. This peptide lacks a carbonyl oxygen so it cannot form the necessary hydrogen-bonding interactions to stabilize the Asx-turn; however, it can achieve an analogous structure. Finally, the thioasparagine-containing[11] peptide, **5**, is a substrate for OT with a $K_M$ similar to that for **1**.

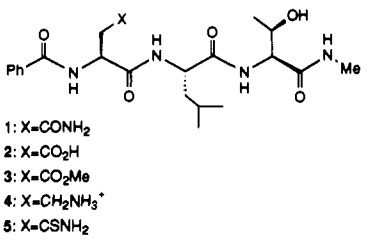


Figure 1.

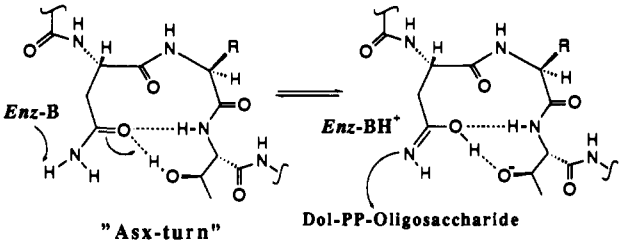


Figure 2.

Table I. Enzyme Kinetic Analysis of Tripeptides[a]

| peptide | apparent $K_M$ (mM) | rel $V$ (%)[b] | $K_i$ (mM) |
|---|---|---|---|
| Bz-Asn-Leu-Thr-NHMe (**1**) | 0.24 | 100 | |
| Bz-Asp-Leu-Thr-NHMe (**2**) | | | >10[c] |
| Bz-Asp(OγMe)-Leu-Thr-NHMe (**3**) | | | >10[c] |
| Bz-Amb-Leu-Thr-NHMe (**4**) | | | 1.0 |
| Bz-Asn(γS)-Leu-Thr-NHMe (**5**) | 0.26 | 8.4 | |

[a] Determined in the presence of 200 000 dpm, 6.1 Ci/mmol $(GlcNAc)_2$-P-P-Dol ($4 \times 10^{-9}$ M).[7] [b] Peptide **1** as standard. [c] No inhibition was observed at concentrations below 5 mM.

Therefore, the important criteria to be considered in formulating a working hypothesis for the catalytic mechanism of OT are the following: the role of the Asx-turn, the lack of evidence for an intermediate bearing a formal negative charge at nitrogen, and the effect of differing proton donors at the carboxamide site.

A mechanism consistent with these considerations is shown in Figure 2. It is proposed that the peptide backbone interactions and the essential hydroxy amino acid provide the necessary machinery for protonation of the carboxamido oxygen and, simultaneously, a general base at the active site abstracts a proton from the nitrogen forming the imidate tautomer, which is a competent nucleophile. It is intriguing to note that the Asx-turn interaction juxtaposes the critical hydroxyl group with the syn lone pair of electrons on the carboxamido oxygen.[12] Rate acceleration may be affected either by catalysis of a rate-determining tautomerization or by stabilization of the imidate tautomer in the environment created by the peptide. Modifications which affect either the $pK_a$ of the nitrogen protons or the basicity at the oxygen site should influence the binding to OT.

(1) Yan, S. C. B.; Grinnell, B. W.; Wold, F. *Trends Biochem. Sci.* **1989**, *14*, 264.
(2) Kaplan, H. A.; Welply, J. K.; Lennarz, W. J. *Biochim. Biophys. Acta* **1987**, *906*, 161.
(3) Imperiali, B.; Shannon, K. L.; Rickert, K. W. *J. Am. Chem. Soc.*, preceding paper in this issue.
(4) Abbadi, A.; Mcharfi, M.; Aubry, A.; Premilat, S.; Boussard, G.; Marraud, M. *J. Am. Chem. Soc.* **1991**, *113*, 2729.
(5) Marshall, R. D. *Ann. Rev. Biochem.* **1972**, *41*, 673.
(6) Bause, E.; Legler, G. *Biochem. J.* **1981**, *195*, 639.
(7) Imperiali, B.; Shannon, K. L. *Biochemistry* **1991**, *30*, 4374. Kinetic results are generated from a single crude microsomal preparation in order to circumvent inherent variations in the absolute activity.
(8) Gavel, Y.; von Heijne, G. *Protein Eng.* **1990**, *3*, 433.
(9) Bause, E. *FEBS Lett.* **1979**, *103*, 296.
(10) Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271.
(11) The steric and electronic implications of this substitution have been discussed by Sherman and Spatola: Sherman, D. B.; Spatola, A. F. *J. Am. Chem. Soc.* **1990**, *112*, 433.
(12) As suggested by studies on the carboxyl functional group (Gandour, R. D. *Bioorg. Chem.* **1981**, *10*, 169), the precise directionality of the proposed protonation step may be important for allowing the tautomerization to pick the path of minimum resistance.

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