Structural and Functional Characterization of a Constrained Asx-Turn Motif

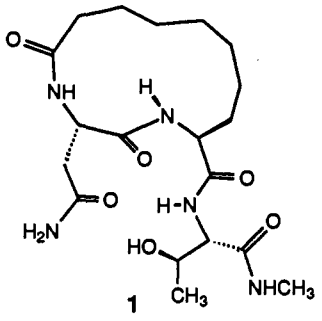
Barbara Imperiali,* Jeffrey R. Spencer, and Mary D. Struthers

Contribution No. 8954
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125

Received June 7, 1994

The specific and timely glycosylation of proteins is associated with the proper structure, function, and targeting of glycoprotein conjugates.[1] For N-linked glycosylation, catalyzed by the enzyme *oligosaccharyl transferase* (OT), we have recently proposed[2,3] that the fidelity of this modification may be associated with particular conformational tendencies of polypeptide substrates which contain the consensus sequence Asn-Xaa-Thr/Ser.[4] The presence of a local Asx-turn,[5] about the Asn-Xaa-Thr/Ser sequence, is central to this proposal, which was based on studies of the glycosyl acceptor properties of linear and cyclic peptides.[3] Herein, we describe a new substrate for N-linked glycosylation which is conformationally constrained to adopt an Asx-turn and specifically designed to test this proposal.

Critical backbone dihedral angles that must be defined in an Asx-turn motif are the asparagine ψ and the Xaa ϕ. Therefore, compounds with main-chain to side-chain lactam cyclizations between the N-terminus and the Xaa side chain were evaluated. Modeling studies revealed that the cyclic compound *cyclo*[Asn-Add]-Thr-NHMe (**1**), which contains the amino acid (*S*)-2-aminodecanedioic acid (Add), shows ideal Asx-turn properties in a minimum energy conformer.[6] Compound **1** allows for detailed structural characterization in aqueous media. Additionally, the properties of this peptide as a substrate for OT can be directly compared with those of simple, unconstrained analogs thereby allowing for direct assessment of the role of the Asx-turn in N-linked glycosylation.



1

The N^{α}-acetylated racemic amino acid, Add, was synthesized by a modified Sorensen procedure[7] and enzymatically resolved with acylase I.[8] Synthesis of the linear tripeptide was carried out by standard solution-phase methods. The final cyclization of

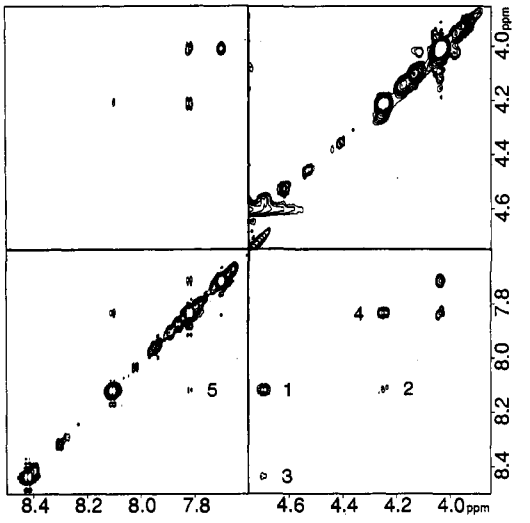


Figure 1. Composite spectrum of *cyclo*[Asn-Add]-Thr-NHMe (**1**) obtained by 600-MHz ^{1}H NMR ROESY experiment. The sample had a peptide concentration of 6.7 mM in 700 μL of 43% aqueous methanol-d_4, pH 4.5 (uncorrected), at 7 °C. A mixing time of 200 ms was used. The corresponding transpose signals for NOEs 1 and 2 are not seen due to the eradication of the initial α-proton magnetization by the water suppression technique.

N^{α}-Boc-Asn-Add-Thr(tBu)-NHMe was carried out *via* activation of the side chain carboxylic acid of Add as the pentafluorophenyl ester, followed by acidolysis of the *tert*-butyl based protecting groups and neutralization with triethylamine.[9] For comparison, the linear tripeptides N^{α}-Bu-Asn-Leu-Thr-NHMe (**2**) and N^{α}-Bu-Gln-Leu-Thr-NHMe (**3**) were also synthesized.

Kinetic analysis of the asparagine-containing peptides with yeast OT clearly demonstrates that the preorganization[10] of **1** to an Asx-turn conformer greatly improves enzyme affinity. The cyclic compound, **1**, has an apparent K_M of 78 μM, while the corresponding value for **2** is 800 μM.[11] The relative maximum velocities for the two compounds are similar (**1**, V_{rel} 100%; **2**, V_{rel} 90%). Peptide **3** is not a substrate for OT.[4] These results indicate the importance of the Asx-turn as a recognition motif in the glycosylation process.

The conformation of peptide **1** in aqueous methanol (57:43 water/methanol) was investigated by 2D ^{1}H NMR using a ROESY[12] experiment. The structure of the macrocycle can be defined by three key NOE cross peaks (Figure 1). The relative intensities of these NOEs [(1) strong, (2, 3) weak] establish the *trans* amide geometries within the ring. The sequential NOE (4) and the medium-range NOE (5) provide evidence for a nonextended conformation consistent with the Asx-turn as depicted in Figure 2.

The adoption of an Asx-turn is further supported by observation of a reduced amide proton temperature coefficient for the Thr-NH (–4.3 ppb/K). This amide proton would be expected to be solvent shielded due to hydrogen bonding with the Asn C=O$^{\delta 1}$. Table 1 documents amide proton temperature coefficients and $^3J_{HN\alpha}$ coupling constant values for peptides **1–3**.

Conformational analysis reveals that while peptide **1** favors the Asx-turn, this motif is still accessible to the linear peptide **2**. However, the analogous conformation for the glutamine-containing peptide is significantly less stable.[13] Although a weak XaaN^{α}H-ThrN^{α}H NOE cross peak is observed for all three peptides, the amide proton temperature coefficients reveal

(1) Horak, J. *Folia Microbiol.* **1991**, *36*, 3.
(2) Imperiali, B.; Shannon, K. L. *Biochemistry* **1991**, *30*, 4374.
(3) Imperiali, B.; Shannon, K. L.; Rickert, K. W. *J. Am. Chem. Soc.* **1992**, *114*, 7942.
(4) Marshall, R. D. *Biochem. Soc. Symp.* **1974**, *40*, 17.
(5) Abbadi, A.; Mcharfi, M.; Aubry, A.; Premilat, S.; Boussard, G.; Marraud, M. *J. Am. Chem. Soc.* **1991**, *113*, 2729.
(6) Macrocyclic compounds of varying bridge length were modeled using Discover (Biosym, Version 2.9). Minimizations were carried out through 100 steps of steepest descent followed by 1000 steps of conjugate gradient. Low-energy conformers were compared. The macrocyclic compound **1** featured *trans* amide geometries and very little ring strain, while simultaneously favoring the Asx-turn.
(7) Albertson, N. F. *J. Am. Chem. Soc.* **1946**, *68*, 450.
(8) Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 6354.
(9) Evans, D. A.; Ellman, J. A. *J. Am. Chem. Soc.* **1989**, *111*, 1063.
(10) Hruby, V. J.; Mosberg, M. I. *Peptides* **1982**, *3*, 329.
(11) Yeast OT was assayed as described for the pig liver enzyme (ref 2). Kinetic results are generated from a single solubilized microsomal preparation in order to circumvent inherent variations in the absolute activity.
(12) Bothner-By, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811.

0002-7863/94/1516-8424$04.50/0

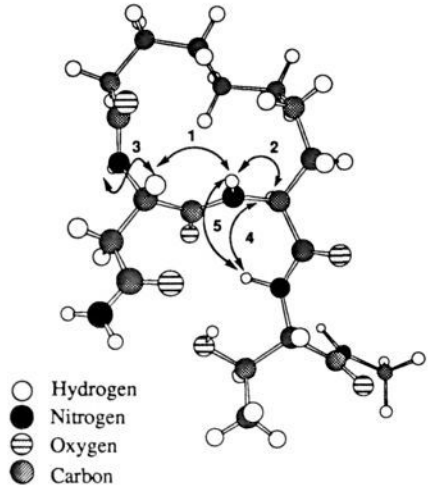


Figure 2. Energy-minimized structure of **1**. From an extended backbone conformation **1** was minimized with Discover, using 200 steps of steepest descent followed by 1000 steps of conjugate gradient minimization. All observed NOE data were included in the computation as distance constraints with 0.5–3.5 Å limits.

Table 1. Summary of NH Temperature Coefficients and $^3J_{HN\alpha}$ Coupling Constant Values for **1**–**3** in 43% Aqueous Methanol-d_4, pH 4.5 (Uncorrected)

	Asn/Gln		Xaa		Thr	
	$-\Delta\delta/\Delta T$ (ppb/K)	$^3J_{HN\alpha}$ (Hz)	$-\Delta\delta/\Delta T$ (ppb/K)	$^3J_{HN\alpha}$ (Hz)	$-\Delta\delta/\Delta T$ (ppb/K)	$^3J_{HN\alpha}$ (Hz)
1	5.6	8.84	7.8	8.03	4.3	7.96
2	7.5	7.91	8.6	6.78	5.3	7.39
3	6.8	6.78	7.5	6.77	5.9	8.06

differences among these compounds which correlate with the ability to adopt an Asx-turn. This trend is also demonstrated in the variation of the $^3J_{HN\alpha}$ values (Table 1), which are related to the ϕ torsional angle.[14] Coupling constant values in the range 8–10 Hz are expected for an Asx-turn. The large $^3J_{HN\alpha}$ value observed in peptide **1** (>8 Hz) is again consistent with the conformational model for the compound.

Circular dichroism (CD) studies further support the structural differences between the three peptides. The spectrum of peptide **3** closely resembles that of a random coil structure with a single, large, negative ellipticity between 195 and 200 nm (Figure 3).[15] In contrast, the spectrum of compound **1** exhibits a strong negative ellipticity at 218 nm and a diminished signal at 198 nm indicative of a nonrandom conformation.[16] The CD spectrum of **2** shows intermediate properties. Significantly, in spite of the greater

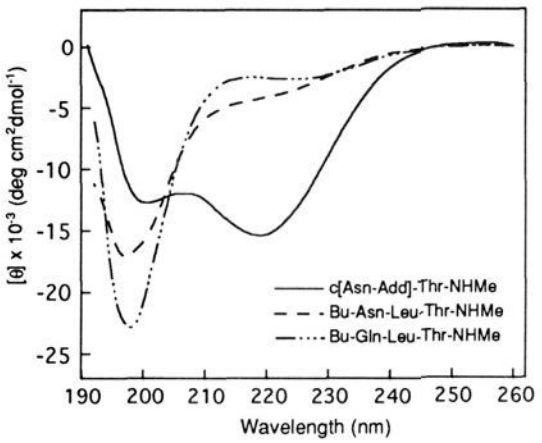


Figure 3. CD spectra of *cyclo*[Asn-Add]-Thr-NHMe (**1**), N^{α}-Bu-Asn-Leu-Thr-NHMe (**2**), and N^{α}-Bu-Gln-Leu-Thr-NHMe (**3**) in 43% aqueous methanol, pH 4.5 (uncorrected). Peptide concentrations were 495, 530, and 515 μM, respectively.

tendency of aqueous solvents to compete for hydrogen bonding, the cyclic constraint in **1** promoted ordered secondary structure, which was observed in 43% aqueous methanol and persisted in as little as 2% aqueous methanol.[16]

The data presented indicate that the Asx-turn is a recognition motif for N-linked glycosylation and support the proposal that the peptide backbone may play a functional role in orchestrating the glycosylation reaction. This proposal[17] implicates the conformational features of the Asx-turn in bringing the hydroxyl functionality of the essential threonine or serine residue into proximity with the asparagine side chain, thereby modulating the amide nucleophilicity. The conformational properties of peptides **1**, **2**, and **3** highlight the differences between asparagine and glutamine in contributing toward polypeptide secondary structure and suggest the origin of the unique chemical reactivity of asparagine in the glycosylation reaction.

Acknowledgment. This research was supported by National Institutes of Health Grant GM39334 and an NSF predoctoral fellowship to M.D.S. B.I. also gratefully acknowledges support from the Alfred P. Sloan Foundation, the Camille and Henry Dreyfus Teacher-Scholar Program, and Zeneca Inc. We acknowledge the Dorothy Chandler, Camilla Chandler Frost Laboratory in Biology at Caltech for use of NMR instrumentation (Varian Unity Plus 600 Spectrometer).

Supplementary Material Available: Synthetic details and spectroscopic data for **1**–**3**, including the complete ROESY spectrum for **1**, and enzyme kinetic data for substrates **1** and **2** (14 pages). This material is contained in many 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.

(13) Baker, E. N.; Hubbard, R. E. *Prog. Biophys. Mol. Biology* **1984**, *44*, 97. This statistical survey provides compelling evidence for the differences between the hydrogen-bonding tendencies of glutamine and asparagine. Of the short-range interactions examined, 55% of the hydrogen bonds to the carbonyl group of the asparagine side chain are provided by the $(i + 2)$ residue backbone NH, while, in contrast, glutamine engages in very few short-range contacts, and none involve the $(i + 2)$ residue.

(14) Pardi, A.; Billeter, M.; Wuthrich, K. *J. Mol. Biol.* **1984**, *180*, 741.

(15) Johnson, W. C. J. *Proteins: Struct., Funct., Genet.* **1990**, *7*, 205.

(16) These spectral properties were independent of the methanol content of the solvent mixture (2–43%), peptide concentration (50–500 μM), and pH (4.5–7.0, uncorrected). Therefore the NMR data can be directly compared with a predominantly aqueous and neutral environment.

(17) Imperiali, B.; Shannon, K. L.; Unno, M.; Rickert, K. W. *J. Am. Chem. Soc.* **1992**, *114*, 7944.