Yeast Oligosaccharyltransferase: Glycosylation of Peptide Substrates and Chemical Characterization of the Glycopeptide Product[1]

Richard S. Clark, Shyamal Banerjee, and James K. Coward*

Departments of Medicinal Chemistry and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

Received March 5, 1990

The product of the reaction catalyed by yeast oligosaccharyltransferase was examined in order to determine the nature of the chemical linkage between the sugar and peptide. Biosynthetic donor lipid [^{3}H]oligosaccharide was prepared and used as a substrate for yeast oligosaccharyltransferase together with a chemically synthesized peptide acceptor, *N*-benzoyl-Asn-Leu-Thr-NH_2. The glycosylated peptide product of the in vitro reaction was isolated and hydrolyzed with *endo*-β-*N*-acetylglucosaminidase-H to yield a large oligosaccharide and the glycotripeptide, *N*-benzoyl-Asn(GlcNAc)-Leu-Thr-NH_2. This glycopeptide was purified using gel filtration, affinity binding, and reverse-phase high-performance liquid chromatography. The biosynthetic glycopeptide was compared with chemically synthesized glycopeptides in which a 1-amino-GlcNAc moiety was linked to either the α- or β-carboxyl of aspartate. It was determined that the sole biosynthetic product has the structure in which the carbohydrate is linked to the peptide through the β-carbonyl of asparagine, i.e., a normal α-peptide. These experiments provide an unambiguous structural proof of the protein–carbohydrate linkage in the glycoprotein product of the oligosaccharyltransferase-catalyzed reaction.

N-Linked protein glycosylation is a universal process in eukaryotes and is intimately related to intracellular protein sorting and secretion. This cotranslational process, which takes place in the rough endoplasmic reticulum,[2] is fundamental to eukaryotic cells as evidenced by the ubiquity of oligosaccharyltransferase, (OST),[3] the enzyme that transfers en bloc the initial oligosaccharide to the growing peptide chain.[4] In addition, although many N-linked oligosaccharides are present in cells, one oligosaccharide [$Glc_3Man_9(GlcNAc)_2$] is preferentially transferred during cotranslational glycosylation.[4-8]

OST, the key enzyme in the transfer of oligosaccharide from lipid oligosaccharide to the carboxamido nitrogen of asparagine is biochemically interesting for the following reasons: first, OST activity may be a prime site for regulation of N-linked protein glycosylation since OST catalyzes the transfer of the oligosaccharide product of the dolichol cycle to Asn in the tripeptide sequon Asn-Xaa-Thr/Ser during the translation of mRNA. Both of these processes, the dolichol cycle and mRNA translation, utilize large amounts of cellular energy in the form of nucleotide-driven reactions. Second, the asparagine residue of the tripeptide sequon Asn-Xaa-Thr/Ser is the site of glycosylation in all N-linked glycoproteins sequenced to date. However, not all sequon sites are glycosylated. Third, the reaction involves the β-carboxamido nitrogen of asparagine, effecting overall a nucleophilic displacement at sp^3 carbon, an unusual reaction for which the mechanism has not been elucidated. In addition, processing of the oligosaccharide attached to asparagine begins immediately after transfer and continues through the endo membrane system. This processing results in the formation of many different complex oligosaccharides attached to the protein after initial transfer of $Glc_3Man_9(GlcNAc)_2$.

Although OST occupies a central position in the overall process of N-linked protein glycosylation, uncertainty still exists regarding the linkage between carbohydrate and protein. Zinn et al.[9] and Neuberger et al.[10] have written excellent reviews on the nature of the *N*-acetylglucosaminylasparagine (GlcNAc-Asn) linkage. However, previous work on determination of the GlcNAc-Asn linkage involved successive proteolytic and chemical degradation steps, among which was a step consisting of heating a carbohydrate-containing compound in 2 N HCl at 100 °C for 12–30 min. These severe conditions, utilized to hydrolyze the carbohydrate portion from the glycopeptide (or glycoasparaginyl residue), led to very low yields of the final degradation product after purification from a variety of undesired byproducts. Even recent studies on the structure of N-linked glycopeptides using 2D-NMR techniques have not addressed the regiochemistry of the peptide–carbohydrate linkage.[11] It has been noted that isoaspartyl linkages in proteins are not cleaved during Edman degradation.[12] Since N-linkaged glycoproteins can be sequenched using the Edman procedure,[13] it has been concluded that the linkage from protein to carbohydrate is through the β-carbonyl of asparagine. However, a rigorous proof based on an independent synthesis of both regioisomers of the N-linked glycopeptide has not been reported. Therefore, we have developed a high-yield method to isolate and characterize the glycopeptide product of the OST-catalyzed glycosylation reaction for comparison with the synthetic isomers. The method involves two chromatographic steps, a single enzymatic digestion, and affinity binding which leads to isolation of a GlcNAc-

(1) This paper is deduced to the memory of Emil Thomas Kaiser, a stimulating scientist and an extraordinary human being.

(2) Lennarz, W. J. *Biochemistry* **1987**, *26*, 7205–7210.

(3) Abbreviations used: OST, oligosaccharyltransferase, EC 2.4.1.119; Glc, glucose; Man, mannose, GlcNAc, *N*-Acetylglucosamine; LOS, lipid oligosaccharide (dolichylpyrophsophate–oligosaccharide); P_{40}, the pellet obtained at 40000*g*; Endo H, *endo*-β-*N*-acetylglucosaminidase H, EC 3.2.1.96; Con A, concanavalin A; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, 1-hydroxybenzotriazole.

(4) Kaplan, H. A.; Welply, J. K.; Lennarz, W. J. *Biochim. Biophys. Acta* **1987**, *906*, 161–173.

(5) Hubbard, S. C.; Ivatt, R. J. *Annu. Rev. Biochem.* **1981**, *50*, 555–583.

(6) Kukuruzinska, M. A.; Bergh, M. L. E.; Jackson, B. J. *Annu. Rev. Biochem.* **1987**, *56*, 915–944.

(7) Kornfeld, R.; Kornfeld, S. *Annu. Rev. Biochem.* **1985**, *54*, 631–664.

(8) Presper, K. A.; Heath, E. C. *The Enzymes XVI* **1983**, 449–487.

(9) Zinn, A. B.; Plantner, J. J.; Carlson, D. M. In *The Glycoconjugates*; Horowitz, M. I., Pigman, W., Ed.; Academic Press: New York, 1977; Vol. 1, pp 69–85.

(10) Neuberger, A.; Gottschalk, A.; Marshall, R. D.; Spiro, R. G. In *Glycoproteins: Their Composition, Structure and Function*; Gottschalk, A., Ed.; Amsterdam: Elsevier, 1972; pp 450–490.

(11) Berman, E.; Dabrowski, U.; Dabrowski, J. *Carbohydr. Res.* **1988**, *176*, 1–15.

(12) Chazin, W. J.; Kordel, J.; Thulin, E.; Hoffman, T.; Drakenberg, T.; Forsen, S. *Biochemistry* **1989**, *28*, 8646–8653 and references therein.

(13) Paxton, R. J.; Mooser, G.; Pande, H.; Lee, T. D.; Shively, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 920–924. During sequencing, yields of the derivatized amino acids decrease dramatically following any modification site, e.g., glycosylation, phosphorylation, etc. See, for example: Reddy, V. A.; Johnson, R. S.; Biemann, K.; Williams, R. S.; Ziegler, F. D.; Trimble, R. B.; Maley, F. *J. Biol. Chem.* **1988**, *263*, 6978–6985.

0022-3263/90/1955-6275$02.50/0

Table I. Properties of Synthetic Peptides and Intermediates

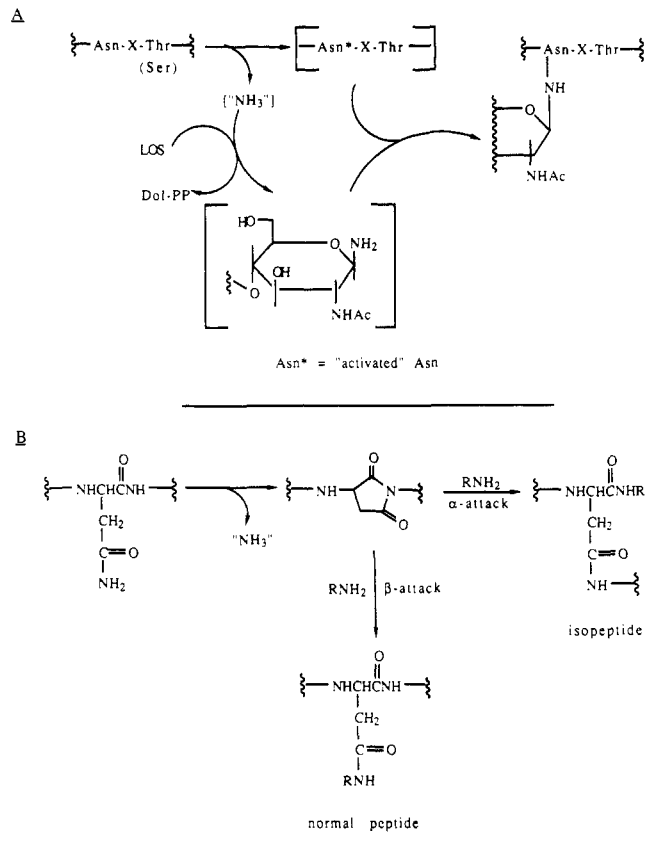
compound	method[a]	yield, %	mp, °C (lit.)	formula[b]
$BocAsnLeuThrNH_2$	A[c]	67	191–193 (195–196)[d]	nd
$CH_3C(O)AsnLeuThrNH_2$	D	66	263–264 (262–264)[d]	$C_{16}H_{30}N_5O_6$[e]
$C_6H_5C(O)AsnLeuThrNH_2$	D	72	237–239 (239–241)[d]	$C_{21}H_{32}N_5O_6$[e]
$C_7H_{15}C(O)AsnLeuThrNH_2$	D	75	259–261 (255–257)[d]	$C_{22}H_{42}N_5O_6$[e]
$Z\text{-}isoAsnLeuThrNH_2$	A	84	235–237	$C_{22}H_{34}N_5O_7$
$CH_3C(O)isoAsnLeuThrNH_2$	D	64	209–211	$C_{16}H_{30}N_5O_6$
$C_6H_5C(O)isoAsnLeuThrNH_2$	D	69	215–217	$C_{21}H_{32}N_5O_6$

[a] Methods A–D refer to synthetic procedures described in the Experimental Section. [b] High-resolution mass spectroscopy (MH^+). [c] Peptide product is insoluble in EtOAc; workup procedure was modified to reflect this fact and desired product was isolated by filtration after EtOAc trituration of initial crude reaction residue. [d] Welply et al. (1983). [e] Satisfactory elemental analysis also obtained for partial hydrates.

containing tripeptide for use in further characterization of the OST-catalyzed reaction.

One possible mechanism for the OST-catalyzed reaction would involve nucleophilic activation of the carboxamido nitrogen of asparagine through the participation of an activated asparagine (Asn*) with concomitant generation of "NH_3" at the active site of the enzyme. This "NH_3" could then attack the anomeric carbon of the proximal GlcNAc in the LOS also bound at the active site to give an intermediate 1-amino oligosaccharide together with release of the dolicholpyrophosphate (Scheme IA). This posulated mechanism, leading to formation of "NH_3" at the active site, is analogous to the well-established mechanism for glutamine-dependent amino transfer reactions.[14] The reaction between an amido N atom and the C_1 of a sugar involved in a pyrophosphate linkage is more specifically analogous to the reaction catalyzed by 5-phosphoribosyl pyrophosphate (PRPP) amidotransferase between Gln and PRPP in which 5-phosphoribosylamine is formed.[15] Among several possible candidates for Asn* is a cyclic imide intermediate arising from attack of the peptidyl nitrogen of the Xaa in the adjacent amino acid in the sequon Asn-Xaa-Thr/Ser (Scheme IB). This type of cyclic imide intermediate is known to occur, e.g, in the spontaneous deamidation of asparagine residues in proteins,[16–18] leading to rearranged isoaspartate-containing proteins which can be repaired by protein carboxyl *O*-methyltranferase.[19,20] If this cyclic intermediate occurred during the enzymatic transfer of oligosaccharide to protein, an isoglycopeptide product could arise if the amino sugar generated attacked at the α-carbonyl of the cyclic imide intermediate. We have synthesized simple glycopeptides, **1** and **2**, related to both possible products, and have examied the biosynthetic product in order to compare it with the chemically synthesized glycopeptides. Our results show that the product of OST-catalyzed reaction is the unrearranged glycopeptide. In addition, the isopeptide, *N*-benzoyl-isoAsn-Leu-Thr-NH_2, is neither a substrate nor an inhibitor of the enzyme-catalyzed reaction, also suggesting, by microscopic reversibility, either that no cyclic imide intermediate is formed, or that nucleophilic attack on a cyclic imide occurs only at the β-carbonyl.

Scheme I



Experimental Section

N-Cbz L-aspartic acid α-*tert*-butyl ester dicyclohexylamide (DCHA) salt and the corresponding β-*tert*-butyl ester DCHA salt were obtained from Chemical Dynamics. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine was prepared from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (Sigma) via the intermediate azide, as previously described[21,22] with the exception that reduction of the azide to the amine was effected in 75% yield using the Staudinger reaction (Ph_3P/NH_4OH).[23] Cloned endo H^{24} and ^{14}C-labeled oligosaccharides, $Glc_3[^{14}C]Man_9(GlcNAc)_2$ and $[^{14}C]Man_9(GlcNAc)_2$, were generous gifts of Dr. Robert Trimble, New York State Department of Health. Bio-Gel P-4 (–400 mesh) was purchased from Bio-Rad. Nucleotide sugars and concanavalin A-Sepharose 4B (11 mg Con A/mL packed gel) were obtained from Sigma Chemical Co. GDP-[^{3}H]Man (29.1 Ci/mmol) and UDP-[^{3}H]GlcNAc (26.8 Ci/mmol) were purchased from DuPont NEN. All other reagents were of reagent grade or better unless otherwise specified.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 MHz or 360 MHz spectrometer, unless otherwise noted. Mass spectra were recorded on a VG Analytical, Model 70-250S. All new synthetic compounds were fully characterized by NMR (^{1}H,

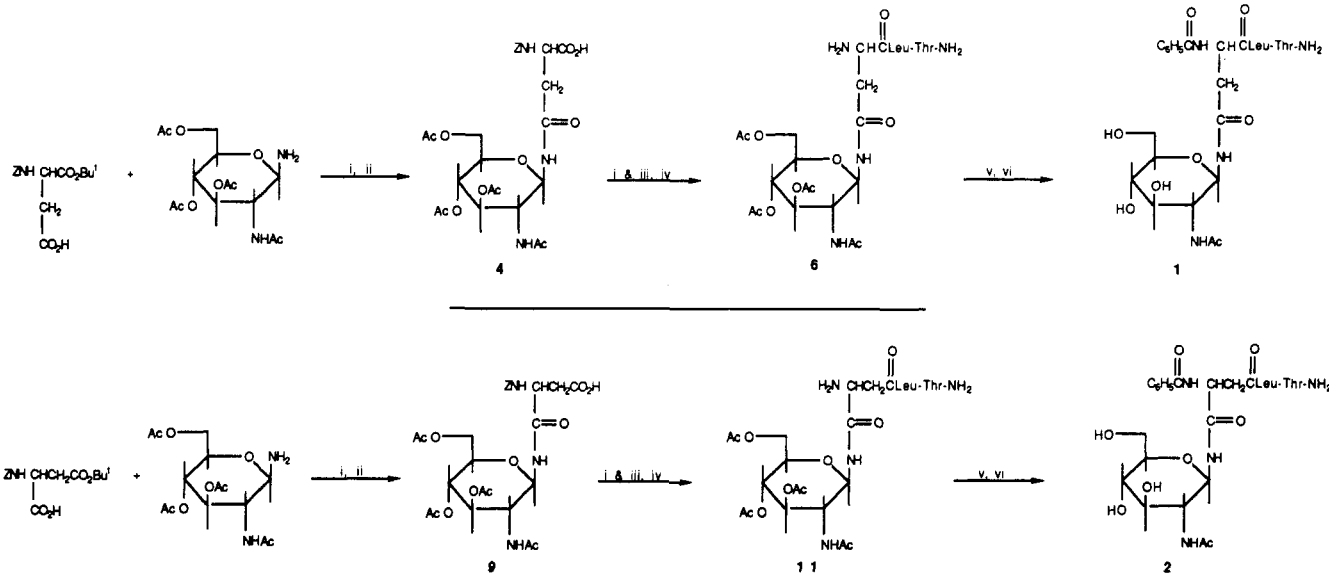
(14) Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman & Co.: San Francisco, 1979; pp 138–156.
(15) Schendel, F. J.; Cheng, Y. S.; Otvos, J. D.; Wehrli, S.; Stubbe, J. *Biochemistry* **1988**, *27*, 2614–2623.
(16) Kossiakoff, A. A. *Science* **1988**, *240*, 191–194.
(17) Geiger, T.; Clarke, S. *J. Biol. Chem.* **1987**, *262*, 785–795.
(18) Galletti, P.; Ciardiello, A.; Ingrosso, D.; D'Alessio, G. *Biochemistry* **1988**, *27*, 1752–1757.
(19) McFadden, P. N.; Clarke, S. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2595–2599.
(20) Clarke, S. *Annu. Rev. Biochem.* **1985**, *54*, 479–506.
(21) Michael, M.; Wulff, H. *Chem. Ber.* **1956**, *89*, 1521–1530.
(22) Bolton, C. H.; Jeanloz, R. W. *J. Org. Chem.* **1963**, *28*, 3228–3230.
(23) Valtier, M.; Knouz, N.; Carrie, R. *Tetrahedron Lett.* **1983**, *24*, 763–764.
(24) Trumbly, R. J.; Robbins, P. W.; Belfort, M.; Ziegler, F.; Maley, F.; Trimble, R. B. *J. Biol. Chem.* **1985**, *260*, 5683–5690.

Table II. Properties of Synthetic Glycopeptides and Intermediates

compound	method[a]	yield,[b] %	mp, °C	formula[c]
Z-Asnβ-(Ac_3GlcNAc)α-OBu^t (3)	A	59	216–218	$C_{30}H_{41}N_3O_{13}$
Z-Asnβ-(Ac_3GlcNAc)α-OH (4)	B	70	220–222	$C_{26}H_{33}N_3O_{13}\cdot 0.5H_2O$[d]
Z-Asnβ-(Ac_3GlcNAc)LeuThrNH_2 (5)	A	74	275–277	$C_{36}H_{53}N_6O_{15}$ (MH^+)
H-Asnβ-(Ac_3GlcNAc)LeuThrNH_2 (6)	C	79	206–209	nd
$C_6H_5C(O)$Asnβ-(Ac_3GlcNAc)LeuThrNH_2 (7)	D	74	279–281	$C_{35}H_{50}N_6O_{14}$
$C_6H_5C(O)$Asnβ-(GlcNAc)LeuThrNH_2 (1)	E	66	264–266	$C_{29}H_{45}N_6O_{11}$ (MH^+)
Z-Asnα-(Ac_3GlcNAc)β-OBu^t (8)	A	58	169–170	$C_{30}H_{41}N_3O_{13}$
Z-Asnα-(Ac_3GlcNAc)β-OH (9)	B	78	195–197	$C_{26}H_{33}N_3O_{13}\cdot 0.75H_2O$[d]
Z-Asnα-(Ac_3GlcNAc)β-LeuThrNH_2 (10)	A	75	285–286	$C_{36}H_{53}N_6O_{15}$ (MH^+)
H-Asnα-(Ac_3GlcNAc)β-LeuThrNH_2 (11)	C	78	196–199	nd
$C_6H_5C(O)$Asnα-(Ac_3GlcNAc)β-LeuThrNH_2 (12)	D	65	283–286	$C_{35}H_{50}N_6O_{14}\cdot 2H_2O$
$C_6H_5C(O)$Asnα-(GlcNAc)LeuThrNH_2 (2)	E	66	268–270	$C_{29}H_{45}N_6O_{11}$ (MH^+)

[a] Methods A–E refer to synthetic procedures described in the Experimental Section. [b] Yields given are of purified products. [c] Satisfactory elemental analysis or high-resolution mass spectra (MH^+). [d] High-resolution mass spectra (MH^+) also obtained.

Scheme II. Synthesis of Glycopeptides[a]



[a] Reagents: (i) DCC-HOBt; (ii) TFA; (iii) H-Leu-Thr-NH_2; (iv) H_2/Pd–C; (v) Bz_2O; (vi) Et_3N–aqueous CH_3OH.

^{13}C) and mass spectra. In addition, all compounds listed in Tables I and II gave satisfactory combustion analysis and/or high-resolution mass spectra.

Synthesis of Tripeptide Substrates for Oligosaccharyltransferase. (1) The synthesis of *N*-acyl derivatives of the tripeptide Asn-Leu-Thr-NH_2 was accomplished using various protected amino acids and the DCC-HOBt solution method. The TFA salt of Leu-Thr-NH_2 was prepared essentially as described by Welply et al.[25] and was coupled with Boc-protected Asn in the presence of DCC-HOBt to give Boc-Asn-Leu-Thr-NH_2. Treatment with TFA gave the TFA salt of the tripeptide Asn-Leu-Thr-NH_2. The acyl tripeptide substrates were prepared by reacting the appropriate acid anhydride (i.e., acetic, benzoic, or *n*-octanoic anhydride) with the TFA salt of Asn-Leu-Thr-NH_2 in the presence of Et_3N. (2) For the synthesis of *N*-acyl derivatives of isoAsn-Leu-Thr-NH_2, (*S*)-3-Cbz-5-oxo-4-oxazolidineacetic acid[26] was utilized. Cbz-isoAsn-Leu-Thr-NH_2 was prepared via two routes: (a) by aminolysis of the oxazolidine acetic acid in MeOH with dry NH_3 to give Cbz-isoAsn,[26] which was then coupled with the TFA salt of Leu-Thr-NH_2 using DCC-HOBt (method A, see below) to yield the Cbz-protected tripeptide Cbz-isoAsn-Leu-Thr-NH_2; or (b) by directly coupling the Cbz-protected oxazolidineacetic acid with the TFA salt of Leu-Thr-NH_2 using DCC-HOBt, followed by aminoolysis (NH_3/MeOH) to give Cbz-isoAsn-Leu-Thr-NH_2. Hydrogenolysis of Cbz-isoAsn-Leu-Thr-NH_2 (method C, see below) gave the free amine, which was acylated with either acetic anhydride or benzoic anhydride in the presence of Et_3N (method D, see below) to give *N*-acetyl-isoAsn-Leu-Thr-NH_2 and *N*-benzoyl-isoAsn-Leu-Thr-NH_2, respectively. In all cases, the acyl tripeptide products were isolated by passing the reaction mixture through Dowex 50W-X8 cation (H^+) resin. The products were crystallized and characterized as summarized in Table I.

Synthesis of Glycotripeptides (Scheme II). 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-(α-(carbobenzyloxy)-β-aspartyl)-2-deoxy-β-D-glucopyranosylamine (4). *N*-Cbz-L-aspartic acid α-*tert*-butyl ester DCHA salt (750 mg, 1.48 mmol) was dissolved in EtOAc (25 mL), followed by washing of the resulting solution with 2 M aqueous $NaHSO_4$ (2 × 10 mL) in a separatory funnel. The organic layer was washed three times with brine, dried ($MgSO_4$), and concentrated in vacuo to give 440 mg (1.36 mmol, 92%) of the free acid. This material was dissolved in freshly distilled THF (40 mL), followed by the addition (−10 °C) of 375 mg (2.7 mmol) of HOBt and 309 mg (1.5 mmol) of DCC. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine (472 mg, 1.36 mmol) was then added, and stirring of the reaction mixture was initiated at −10 °C for 30 min and continued for 15 h at ambient temperature. DCU was removed by filtration, and the filtrate was concentrated in vacuo. The resulting residue was dissolved in EtOAc, additional DCU was removed by filtration, and the filtrate was washed with 1 N HCl–saturated NaCl (1:1) (3 × 20 mL) and saturated $NaHCO_3$ (3 × 10 mL). After drying over $MgSO_4$, the organic solution was concentrated in vacuo to give 705 mg (79.5%) of the desired product contaminated with a small amount of DCU. This material was suitably pure for use in further transformations. Purification of the crude product was accomplished by flash chromatography on silica gel using EtOAc as the eluant to give the desired α-*tert*-butyl ester intermediate,

(25) Welply, J. K.; Shenbagamurthi, P.; Lennarz, W. J.; Naider, F. *J. Biol. Chem.* **1983**, *258*, 11856–11863.
(26) Itoh, M. *Chem. Pharm. Bull.* **1969**, *17*, 1679–1686.

3 (525 mg, 59%): mp 216–218 °C; TLC R_f = 0.72 (system A, see Chromatographic Methods); ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9 H, $(CH_3)_3$), 1.98, 2.03, 2.04, 2.10 (4 s, 12 H, 4 CH_3CO), 2.64–2.77 (dd, 2 H, β-CH_2), 4.50 (t, 1 H, α-CH), 5.11 (s, 2 H, $PhCH_2$), 3.44–4.31 (m, 4 H, sugar protons), 5.07–5.10 (m, 3 H, sugar protons), 5.85 (d, 1 H, NH), 6.09 (d, 1 H, NH), 7.13 (d, 1 H, NH), 7.31 (s, 5 H, C_6H_5). This procedure is listed as method A in Tables I and II.

The *tert*-butyl ester, 3 (500 mg, 0.76 mmol), was dissolved in 10 mL of TFA–CH_2Cl_2 (1:1), and the solution was allowed to stand at ambient temperature for 30 min. The solvent was then removed in vacuo to yield a solid white product which was crystallized from CH_3OH–EtOAc to give 315 mg (70%) of 4: mp 220–222 °C; TLC R_f = 0.50 (system A); ^{1}H NMR ($CDCl_3$) same as 3 except that the *tert*-butyl protons are absent and the β-CH_2 protons are a less well-defined multiplet centered at 2.79 ppm. This procedure is listed as method B in Table II.

2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[(aspartylleucyl-threoninamid)-4.1-yl]-2-deoxy-β-D-glucopyranosylamine (6). A mixture of the *N*-Cbz derivative, 5 (240 mg, 0.29 mmol, prepared from 4 and the TFA salt of Leu-Thr-NH_2 using DCC-HOBt (method A), see Table II), in CH_3OH (25 mL), HOAc (1 mL), and H_2O (4 mL) was subjected to hydrogenation over Pd–C (30 mg) for a period of 15 h. Upon completion of the reaction, the catalyst was removed by filtration, and the filtrate was concentrated in vacuo to give a solid white product which was crystallized from CH_3OH–EtOAc to give 158 mg (79%) of 6: mp 206–209 °C; TLC R_f = 0.16 (system A); ^{1}H NMR ($CDCl_3$–CD_3OD) δ 0.95, 0.99 (6 H, 2 d, Leu-$(CH_3)_2$), 1.18 (d, 3 H, Thr-CH_3), 1.64–1.75 (m, 3 H, Leu-CH_2 and CH), 1.92, 2.01, 2.04, 2.07 (4 s, 12 H, 4 CH_3CO), 2.49–2.77 (2 sets of d, 2 H, Asn-β-CH_2), 3.65–5.21 (sets of m, 8 H, 1 H for αCH Asn, 7 H for sugar); mass spectrum (FAB) (rel intensity) 675 (M + 1, 100), 673 (M - 1, 100). This procedure is listed as method C in Table II.

2-Acetamido-1-*N*-[(*N'*-benzoylaspartylleucylthreonin-amid)-4.1-yl]-2-deoxy-β-D-glucopyranosylamine (1). The free amine 6 (140 mg, 0.21 mmol) was dissolved in dioxane (10 mL) and Et_3N (41 μL, 0.30 mmol). To the resulting solution at ice-bath temperature was added 84 mg (0.37 mmol) of benzoic anhydride, and the reaction solution was allowed to stir for 1 h at ice-bath temperature and for an additional 1 h at ambient temperature. The solvent was removed in vacuo, and the resulting residue was dissolved in 50% aqueous THF (25 mL) and mixed with Dowex 50W-X8 (H^+), the supernatant was decanted off, and the resin was washed with additional 50% aqueous THF. Concentration of the combined aqueous THF solutions yielded a solid white product which was crystallized from THF–H_2O to give 110 mg (74%) of 7: mp 279–281 °C; TLC R_f = 0.59 (system A); ^{1}H NMR and ^{13}C NMR, see Table III and text; mass spectrum (FAB) (rel intensity) 779 (M + 1, 14), 777 (M - 1, 100). Anal. Calcd for $C_{35}H_{50}N_6O_{14}$: C, 53.97; H, 6.47; N, 10.79. Found: C, 53.76; H, 6.52; N, 10.66. This procedure is listed as method D in Tables I and II.

Peracetylated product (7) (50 mg, 0.06 mmol) was dissolved in MeOH (10 mL), H_2O (3 mL), and Et_3N (200 μL, 1.43 mmol). The resulting solution was allowed to stir for 8 h at room temperature. The solution was mixed with Dowex 50W-X8 (H^+), the supernatant was decanted off, and the resin was washed with H_2O (10 mL). Concentration of the combined aqueous solution yielded 35 mg (85%) of crude product which was crystallized two times from MeOH–H_2O to give 27 mg (66%) of 1 as a glassy white solid: mp 264–266 °C; TLC R_f = 0.33 (system A); ^{1}H NMR and ^{13}C NMR, see Table III and text; HR mass spectrum calcd for $C_{29}H_{45}N_6O_{11}$ (MH^+) *m/e* 653.3146, obsd *m/e* 653.3147. This procedure is listed as method E in Table II.

NMR Spectroscopy. (a) 2D heteronuclear-correlated NMR spectra: C-H shift correlation with broadband proton homonuclear decoupling[27] was done on a General Electric GN 500-NB spectrometer operating at 11.8 Ts (500.1 MHz for ^{1}H, 125.7 MHz for ^{13}C). The delays before and after the last 90° (^{13}C) mixing pulse were optimized with $J_{C,H}$ = 140 Hz. The spectra were recorded with spectral windows of 8474 Hz with 2048 data points for ^{13}C and 2906 Hz with 256 data points for ^{1}H. A total of 256 experiments were acquired each with ca. 300 transients and a relaxation delay of ca. 2 s. Glycopeptides were dissolved in D_2O.

(b) 2D homonuclear-correlated NMR spectra: Hypercomplex COSY[28] was done on GN 500-NB spectrometer with the same sample solution as in (a). Spectra were recorded with a spectral width of 6535 Hz and a 512 × 1024 data matrix was used. Chemical shifts were referenced to Me_4Si. Glycopeptides were dissolved in a mixture of 95% H_2O and 5% D_2O.

Growth of Yeast. *Saccharomyces cerevisiae* (S-288C) were grown in 1-L batches of medium containing 10 g of yeast extract, 20 g of bactopeptone, and 20 g of glucose (rich medium) in 2-L Erlenmeyer flasks at 27 °C in a rotary shaker (225 rpm) and were collected in mid-log phase growth as described by Trimble et al.[29]

Preparation of Yeast Microsomes (P_{40}). Yeast microsomes were isolated from the pellet obtained at 40000*g* by differential centrifugation of a yeast homogenate essentially as described by Trimble et al.,[29] with the exception that a Bead-Beater (Biospec

Table III. ^{1}H and ^{13}C NMR Assignments for 1 and 2

^{1}H, ppm	^{13}C, ppm	assignment (1)	$J_{H,H}$, Hz
0.84	20.44	Leu-CH_3	5.75
0.87	22.31	Leu-CH_3	5.79
1.18	18.86	Thr-CH_3	6.45
1.62	24.45	Leu-γCH	m
1.66	39.52	Leu-βCH_2	m
1.68	21.78	NHCOCH_3	s
2.86	36.50	Asn-βCH_2	−15.81 ($J_{\beta,\beta'}$), 5.59 ($J_{\beta,\alpha}$) −15.82 ($J_{\beta',\beta}$), 8.64 ($J_{\beta',\alpha}$)
3.44	69.56	GlcNAc-4CH	8.81 ($J_{4,5}$), 9.70 ($J_{4,3}$)
3.49	77.66	GlcNAc-5CH	m
3.57	74.37	GlcNAc-3CH	9.35 ($J_{3,4}$), 9.35 ($J_{3,2}$)
3.86	60.62	GlcNAc-6CHa	−12.36 ($J_{Ha,Hb}$), 1.77 ($J_{Ha,5}$)
3.71	60.62	GlcNAc-6CHb	−12.36 ($J_{Hb,Ha}$), 4.8 ($J_{Hb,5}$)
3.78	54.31	GlcNAc-2CH	9.94 ($J_{2,3}$), 9.90 ($J_{2,1}$)
4.26	67.07	Thr-βCH	m
4.30	58.83	Thr-αCH	4.04 ($J_{\alpha,\beta}$)
4.41	52.94	Leu-αCH	3.92 ($J_{\alpha,\beta}$), 10.37 ($J_{\alpha,\beta'}$)
4.94	50.73	Asn-αCH	5.57 ($J_{\alpha,\beta}$), 8.47 ($J_{\alpha,\beta'}$)
5.04	78.32	GlcNAc-1CH	9.74 ($J_{1,2}$)
8.85	–	AsnNH	7.65
8.44	–	LeuNH	6.86
8.08	–	ThrNH	7.39
7.55, 7.20	–	$CONH_2$	–
8.69	–	CONHGlcNAc	8.94
8.10	–	NHAc	9.36
7.51–7.76	–	aryl	m

^{1}H, ppm	^{13}C, ppm	assignment (2)	$J_{H,H}$, Hz
0.58	20.24	Leu-CH_3	6.53
0.71	22.41	Leu-CH_3	6.59
1.18	18.96	Thr-CH_3	6.36
1.48	24.35	Leu-γCH	m
1.64	39.75	Leu-βCH_2	m
1.65	21.75	NHCOCH_3	s
2.94	36.07	Asn-βCH_2	−14.72 ($J_{\beta,\beta'}$), 5.39 ($J_{\beta,\alpha}$) −14.72 ($J_{\beta',\beta}$), 9.40 ($J_{\beta',\alpha}$)
3.46	69.66	GlcNAc-4CH	9.33 ($J_{4,3}$), 9.33 ($J_{4,5}$)
3.51	77.66	GlcNAc-5CH	m
3.58	74.30	GlcNAc-3CH	9.52 ($J_{3,2}$), 9.52 ($J_{3,4}$)
3.91	60.55	GlcNAc-6CHa	−12.31 ($J_{Ha,Hb}$), 1.75 ($J_{Ha,5}$)
3.76	60.55	GlcNAc-6CHb	−12.36 ($J_{Hb,Ha}$), 5.11 ($J_{Hb,5}$)
3.81	53.83	GlcNAc-2CH	9.97 ($J_{2,1}$), 9.97 ($J_{2,3}$)
4.25	67.06	Thr-βCH	m
4.31	58.80	Thr-αCH	3.97 ($J_{\alpha,\beta}$)
4.34	52.75	Leu-αCH	4.24 ($J_{\alpha,\beta}$), 11.04 ($J_{\alpha,\beta'}$)
4.75	51.66	Asn-αCH	5.40 ($J_{\alpha,\beta}$), 9.32 ($J_{\alpha,\beta'}$)
5.02	78.97	GlcNAc-1CH	9.81 ($J_{1,2}$)
8.88	–	AsnNH	7.64
8.48	–	LeuNH	6.93
8.08	–	ThrNH	6.70
7.49, 7.15	–	$CONH_2$	–
8.55	–	CONHGlcNAc	8.66
8.06	–	NHAc	9.05
7.54–7.82	–	aryl	m

(27) Bax, A. *J. Magn. Reson.* **1983**, *53*, 517–520.

(28) States, D.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286–292.

(29) Trimble, R. B.; Maley, F.; Tarentino, A. L. *J. Biol. Chem.* **1980**, *255*, 10232–10238.

Products, Model 909) filled to half capacity with 0.5-mm glass beads was used for homogenization. The homogenizing vessel was cooled in a NaCl–ice bath for 1 min between five 1-min pulses with the Bead-Beater. All other procedures were as described.[29] Microsomes were stored at −80 °C as ca. 30-μL beads formed by dropwise addition of the microsomal preparation into liquid nitrogen. The P_{40} protein concentration was determined by the method of Bradford[30] using bovine γ-globulin as standard after first solubilizing the microsomes with 0.1 N NaOH at ambient temperature for 30 min.

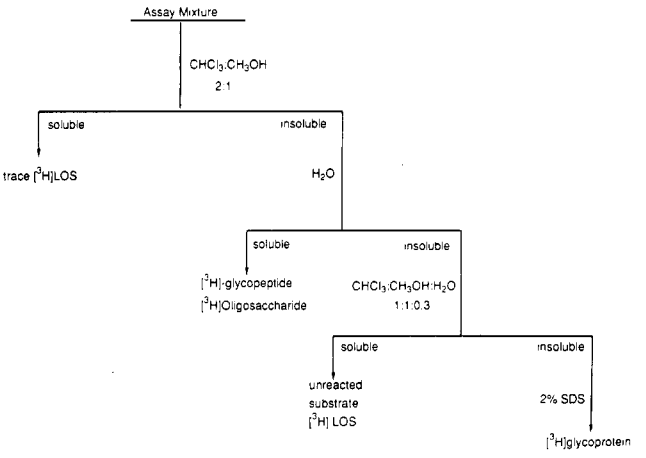
In Vitro Biosynthesis of ^{3}H-Labeled Lipid Oligosaccharide ([^{3}H]LOS). [^{3}H]LOS was prepared biosynthetically using exogenous nucleotide sugars (UDP-GlcNAc, GDP-Man, and UDP-Glc) and endogenous lipid acceptor from the P_{40} fraction of yeast essentially as described by Trimble et al.[29] Incorporation of [^{3}H]mannose from GDP-[^{3}H]Man was performed in a volume of 160 μL containing: 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 1 mM UDP-GlcNAc, 100 μM UDP-Glc, and 12 μM GDP-[^{3}H]Man (500 mCi/mmol) and 1–3-mg yeast microsomal protein. After incubation for 10 min at 27 °C the reaction was terminated by the addition of 4.8 mL of cold $CHCl_3$–CH_3OH (2:1). Glycolipids were then extracted by the method of Lucas et al.[31] as follows. The reaction mixtures were left on ice for 30 min and then centrifuged for 10 min at 2000*g* at 4 °C. The supernatant was removed, the $CHCl_3$–CH_3OH (2:1) insoluble pellet was extracted a second time with 3 mL of cold $CHCl_3$–CH_3OH (2:1), and the supernatant was discarded. The pellet was then carefully dried with a stream of N_2 to remove any residual organic solvent. The residue was suspended in 3 mL of H_2O by sonication, and the mixture was centrifuged. The supernatant was discarded, and the H_2O-insoluble pellet was washed two more times with 3 mL of cold H_2O to remove unincorporated GDP-[^{3}H]Man. Lipid [^{3}H]oligosaccharide was then isolated by extraction of the H_2O-insoluble pellet with 3 mL of $CHCl_3$–CH_3OH–H_2O (1:1:0.3).

Labeling with [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc was performed using a modification of the method of Elting and Lennarz.[32] The procedure involved incubation of yeast microsomes with 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, and 10 μM UDP-[^{3}H]GlcNAc (500 mCi/mmol) for 10 min after which GDP-Man (500 μM) and UDP-Glc (100 μM) were added and incubation continued for 15 min. [^{3}H]Glycolipids were then isolated exactly as described above.

Yeast Oligosaccharyltransferase (OST) Assay. The standard yeast OST assay contained in a volume of 150 μL 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM DTT, 1% (v/v) Triton X-100, 4000–9000 dpm [^{3}H]Man- or [^{3}H]-GlcNAc-labeled LOS, and 1–3 mg of P_{40} protein. For measurement of peptide acceptor capacity, the appropriate peptide dissolved in Me_2SO was added to the assay mixture at the concentration indicated (final Me_2SO concentration 5%, v/v). The reaction mixture was incubated at ambient temperature with vigorous shaking (250 rpm) for 10 min, stopped by the addition of 3 mL cold $CHCl_3$–CH_3OH (2:1), and then left on ice for 30 min. The reaction products were separated by sequential extraction as follows (Scheme III): The $CHCl_3$–CH_3OH (2:1) mixture was centrifuged for 10 min at 2000*g* at 4 °C, the supernatant was carefully removed, and the pellet was then dried with a stream of N_2. The $CHCl_3$–CH_3OH-insoluble material was suspended in H_2O and sonicated briefly to disrupt the pellet, and the mixture was centrifuged. The superantant was discarded, and the H_2O-insoluble pellet was washed two more times with 3 mL of H_2O to remove [^{3}H]glycopeptide. Any unreacted substrate in the H_2O-insoluble pellet was removed by extraction with 2 mL of $CHCl_3$–CH_3OH–H_2O (1:1:0.3). ^{3}H-labeled endogenous microsomal glycoprotein was solubilized by boiling the remaining pellet with 2% (w/v) SDS for 10 min. Radioactivity was determined by counting an aliquot of each fraction in 10 mL of Bio Safe II (RPI) using liquid scintillation procedures and a Packard LS460 liquid scintillation counter.

***endo*-β-*N*-Acetylglucosaminidase-H (Endo H) Hydrolysis.** Endo H was used to remove the large terminal oligosaccharide

Scheme III. Separation of Products of the OST-Catalyzed Glycosylation Reaction



[$Glc_3Man_9(GlcNAc)$] from the biosynthetically prepared glycopeptide (Bz-Asn[$Glc_3Man_9(GlcNAc)_2$]-Leu-Thr-NH_2). Generally, the reaction mixture containing Endo H (38–40 units/mg protein) and the residue from the H_2O-soluble fraction (OST assay above) containing the [^{3}H]oligosaccharide peptide was incubated for 16 h at 37 °C. The reaction was carried out in a volume of 100 μL containing Endo H (200–500 milliunits/mL) and 100 mM Na citrate, pH 5.5. For characterization of the [^{3}H]Man-labeled oligosaccharide, the reaction was stopped by lowering the pH of the reaction mixture to 3.5 with 2 mL of 1 M NaAc/HOAc, pH 3.5. The reaction mixture was then dried in vacuo and taken up in 250 μL of 0.1 M HOAc, and the products were then separated by gel filtration chromatography on a Bio-Gel P-4 column as described below. Alternatively, for characterization of the [^{3}H]GlcNAc-labeled glycopeptide, the reaction was stopped by placing the Endo H reaction mixture on ice. The mixture was then diluted 4:1 with 25 μL of 5x Con A buffer (see below), equilibrated with 50 μL of concanavalin A-Sepharose for 30 min at 4 °C, and the products were separated by lectin affinity binding as described below.

Bio-Gel P-4 Chromatography. Glycopeptides and oligosaccharides were separated by gel filtration chromatography on a Bio-Gel P-4 (−400 mesh) column (1.6 × 68 cm) eluted with 0.1 M HOAc as the solvent. The column, characterized with oligosaccharide and monosaccharide markers described below, was developed at a flow rate of ca. 7 mL/h using a Haake Buchler polystatic pump, and 1.0-mL fractions were collected. Authentic oligosaccharides, $Glc_3[^{14}C]Man_9(GlcNAc)_2$ and $[^{14}C]Man_9(GlcNAc)_2$, and the monosaccharides GlcNAc and Man were used as column markers and eluted at 62, 69, 106, and 117 mL, respectively. Chemically synthesized glycotripeptides (**1** and **2**) eluted at 99–100 mL and were not resolved. Mannose was determined by the phenol–sulfuric acid method as described by Dubois et al.,[33] and *N*-acetylglucosamine was measured using the Morgan–Elson reaction.[34] Total recovery of [^{3}H]glycopeptide and [^{3}H]oligosaccharide in the eluent from the Bio-Gel P-4 column was consistently in the range of 75–85% of applied radioactivity. Less than 2% of the total applied ^{3}H radioactivity was detected in the fractions corresponding to mannose.

Concanavalin A Affinity Binding Experiments. Con A affinity chromatography was performed as described by Rathod et al.,[35] using Con A-Sepharose 4B in Pasteur pipet minicolumns. The resin (0.25 mL) was equilibrated with 1 M NaCl, 25 mM Tris/HCl, pH 7.5, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ (Con A buffer). Next, a solution containing ^{3}H-labeled oligosaccharide or [^{3}H]-glycopeptide in Con A buffer was mixed with the resin and allowed to equilibrate at 4 °C for 30 min. The resin was washed at 4 °C with 15 column volumes and Con A buffer. Bound material was

(30) Bradford, M. E. *Anal. Biochem.* **1976**, *72*, 248–254.
(31) Lucas, J. J.; Waechter, C. J.; Lennarz, W. J. *J. Biol. Chem.* **1975**, *250*, 1992–2002.
(32) Elting, J. J.; Lennarz, W. J. *Methods Enzymol.* **1982**, *83*, 408–415.
(33) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
(34) Dische, Z. *Methods Carbohydr. Chem.* **1962**, *1*, 507–512.
(35) Rathod, P. K.; Tashjian, A. H.; Abeles, R. H. *J. Biol. Chem.* **1986**, *261*, 6461–6469.

then eluted from the resin with 15 column volumes of 5% (w/v) α-methyl mannoside in Con A buffer at ambient temperature. Recovery of applied ^3H radioactivity from the Con A resin was ≥90%.

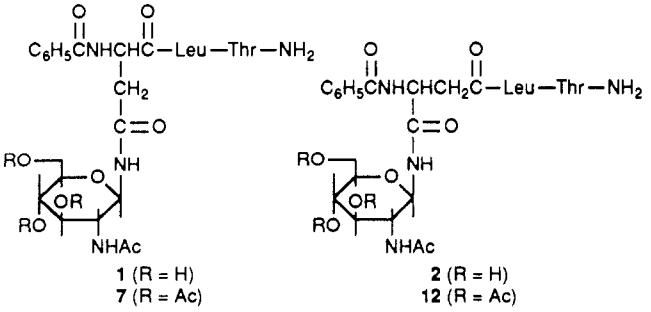
Alternatively, binding to Con A was performed in a batchwise procedure when sample volume had to be kept to a minimum. For example, the product of the Endo H hydrolysis mixture (125 μL in 1x Con A buffer) was mixed with 50 μL of the Con A–Sepharose resin at 4 °C in a 500-μL Eppendorf tube. The mixture was allowed to equilibrate for 30 min at 4 °C. The resin was centrifuged in an Eppendorf microfuge at a setting of 10 for 5 min. The supernatant ([^3H]glycopeptide) was removed and saved for reverse-phase HPLC. The resin was washed five additional times with 250 μL of Con A buffer to completely remove unbound material. Bound [^3H]oligosaccharide was eluted from the resin at ambient temperature with 3 × 250 μL of 5% (w/v) α-methyl mannoside in Con A buffer.

Reverse-Phase HPLC Separation of Isomeric Glycopeptides. Separation of synthetic glycopeptides 1 and 2 was achieved by reverse-phase HPLC using a Beckman/Altex system equipped with a Rheodyne Model 7125 injector, two Model 110 A Altex pumps, and an Altex Model 153 detector with a 254-nm filter on a Vydac C_{18} column (0.46 × 25 cm) eluted isocratically with 32.5% MeOH at a flow rate of 1.2 mL/min. Chemically synthesized glycopeptides, 2 and 1, eluted at 12.0 and 14.2 min, respectively with the column at ambient temperature. Analysis of ^3H-labeled biosynthetic glycopeptide involved the collection of 0.4-mL fractions. Radioactivity was determined by liquid scintillation counting with 10 mL of Bio-Safe II (RPI). Recovery of applied ^3H-radioactivity from the Vydac C_{18} column was ≥80%.

Chromatographic Methods. TLC analysis of synthetic (glyco)peptides was done on silica gel 60F-254 with a 0.2 mm layer thickness using solvent system A (BuOH–HOAc–H_2O, 4:1:5 (v/v/v), upper layer). TLC of ^3H-labeled lipid intermediates was performed on cellulose plates developed in solvent B (BuOH–EtOAc–HOAc–H_2O, 8:6:5:8).[29] Radiographic bands were visualized on Kodak X-Omat AR film by autoradiography of the TLC plates after spraying with EN^{3}HANCE (DuPont NEN). Film images were increased by the use of intensifying screens (Kodak). GPD-[^3H]Man and UDP-[^3H]GlcNAc were characterized by chromatography on polyethyleneimine cellulose thin layers developed with solvent C (0.25 M LiCl).[36]

Results

Synthesis of Glycopeptides. There are several reported syntheses of glycosyl asparagine and related compounds in the literature.[37,38] However, as will be described in greater detail below, some of the synthetic methods found in the literature for the synthesis of glycosyl asparagine lack the regiospecificity which we desired in order to obtain pure regioisomers, 1 and 2, for use in the biochemical work described here, and for planned additional structural studies using 2D-NMR techniques.



Our initial approach to the synthesis of 1 and 2 involved the use of N-protected aspartic anhydrides. *N*-Cbz aspartic anhydride was used initially, and the reaction with cyclohexylamine was found to proceed as described[39] to provide exclusively the α-amide resulting from attack of the amine nucleophile at only one of the two anhydride carbonyls. Unfortunately, attempts to extend this chemistry to an amino sugar, 1-amino-*N*-acetylglucosamine (1-NH_2GlcNAc),[40] produced a mixture of isomers as indicated by TLC and NMR analyses. An alternative using *N*-phthaloyl (Pht) aspartic anhydride to direct nucleophilic attack to the β-carbonyl[41] is not appropriate for this synthesis since the deblocking conditions (N_2H_4, EtOH) are incompatible with the anticipated peracetylated glycosyl asparagine product. Our second approach involved the use of substituted oxazolidinones, in which the α-amino acid functions are tied up in the heterocyclic ring, thus leaving the β-carboxyl free for further transformations. The use of cyclohexylamine as a model amine nucleophile led to the expected β-amide, in agreement with literature reports.[26,42] Reaction of the free β-acid with peracetylated 1-NH_2GlcNAc using DCC–HOBt methodology resulted in a crude product which consisted predominantly of the desired β-amide. Unfortunately, removal of the Cbz protecting group proved to be sluggish with ca. 50% starting material remaining after a 5-h hydrogenation. The use of Boc-aspartic acid β-1,3-dithian-2-ylmethyl (DIM) ester for glycopeptide synthesis has been reported,[43] but again the conditions for removing the DIM group (aqueous base, pH 8.0) are incompatible with the peracetylated intermediate.

Our third, and successful, approach involved the use of selectively protected aspartic acid derivatives as outlined in Scheme II. *N*-Cbz-aspartic acid α- or β-*tert*-butyl esters were condensed with peracetylated 1-NH_2GlcNAc to provide the fully blocked glycosyl asparagine α-ester, 3, and β-ester, 8, which on treatment with TFA gave N-blocked glycosyl asparagines 4 and 9, respectively. Following coupling of 4 and 9 to leucylthreonylamide[25] to give the glycosyl tripeptides, 5 and 10, the N-terminal Cbz group was removed by hydrogenolysis to provide the free amines, 6 and 11. Benzoylation of the N-terminus led to the peracetylated glycopeptides, 7 and 12, which were deacetylated using Et_3N in aqueous MeOH to give the target glycopeptides, 1 and 2. The physical properties of various intermediates and the final products prepared via this synthetic route are given in Table II.

Extensive NMR experiments (^1H, ^{13}C, heteronuclear and homonuclear COSY) were carried out with 1, 2, 7, and 12. Selected ^1H NMR data for both of the isomeric peracetylated glycopeptides, 7 and 12, together with similar spectral data for glycopeptides 1 and 2, are shown in Figure 1. In all cases, the signals due to the methylene protons at the β-position of the Asn residue are isolated from those of all other protons. The diastereotopic β-methylene protons are observed as two doublets of doublets (δ 2.8–3.4) at various chemical shifts and coupling constants depending on the glycopeptide regiochemistry, i.e., 7 vs 12, 1 vs 2. These differences allow for clear distinction between the isomeric glycopeptides (1 and 7 vs 2 and 12). The α-methine proton also can act as a sensitive probe for distinguishing between the isomeric glycopeptides (see below). However, it is not possible to assign all ^1H and ^{13}C signals to a specific proton or carbon without the use of homonuclear and heteronuclear correlation spectroscopy

(36) Randerath, K.; Randerath, E. *J. Chromatogr.* **1964**, *16*, 111–125.
(37) Garg, H. G.; Jeanloz, R. W. *Adv. Carbohydr. Chem. Biochem.* **1985**, *43*, 135–201. Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1987**, 294–308.
(38) Yamamoto, A.; Miyashita, C.; Tsukamoto, H. *Chem. Pharm. Bull.* **1965**, *13*, 1041–1046.
(39) Tamura, M.; Nishizaki, H; Miyazaki, C.; Okai, H. *Bull. Chem. Soc. Jpn.* **1984**, *57*, 3167–3172.
(40) Tamura, M.; Okai, H. *Carbohydr. Res.* **1984**, *133*, 207–218 and references therein.
(41) King, F. E.; Kidd, A. A. *J. Chem. Soc.* **1951**, 2976–2978.
(42) Tanaka, M.; Yamashina, I. *Carbohydr. Res.* **1973**, *27*, 175–183.
(43) Waldmann, H.; Kunz, H. *J. Org. Chem.* **1988**, *53*, 4172–4175.

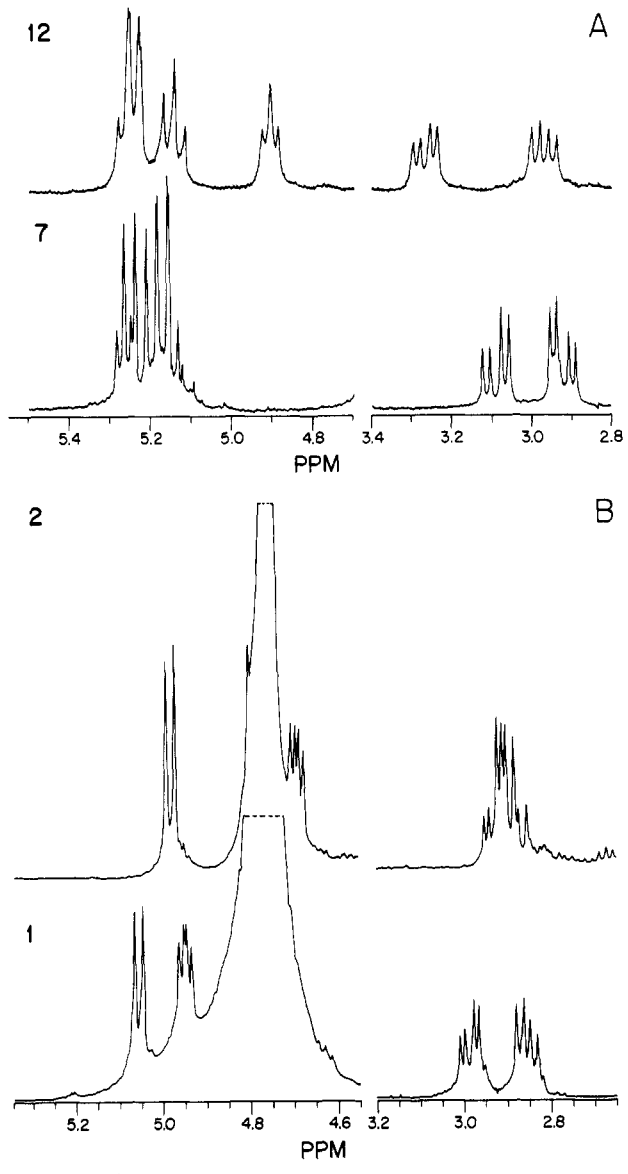


Figure 1. ^1H NMR spectra at 360 MHz of (A) peracetylated glycopeptides **12** (upper) and **7** (lower). (B) 500-MHz spectra of glycopeptides **2** (upper) and **1** (lower). Compounds **7** and **12** (ca. 12–13 mg) were dissolved in 0.5 mL of $CDCl_3$ containing ca. 20 μL of TFA. Compounds **1** and **2** (ca. 10–12 mg) were dissolved in 0.5 mL of D_2O to give final concentrations of 36 and 31 mM, respectively.

(COSY), an example of which is shown in Figure 2.

The assignments made for each ^1H and ^{13}C in **1** and **2** (Table III) revealed significant differences between identical positions in the isomeric glycopeptides. Thus, in the ^{13}C NMR spectra, the Asn β-CH_2 which occurs at δ 36.07 in **2** shifts downfield to δ 36.50 in **1**. Conversely, the Asn α-CH which occurs at δ 51.66 in **2** shifts upfield to δ 50.73 in **1**. In the ^1H NMR spectra (Figure 1B), the Asn β-CH_2 protons are compressed in the ABX spin system of **2** (δ 2.94) vs **1** (δ 2.86). The α-CH proton is observed at δ 4.75 in **2**, shifting downfield to δ 4.94 in **1**. Similar differences were observed in the peracetylated precursors, **7** and **12** (Figure 1A, tabular data not shown).

Characterization of Biosynthetic Compounds. To facilitate the study of the reaction catalyzed by yeast OST it was first necessary to prepare lipid [^3H]oligosaccharide ([^3H]LOS). GDP-[^3H]Man was utilized to obtain yeast [^3H]LOS according to the procedure described by Trimble et al.[29] and typically resulted in 3–8% incorporation of [^3H]Man from GDP-[^3H]Man into [^3H]LOS. UDP-[^3H]-GlcNAc was utilized as a LOS precursor according to the method described by Elting and Lennarz,[32] but the yield of [^3H]GlcNAc-labeled LOS was lower (0.1–0.75%) than that obtained with GDP-[^3H]Man conversion to [^3H]-Man-labeled LOS. The yield of [^3H]GlcNAc-labeled LOS was much lower than expected, even after allowing for the difference in stoichiometry between mannose and *N*-acetylglucosamine in the lipid oligosaccharide, $Glc_3Man_9GlcNAc_2$-PPDol. These low yields presumably reflect disruption of the highly organized multienzyme complex present in the intact endoplasmic reticulum[2] upon formation of the yeast microsomes employed in these biosynthetic experiments.

[^3H]LOS was characterized by two methods. First, thin-layer chromatography of intact [^3H]LOS on cellulose plates (system B) revealed four bands of radioactivity (R_f = 0.5–0.63) when examined by autoradiography. This area of the chromatogram corresponds to high-mannose containing lipid oligosaccharides with a dolichyl pyrophosphate *N,N'*-diacetylchitobiosyl core.[44] Second, to further characterize the [^3H]LOS produced in vitro with the yeast microsome preparation, 5000 dpm of the [^3H]-Man-LOS mixture was subjected to mild acid hydrolysis (0.1 N HCl in 50% *n*-propanol, 50 °C, 90 min) which is known to release oligosaccharides of the type Glc_xMan_y-$(GlcNAc)_2$.[29,31] Gel filtration on a Bio-Gel P-4 column of the aqueous fraction obtained after mild acid hydrolysis of [^3H]LOS revealed four peaks of radioactivity, with the two largest fractions corresponding to $Glc_3Man_9(GlcNAc)_2$ and $Man_9(GlcNAc)_2$ when compared to authentic ^{14}C-labeled oligosaccharides. The earliest eluting peak of radioactivity (59–64 mL), corresponding to the largest [^3H]mannose-containing oligosaccharide, represented approximately 50% of the total radioactivity applied to the column.

The same yeast microsomal (P_{40}) preparations that were utilized for the biosynthetic production of [^3H]LOS were also used as a source of OST activity. In this study, biosynthetic [^3H]mannose-containing LOS was utilized as the oligosaccharide donor to measure in vitro glycosylation by yeast OST of both endogenous microsomal yeast proteins and exogenous synthetic tripeptides. Three *N*-acyl-blocked tripeptides, *N*-acetyl-, *N*-benzoyl-, and *N*-octanoyl-Asn-Leu-Thr-NH_2, had been shown previously to be oligosaccharide acceptors using the OST activity from hen oviduct.[25] In the current work these peptides also were found to be yeast OST substrates. The isolation procedure used in these experiments allows for the rapid separation of glycosylated exogenous tripeptide from glycosylated, endogenous microsomal protein. The separation is based on the difference in solubility of [^3H]LOS, [^3H]glycopeptide, and [^3H]glycoprotein in $CHCl_3$–CH_3OH–H_2O (1:1:0.3), H_2O, and 2% SDS, respectively. [^3H]Glycopeptide formed was recovered in the H_2O-soluble fraction obtained during the assay mixture workup, while unreacted [^3H]LOS substrate was recovered in the $CHCl_3$–CH_3OH–H_2O (1:1:0.3) fraction. [^3H]Glycoprotein was measured as SDS-soluble radioactivity remaining after extraction of [^3H]LOS substrate and [^3H]glycopeptide (Scheme III).

The results from several yeast OST assays using synthetic tripeptides of the basic formula (*N*-acyl)-Asn-Leu-Thr-NH_2 as substrates for yeast OST are shown in Table IV. Yeast OST activity, in the absence of exogenous synthetic acceptor tripeptide, catalyzed the net transfer of 3050 dpm of [^3H]oligosaccharide from [^3H]LOS to endogenous yeast microsomal protein. This represents 38%

(44) Lehle, L.; Tanner, W. *Biochim. Biophys. Acta* **1978**, *539*, 218–229.

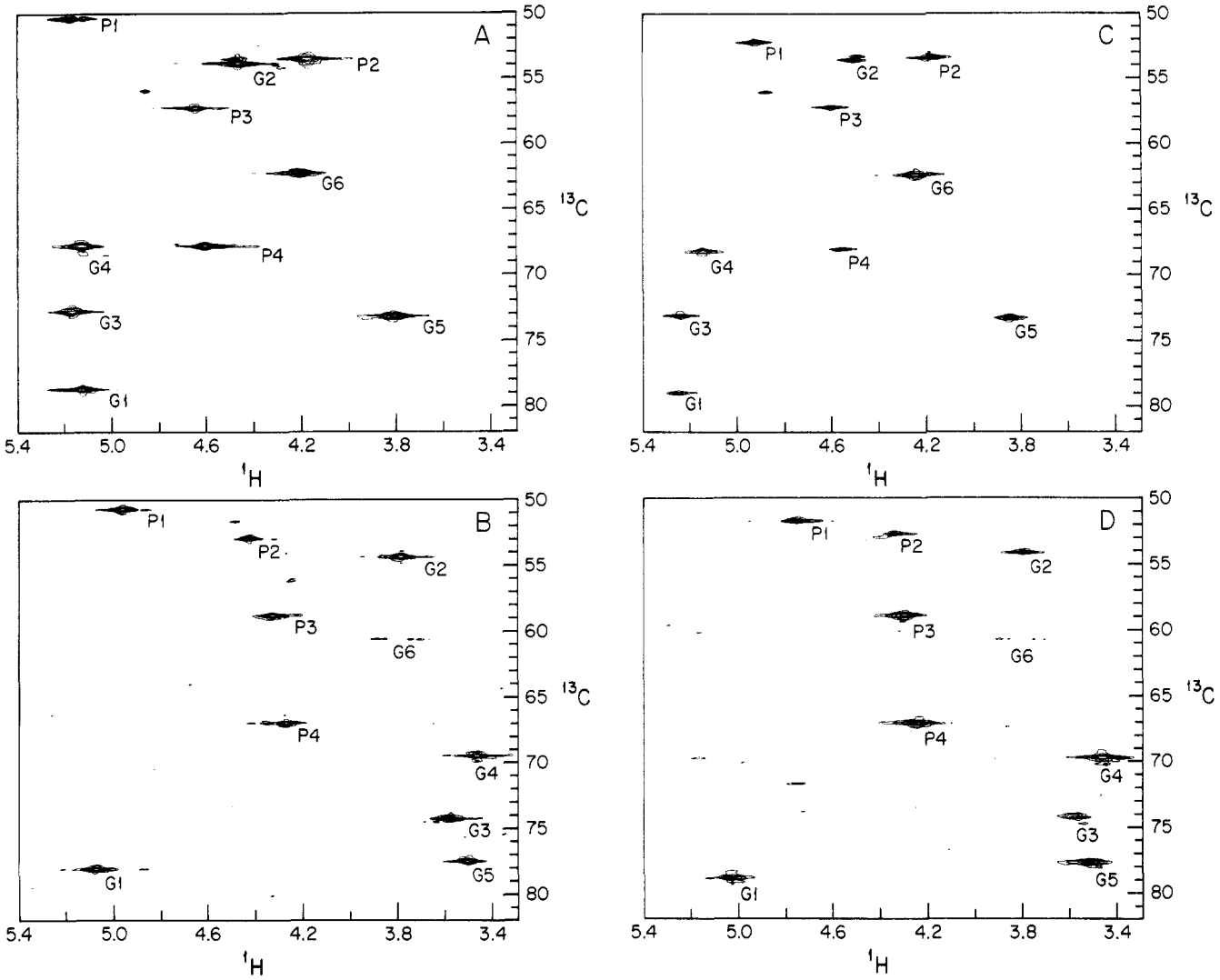


Figure 2. A portion of the heteronuclear carbon–proton COSY contour plots establishing connectivities in the CH region. (A) **7** in $CDCl_3$. (B) **1** in D_2O. (C) **12** in $CDCl_3$. (D) **2** in D_2O. Sample concentrations are identical to those given for Figure 1. Crosspeaks have been assigned (Table III) as follows: G1–G6, C–H of carbons 1–6 of GlcNAc; P1, Asn α-CH; P2, Leu α-CH; P3, Thr α-CH; P4, Thr β-CH.

of the substrate [^{3}H]LOS (8100 dpm) and 89% of the apparent total OST product (3440 dpm); 390 dpm (4.8% of [^{3}H]LOS, 11% of total OST product) was located in the H_2O fraction. The H_2O-soluble radioactivity in the absence of exogenous peptide acceptor was not characterized but is believed to be oligosaccharide resulting from hydrolysis of the pyrophosphate bond in LOS. As previously found in the hen oviduct system,[25] the N-blocking moiety affected the ability of the tripeptide to function as a substrate for OST. The data in Table IV show that the *N*-benzoyl-Asn-Leu-Thr-NH_2 tripeptide was the best oligosaccharide acceptor 4180 net dpm transferred, 52% of [^{3}H]LOS, 94% of total OST product) and the *N*-acetyl-blocked tripeptide the poorest acceptor (1580 net dpm, 20% of [^{3}H]LOS, 64% of total OST product). The *N*-octanoyl-blocked tripeptide was intermediate in acceptor capacity (2530 net dpm transferred (36% of [^{3}H]LOS, 96% of total OST product)), but due to the low solubility of this tripeptide in the assay mixture it was difficult to accurately quantitate its ability to act as an oligosaccharide acceptor.

The oligosaccharide acceptor capacity of the three tripeptides tested was related to their ability to act as alternate substrates and inhibit the yeast OST-catalyzed glycosylation of endogenous microsomal proteins. The data in Table IV show that at a concentration of 360 μM,

Table IV. A Comparison of Various Acceptor Peptides in the Yeast OST Assay[a]

sample	dpm[b] H_2O	dpm[b] SDS
control, no peptide	390 (11%)[d]	3050 (89%)
360 μM benzoyl-Asn-Leu-Thr-NH_2 (n =3)[c]	4570 (94%)	310 (6%)
360 μM octanoyl-Asn-Leu-Thr-NH_2 (n = 1)	2920 (96%)	120 (4%)
360 μM acetyl-Asn-Leu-Thr-NH_2 (n = 2)	1970 (64%)	1130 (36%)
360 μM acetyl-isoAsn-Leu-Thr-NH_2 (n = 2)	370 (14%)	2260 (86%)
360 μM benzoylHisoAsn-Leu-Thr-NH_2 (n = 3)	400 (12%)	2830 (88%)
360 μM benzoyl-isoAsn-Leu-Thr-NH_2, 120 μM benzoyl-Asn-Leu-Thr-NH_2[e] (n = 3)	3830 (88%)	520 (12%)

[a] Assay as described in the Experimental Section with 8100 dpm [^{3}H]Man-LOS and an incubation time of 10 min. [b] Zero-time control (H_2O = 490 dpm and SDS = 500 dpm) is subtracted from all raw data. [c] Number of determinations with variation of <13%. [d] Number in parentheses is OST product expressed as a percent of the total OST product (glycosylated peptide + glycosylated protein). [e] When expressed as the percent total OST product formed in H_2O-soluble glycopeptide, [benzoyl-Asn-Leu-Thr-NH_2] = 120 μM or 360 μM were identical (±10%).

the benzoyl- and octanoyl-blocked tripeptides were approximately equally effective as competitors for the endogenous proteins and reduced the glycosylation of endogenous microsomal protein (2% SDS-solubilized radioactivity) by 90% and 96%, respectively. The *N*-acetyl-blocked tripeptide was less effective as an alternate substrate. At a concentration of 360 μM this tripeptide reduced yeast microsomal [^{3}H]glycoprotein formation by 63%.

In order to assess the possibility that a cyclic imide might be a viable intermediate (Scheme IB) during the OST-catalyzed reaction, isoasparagine (isoAsn) was inserted in place of asparagine in the standard acceptor tripeptide, (N-RC(O)-Asn-Leu-Thr-NH_2) and tested for oligosaccharide acceptor capacity in the standard yeast OST assay. Deamidation of an isoAsn-containing peptide would lead to the same cyclic imide intermediate. The results of such an experiment are shown in Table IV. Radioactivity in the H_2O-soluble fraction in the presence of 360 μM acetyl- and benzoyl-blocked isoAsn-containing tripeptide was the same, within experimental error, as the control. Nor did the isoAsn-linked tripeptides inhibit the OST-catazlyed glycosylation of endogenous microsomal protein as determined by 2% SDS-solubilized radioactivity. Although the absolute amount of SDS-solubilized radioactivity was lower for the acetyl-blocked isoAsn-linked tripeptide than for the minus peptide control, the SDS-soluble radioactivity was nearly identical (86% vs 88%) with that measured in the absence of exogenous tripeptide when expressed as percent total OST product (H_2O + SDS fractions). The lower value for the SDS-soluble radioactivity in the acetyl-blocked isoAsn-containing tripeptide was attributed to a lower overall recovery of radioactivity for that particular series of assays. Variation in total recovery of ^{3}H radioactivity from assay to assay was approximately 10–15%. To further examine whether or not the isoAsn-containing benzoylated tripeptide was able to bind at the active site of yeast OST, 360 μM benzoyl-blocked isoAsn-containing peptide was added to the standard OST assay containing 120 μM benzoyl-Asn-Leu-Thr-NH_2. The results of this competitive assay are shown in Table IV. It was found that the distribution of [^{3}H]Man between glycopeptide and glycoprotein was very similar to that obtained at 120 μM benzoyl Asn-Leu-Thr-NH_2 alone.

Gel Filtration Characterization of ^{3}H-Labeled Glycopeptides. The first step in the characterization of the biosynthetic product of the yeast OST-catalyzed reaction was identification of the H_2O-soluble product obtained from the reaction between biosynthetic [^{3}H]LOS and the benzoyl-blocked tripeptide (*N*-benzoyl-Asn-Leu-Thr-NH_2). ^{3}H radioactivity in the residue obtained from the H_2O-soluble fraction from an OST-catalyzed reaction was examined by gel filtration chromatography both before and after treatment with Endo H. Endo H cleaves the β-1–4 glycosyl bond between the internal GlcNAc residues in high-mannose-containing N-linked oligosaccharides.[45] Hydrolysis with this endoglycosidase would lead to the release of the large oligosaccharide, Glc_3[^{3}H]Man_9(GlcNAc), from the initial oligosaccharide peptide formed (*N*-benzoyl-Asn[$(GlcNAc)_2$[^{3}H]Man_9Glc_3]-Leu-Thr-NH_2). The H_2O-soluble fraction obtained from a yeast OST-catalyzed reaction (Scheme III), containing the intact, fully glycosylated *N*-benzoyl-Asn[$(GlcNAc)_2Man_9Glc_3$]-Leu-Thr-NH_2 product, was taken to dryness in vacuo. The residue was taken up in 250 μL of 0.1 M HOAc and then

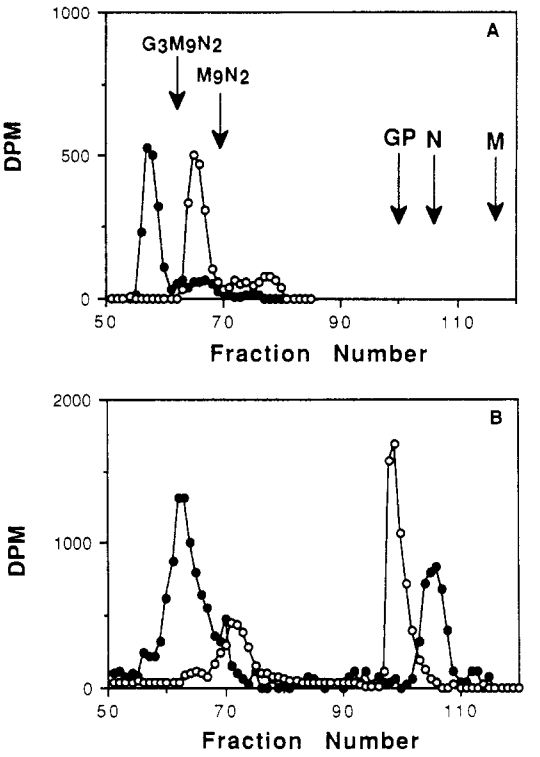


Figure 3. Gel filtration of glycopeptides and/or oligosaccharides before (●) and after (○) Endo H catalyzed hydrolysis of H_2O-soluble glycopeptide (see text for details). (A) [^{3}H]Man-labeled. (B) [^{3}H]GlcNAc-labeled. Fraction volume was 1 mL. G = Glc; M = Man; N = GlcNAc; GP = synthetic glycopeptides 1 and 2.

characterized by gel filtration on a Bio-Gel P-4 column which had previously been standardized with hetero-oligosaccharides and monosaccharides of known composition. As shown in Figure 3A, the major fraction (85%) of radioactivity eluted earlier than the oligosaccharide. $Glc_3Man_9(GlcNAc)_2$, which is known to be initially transferred by yeast OST.[46] In a parallel experiment, an equal amount of radioactivity from the H_2O-soluble fraction of an OST assay was dried in vacuo, the residue was taken up in 100 μL of 0.1 M sodium citrate, pH 5.5, and treated with 50 mU of Endo H for 18 h at 37 °C. The product of the Endo H catalyzed reaction was concentrated and then chromatographed on the Bio-Gel column. As shown in Figure 3A, the major product of the Endo H catalyzed reaction eluted later than the intact [^{3}H]Man-containing glycopeptide and earlier than the elution position of $Man_9(GlcNAc)_2$, consistent with the expected oligosaccharide product, $Glc_3Man_9(GlcNAc)$. Since the radioactivity in the oligosaccharide transferred to the synthetic peptide was located in the mannose residues, it was not possible to follow the fate of the small glycopeptide produced in the Endo H catalyzed reaction. In addition, it was not possible to locate the elution position of the biosynthetic *N*-benzoyl-Asn(GlcNAc)-Leu-Thr-NH_2 peptide on the Bio-Gel P-4 column using UV absorbance of the benzoyl chromophore.

This problem was addressed by substituting [^{3}H]-GlcNAc-labeled LOS as the oligosaccharide donor in the yeast OST-catalyzed reaction. Bio-Gel P-4 gel filtration chromatography of the [^{3}H]GlcNAc-containing oligosaccharide–peptide residue (H_2O-soluble fraction of the yeast OST-catalyzed reaction) before and after Endo H hydrolysis is shown in Figure 3B. Two major peaks of

(45) Tarentino, A. L.; Trimble, R. B.; Maley, F. *Methods Enzymol.* **1978**, *50*, 574–584.

(46) Trimble, R. B.; Byrd, J. C; Maley, F. *J. Biol. Chem.* **1980**, *255*, 11892–11895.

radioactivity were obtained prior to treatment with Endo H. In addition to the expected large [^{3}H]GlcNAc-containing glycopeptide product which eluted early (ca. 60 mL), there was also some free [^{3}H]GlcNAc in the residue from the H_2O-soluble fraction obtained from the OST reaction. Material eluting from the Bio-Gel columns at 57–70 mL (containing presumed [^{3}H]GlcNAc-labeled glycopeptide) was pooled and concentrated in vacuo, the residue was taken up in 100 μL of 0.1 M sodium citrate, pH 5.5, and then subjected to hydrolysis with Endo H for 18 h at 37 °C. The product of the hydrolysis reaction with Endo H was then concentrated to dryness, and the residue was taken up in 250 μL of 0.1 M HOAc and rechromatographed on the Bio-Gel column. The large [^{3}H]GlcNAc-oligosaccharide product of the Endo H reaction eluted later than the intact [^{3}H]GlcNAc-containing glycopeptide. The [^{3}H]GlcNAc-containing glycopeptide product of the Endo H reaction eluted at 99 mL but was poorly resolved from free GlcNAc. The distribution of ^{3}H radioactivity of the Endo H hydrolysis reaction products on the Bio-Gel P-4 column was very nearly 50:50 between the [^{3}H]GlcNAc-containing oligosaccharide and [^{3}H]GlcNAc-containing glycopeptide, as would be expected if the *N,N'*-diacetylchitobiosyl core of the oligosaccharide attached to the peptide was uniformly labeled with [^{3}H]GlcNAc. However, when the [^{3}H]GlcNAc-containing glycopeptide fraction from the second Bio-Gel P-4 separation was concentrated to dryness in vacuo, there was considerable degradation, as evidenced by heterogeneity on the C_{18} reverse-phase column (data not shown).

Affinity Binding of Oligosaccharides and Glycopeptides to Concanavalin A. To circumvent the degradation of the biosynthetic Bz-Asn(GlcNAc)-Leu-Thr-NH_2 noted above, an alternative approach was taken to separate the [^{3}H]GlcNAc-labeled oligosaccharide and glycopeptide products of the Endo H reaction using lectin affinity binding to Con A-Sepharose. This lectin has a specificity for high mannose oligosaccharides[47] and should not retain the [^{3}H]GlcNAc-labeled glycopeptide product of the Endo H reaction. Preliminary experiments demonstrated that both the [^{3}H]mannose-labeled oligosaccharides released by mild acid hydrolysis from [^{3}H]-Man-LOS and the large, intact [^{3}H]Man-containing glycopeptide product of the yeast OST reaction were retained by Con A–Sepharose (data not shown). A new batch of [^{3}H]GlcNAc-labeled glycopeptide was prepared using yeast OST and purified on the Bio-GEl column as described above, to give the desired product (1250 dpm), which was then subjected to digestion with Endo H. The products of the Endo H reaction (1070 dpm) were diluted with Con A buffer and then applied directly to Con A–Sepharose in a batch procedure. From three washes of the Con A–Sepharose, a total of 375 dpm (70% recovery) of glycopeptide was obtained. A total of 460 dpm (85% recovery) was obtained by eluting the bound material (oligosaccharide) with α-methylmannoside. The fraction containing unbound (glycopeptide) radioactivity was filtered through a 0.2-μm nylon filter and a portion (ca. 60%) used immediately for HPLC separation on a Vydac C_{18} reverse-phase column.

Reverse-Phase HPLC Separation of Isomeric Glycopeptides. In order to determine the structure of the products formed during the yeast OST-catalyzed glycosylation reaction, [^{3}H]GlcNAc-containing glycopeptide product purified from the Endo H reaction was subjected to reverse-phase HPLC using a C_{18} column for comparison

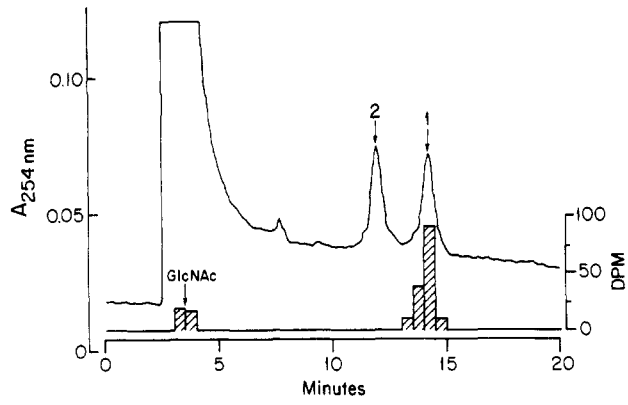


Figure 4. HPLC of synthetic glycopeptides 1 and 2 (solid line) and biosynthetic glycopeptide labeled with [^{3}H]GlcNAc (crosshatch). See text for details on isolation of the ^{3}H-labeled product of OST-catalyzed glycosylation.

with synthetic glycopeptides, 1 and 2. As shown in Figure 4 (solid line), this column allows compete resolution of the glycopeptides prepared by chemical synthesis. The [^{3}H]GlcNAc-containing fraction which was not retained by the Con A–Sepharose was applied directly to the reverse-phase column together with a mixture of the chemically synthesized isomeric glycopeptides and eluted with 32.5% MeOH. Results from the reverse-phase HPLC analysis of the OST reaction product are shown in Figure 4. The biosynthetic [^{3}H]GlcNAc-glycopeptide coeluted with the chemically synthesized glycopeptide 1. No radioactivity was detected in the HPLC fraction corresponding to the isoglycopeptide 2. The overall recovery of radioactivity was ca. 80% of applied ^{3}H.

Discussion

Little is known mechanistically about OST for the following reasons: Due to the location of the enzyme in the lipid environment of the endoplasmic reticulum it has been very difficult to purify the enzyme using classical protein purification techniques. In addition, the enzyme from all sources other than yeast has a very limited stability once removed from the lipid milieu of the rough endoplasmic reticulum.[4] Only limited success has been achieved with enzyme solubilization using nonionic detergents.[46,48–52] Recent evidence obtained with synthetic, photoaffinity peptide analogues has suggested the existence of two or more proteins in hen oviduct involved in peptide acceptor binding and oligosaccharide transfer.[53–56]

The present study has shown that in N-linked glycoproteins synthesized via the OST-catalyzed reaction the linkage between asparagine and *N*-acetylglucosamine is exclusively through the β-amido group of asparagine. This

(47) Blake, D. A.; Goldstein, I. J. *Methods Enzymol.* **1982**, *83*, 127–132.
(48) Das, R. C.; Heath, E. C. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3811–3815.
(49) Sharma, C. B.; Lehle, L.; Tanner, W. *Eur. J. Biochem.* **1981**, *116*, 101–108.
(50) Aubert, J. P.; Chiroutre, M.; Kerckaert, J. P.; Helbecque, N.; Loucheux-Lefebvre, M. H. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 1550–1559.
(51) Franc, J.-L.; Bouchilloux, S. *Biochem. Biophys. Acta* **1984**, *800*, 166–170.
(52) Chalifour, R. J.; Spiro, R. G. *J. Biol. Chem.* **1988**, *263*, 15673–15680.
(53) Geetha-Habib, M.; Noiva, R.; Kaplan, H. A.; Lennarz, W. J. *Cell* **1988**, *54*, 1053–1060.
(54) Kaplan, H. A.; Naider, F.; Lennarz, W. J. *J. Biol. Chem.* **1988**, *263*, 7814–7820.
(55) Welply, J. K.; Kaplan, H. A.; Shenbagamurthi, P.; Naider, F.; Lennarz, W. J. *Arch. Biochem. Biophys.* **1986**, *246*, 808–819.
(56) Welply, J. K.; Shenbagamurthi, P.; Naider, F.; Park, H. L.; Lennarz, W. J. *J. Biol. Chem.* **1985**, *260*, 6459–6465.

finding is based on direct HPLC comparison of two synthetic glycopeptides, 1 and 2, with a biosynthetic glycotripeptide prepared in vitro and isolated using a novel combination of well-defined purification steps. Formation of an alternative isopeptide linkage was ruled out based on the following criteria: (1) From an estimate of the amount of glycopeptide formed in vitro and purified to homogeneity, the level of isoglycopeptide formed, if any, is less than 5% of the total glycopeptide product. (2) The use of strong acid or base and high temperatures was eliminated in our isolation procedure. The harsh conditions used previously, 2 N HCl, 100 °C, 12–30 min, for removal of the oligosaccharide from the small glycopeptides generated by exhaustive proteolytic digestion[9,10] left open the possibility of chemical degradation and rearrangements of the primary OST product(s).

Use of synthetic tripeptides containing the obligatory acceptor sequon (Asn-Xaa-Thr/Ser) for the production of model glycopeptides in vitro rather than isolation of similar glycopeptides from purified glycoproteins, e.g., ovalbumin, facilitated the purification of the glycopeptide product of the OST-catalyzed reaction. The oligosaccharide acceptor capacity of these tripeptides has been shown to be as good as the same sequence of amino acids in proteins N-glycosylated in vivo or in vitro by OST.[25,35,56] Our data, obtained from measurements of oligosaccharide capacity with yeast OST activity, agrees well with results from similar experiments using the hen oviduct or thyroid OST and the same[25,56] or a similar (i.e., *N*-methylamides[35]) set of acceptor tripeptides. The fact that the isoAsn-linked tripeptides are neither substrates nor inhibitors of yeast OST activity indicates that these modified peptides do not bind (or bind very weakly) at the active site of the enzyme.

Synthesis of both isomers of the *N*-acetylglucosaminylasparagine linkage (1 and 2) was performed in a series of unambiguous chemical steps. Initial ^{1}H NMR experiments indicated that the α- and β-proton resonances of Asn and isoAsn were clearly distinguishable, and this difference was exploited to characterize intermediates in the synthesis of Bz-Asn(GlcNAc)-Leu-Thr-NH_2 (1) and Bz-isoAsn(GlcNAc)-Leu-Thr-NH_2 (2). Most synthetic intermediates were examined with either ^{1}H or a combination of ^{1}H and ^{13}C NMR in which the resonances from the α- and β-protons of asparagine were clearly identifiable. This allowed complete characterization of the α- and β-carbonyl substituents of the *N*-acetylglucosaminyl-asparagine bond at all stages of the synthetic route leading to formation of 1 and 2.

The combination of yeast OST-catalyzed in vitro formation of a biosynthetic glycopeptide, subsequent hydrolytic removal of the major portion of oligosaccharide ($Glc_3Man_9GlcNAc$) with Endo H, and a combination of gel filtration, affinity binding, and reverse-phase HPLC to analyze the biosynthetic product fragment represents a new approach to the characterization of the *N*-acetylglucosaminylasparagine bond. Use of the tripeptide substrate for OST eliminated the extensive proteolytic hydrolysis required for isolation of a similar glycopeptide obtained from glycoproteins (e.g., ovalbumin, α_1 acid glycoprotein)[38,57,58] and oligosaccharide hydrolysis with Endo H eliminated the possibly destructive acid hydrolysis step previously used to remove the oligosaccharide from the glycopeptide.[9,10] To our knowledge the only other report in the literature where the structure of biosynthetically derived AsnGlcNAc was determined by conditions not including strong acid hydrolysis (2 N HCl, 100 °C) was a report by Tarentino et al.[59] These authors first isolated $Asn(GlcNAc_2)Man_6$ from ribonuclease B by exhaustive proteolytic digestion which was followed by removal of five out of six mannose residues with α-mannosidase. The remaining mannose residue was removed by a periodate oxidation (Smith degradation) followed by $NaBH_4$ reduction. The final step was a digestion of $Asn(GlcNAc)_2$ with β-*N*-acetylglucosaminidase to generate AsnGlcNAc, which was shown to be identical with a chemically synthesized standard.

Although the data indicate that the yeast OST reaction mechanism does not likely proceed through a cyclic imide intermediate, this mechanism cannot be ruled out completely. It is possible that the cyclic imide, if formed, could open exclusively as a result of attack on the β-carbon by the nucleophilic amino sugar (Scheme IA) appropriately positioned in the active site of the enzyme. An alternative mechanism involving the β-hydroxyl group of Thr/Ser has been proposed by Bause.[60] This mechanism requires a general base at or near the active site of OST to remove the proton from the β-hydroxyl oxygen of Thr/Ser. The resulting strongly nucleophilic oxyanion must then abstract a proton from the β-carboxyamido nitrogen of Asn which would then be sufficiently nucleophilic to attack the C-1 of the terminal GlcNAc residue in the oligosaccharide donor. The requirement for a hydroxyl-containing amino acid (Ser/Thr) is confirmed in the peptide series studied in this manuscript. A tripeptide in which the hydroxyl group is removed, $BzAsnLeuAlaNH_2$, is neither a substrate for, nor an inhibitor of, the OST-catalyzed reaction (L. Schretzman and J. K. Coward, unpublished results). Other mechanisms involving formation of Asn* (Scheme IA) are currently under investigation in our laboratory using isotopically labeled peptide substrates.

Acknowledgment. The research was supported by a grant from the USPHS, GM30286. We thank Drs. Robert Trimble and Fred Ziegler of the New York State Department of Health for helpful advice and discussions, and for hospitality extended to J.K.C. during a sabbatical leave. Numerous discussions with Mr. Jung Lee were helpful in analyzing the NMR spectral data and in revising several drafts of this paper. We are grateful to Dr. Miriam Greenberg, Department of Biological Chemistry, The University of Michigan, for advice in areas of yeast biology and for use of her laboratory facilities in growing large amounts of yeast. We also thank Dr. Christopher Kojiro for help in obtaining the 2D-NMR spectra and Ms. Jane LeBlanc for preparation of the manuscript.

Registry No. 1, 129835-72-9; 2, 129835-73-0; 3, 129835-74-1; 4, 38877-33-7; 5, 129835-75-2; 6, 129835-76-3; 7, 129835-77-4; 8, 129835-78-5; 9, 38877-35-9; 10, 129835-79-6; 11, 129835-80-9; 12, 129835-81-0; OST, 75302-32-8; Z-Asp-OBu-*t*·DCHA, 17335-87-4; Z-Asp(OBu-*t*)-OH·DCHA, 23632-70-4; H-Leu-Thr-NH_2·TFA, 75671-57-7; BOC-Asn-Leu-Thr-NH_2, 88053-72-9; Z-isoAsn-Leu-Thr-NH_2, 129835-82-1; Ac-Asn-Leu-Thr-NH_2, 87980-75-4; PhCO-Asn-Leu-Thr-NH_2, 87980-77-6; $CH_3(CH_2)_6CO$-Asn-Leu-Thr-NH_2, 87980-76-5; Ac-isoAsn-Leu-Thr-NH_2, 129835-83-2; PhCO-isoAsn-Leu-Thr-NH_2, 129835-84-3; BOC-Asn-OH, 7536-55-2; Z-isoAsn, 23632-73-7; 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine, 4515-24-6; (*S*)-3-Cbz-5-oxo-4-oxazolidineacetic acid, 23632-66-8.

(57) Johansen, P. G.; Marshall, R. D.; Neuberger, A. *Biochem. J.* **1960**, *77*, 239–247.
(58) Wagh, P. V.; Bornstein, I.; Winzler, R. J. *J. Biol. Chem.* **1969**, *244*, 658–666.
(59) Tarentino, A.; Plummer, T. H., Jr.; Maley, F. *J. Biol. Chem.* **1970**, *245*, 4150–4157.
(60) Bause, E. *Biochem. Soc. Trans.* **1984**, *12*, 514–517.