Oligosaccharyltransferase: Synthesis and Use of Deuterium-Labeled Peptide Substrates as Mechanistic Probes†

Jung Lee and James K. Coward*

Interdepartmental Program in Medicinal Chemistry, College of Pharmacy, and Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

Received December 29, 1992; Revised Manuscript Received April 8, 1993

ABSTRACT: Chemically synthesized peptide and lipid disaccharide substrates have been used to investigate two possible mechanisms for enzyme-catalyzed N-glycosylation. Using microsomal oligosaccharyltransferase isolated from yeast, the fate of the deuterium in three stereospecifically deuterated peptides has been investigated. In all three cases, the deuterium present in the peptide substrate was retained in the glycopeptide product, as shown clearly by ^{1}H NMR spectral comparisons. The lack of deuterium wash-out during catalysis provides strong evidence against either enol lactone or ketene formation as an intermediate in this reaction.

The biosynthesis of N-linked glycoproteins is unique in eukaryotes, providing the major class of cellular glycoproteins including such important components as hormones, immunoglobulins, and viral coat proteins. Although N-linked glycoprotein biosynthesis has been studied in detail during the past 2 decades, the regulation and mechanism of reactions catalyzed by the biosynthetic enzymes have been studied in less detail due to the difficulty of purifying the membrane-bound enzymes (Kukuruzinska et al., 1987; Kornfeld & Kornfeld, 1985; Lennarz, 1987). Oligosaccharyltransferase (OST,[1] EC 2.4.1.119) catalyzes a central reaction in the biosynthesis of N-linked glycoproteins as it transfers the core oligosaccharide of a lipid-linked oligosaccharide to a growing peptide in a cotranslational process occurring in the endoplasmic reticulum. Lennarz and co-workers have shown that protein glycosylation occurs only after at least 32 amino acids have been assembled into a growing polypeptide in the ribosomal complex (Glabe et al., 1980). Recently, OST has been purified from canine pancreas and shown to be a multiprotein complex which includes ribophorins I and II and a 48-kDa protein (Kelleher et al., 1992). The reaction catalyzed by OST is shown in Figure 1.

The substrate specificity for OST-catalyzed peptide glycosylation is very intriguing. A sequence containing the three amino acid peptide sequon, Asn-X-Thr/Ser, where X can be any natural amino acid except proline, is necessary but not sufficient for glycosylation to occur. It has been shown that a tripeptide, blocked at both the N- and C-termini and containing the required sequon, can be glycosylated by OST *in vitro* (Welply et al., 1983; Rathod et al., 1986; Clark et al., 1990). Imperiali and co-workers (1991, 1992a,b) have suggested that a secondary structural motif, involving interactions between the asparagine side-chain carboxamide and the backbone amide/side-chain hydroxyl of the threonine, might be critical for the distinct substrate activity of certain peptides. We have recently shown that a chemically synthesized lipid-linked disaccharide can substitute for the more complex "core" lipid-linked oligosaccharide (Figure 1) as the second substrate. The product of the enzyme-catalyzed reaction was verified to be a disaccharide-containing glycopeptide by comparison with material prepared by an independent chemical synthesis (Lee & Coward, 1992). Thus, it is possible to use synthetically accessible substrates (peptides and lipid disaccharide) in mechanistic studies of the OST-catalyzed reaction.

OST catalyzes an interesting chemical reaction. In the enzyme-catalyzed reaction, a nonnucleophilic carboxamido nitrogen of an asparagine residue displaces dolichyl pyrophosphate from the lipid oligosaccharide (LOS) in an S_N2 fashion. Amides are very weak acids (pK_a = ca. 15). Thus, in nonenzymatic nucleophilic displacement reactions, an amide must be converted to its conjugate base using a strong base such as NaH. The conjugate base then can displace a leaving group on an electrophilic carbon in an S_N2 fashion. Similarly, the carboxamide nitrogen of the OST peptide substrate must be activated in some way in order to displace the dolichyl pyrophosphate of LOS. The OST-catalyzed reaction is very similar to many glutamine-dependent amino transferases (Buchanan, 1973; Schendel et al., 1988). The reactions catalyzed by OST and glutamine-dependent enzymes are similar in that the amide functionalities of both asparagine and glutamine are involved in the enzymatic reactions. However, they differ in a major way since the parent amino acid, glutamate, is not incoporated in the aminated product in the latter case, whereas the aspartyl moiety is incorporated in the glycopeptide product of the OST-catalyzed reaction (Figure 1).

Since the two OST substrates are not inherently reactive molecules, it is of interest to investigate various mechanisms of substrate activation. One possibility is a nucleophilic activation which would make the carboxamido nitrogen of the peptide substrate a reactive nucleophile. In an nucleophilic activation mechanism, the resonance stabilization of an amide can be broken by various modifications at the amide, leading to an activated aspartate (Asp*) and the generation of NH_3

† This research was supported by a National Research Service Award (T32 GM07767) to J.L. and by a fellowship to J.L. from the Program in Protein Structure and Design, University of Michigan.

[1] Abbreviations: OST, oligosaccharyltransferase; LOS, lipid oligosaccharide; LDS, lipid disaccharide; Boc, *tert*-butoxycarbonyl; PLP, pyridoxal phosphate; GlcNAc, *N*-acetyl-D-glucosamine; Man, mannose; Glc, glucose; AAT, aspartic aminotransferase; pCMB, *p*-(chloromercuri)-benzoic acid; diH_2O, deionized H_2O.

0006-2960/93/0432-6794$04.00/0

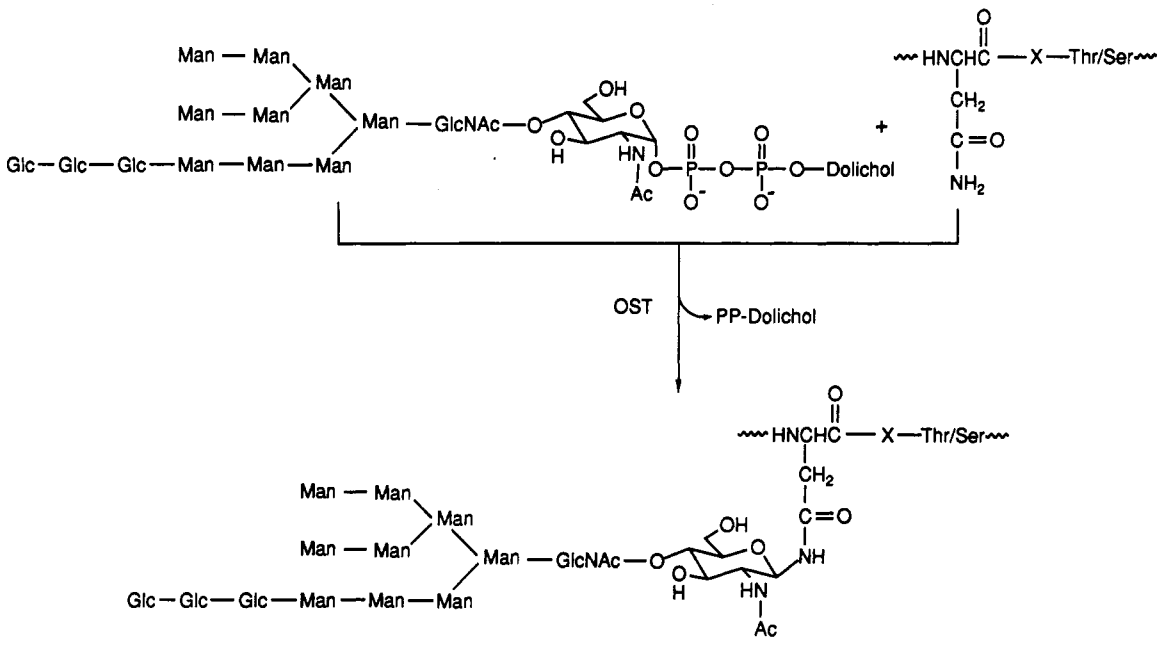
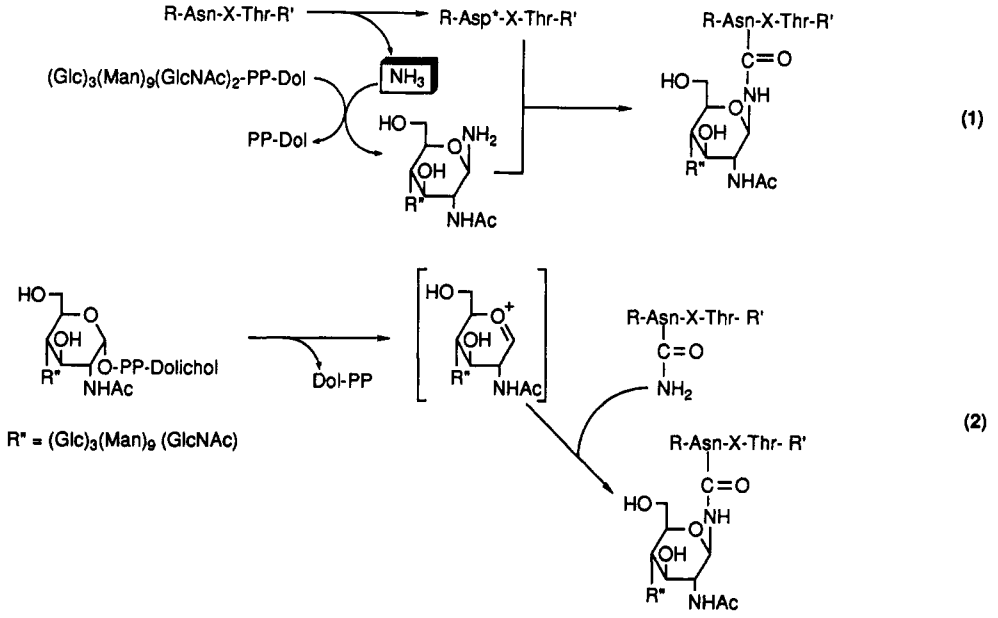


FIGURE 1: Reaction catalyzed by OST showing the core lipid oligosaccharide and the growing peptide with the sequon Asn-X-Thr/Ser.



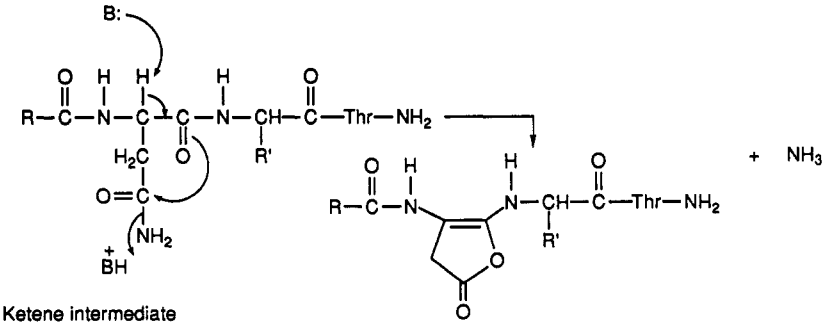
at the active site (eq 1). At the extreme, attack by free NH_3 on LOS leads to an amino sugar derivative which is then able to react with Asp* to form the new bond between the peptide/protein and carbohydrate.[2] This mechanism, although having considerable precedent in the glutamine-dependent reactions noted above, represents one end of the C–N bond breaking spectrum. Mechanisms which involve the loss of amide resonance stabilization without breaking of the C–N bond include attack by an enzyme nucleophile (e.g., Scheme Id) and subsequent attack on LOS by the amino function of an enzyme-bound tetrahedral intermediate. Alternatively, simple substrate binding induced rotation about the C–N bond would result in decreased delocalization of the nitrogen electrons into the carbonyl group, thereby rendering the amide nitrogen more nucleophilic. The latter two mechanistic possibilities are not addressed in the research described herein.

Alternatively, an electrophilic activation of LOS could occur in the OST-catalyzed reaction (eq 2). Similar to many glycosyl transferases (Kirby, 1987; Sinnott, 1987), participation of the lone pair electrons on the oxygen in the sugar ring could facilitate the expulsion of dolichyl pyrophosphate with concomitant formation of an oxocarbocation-containing oligosaccharide Generation of this electrophilic cationic species would facilitate attack by the asparagine amide to form the glycopeptide. However, it should be noted that solution alkylation of amides by such reactive electrophiles as $Me_3O^+BF_4^-$ results in O-alkylation (March, 1985). The OST protein could presumably enforce N-alkylation by proper positioning of the two substrates. Since this type of mechanism involves a rehybridization at C-1 from sp^3 in LOS to sp^2 in the carbocation, the best probe of this possible mechanism

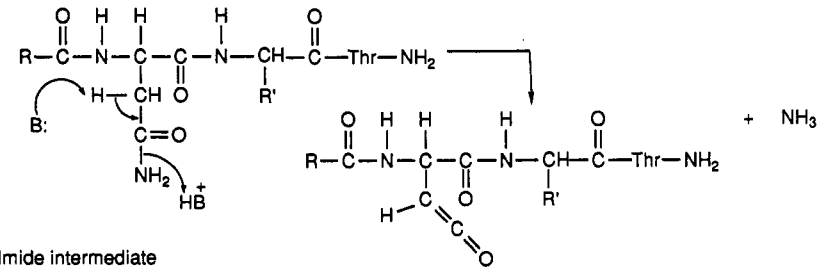
[2] By analogy with glutamine-dependent amino transferases, the nucleophilic activation mechanism (eq 1) suggests that exogenous $^{15}NH_3$ could be used in combination with [β-^{13}C]asparagine in the peptide substrate. Incorporation of exogenous $^{15}NH_3$ would lead to $^{13}C(O)$-^{15}NH in the glycopeptide product, which would be detected by ^{13}C–^{15}N coupling in the appropriate NMR spectra. Similary, use of double labeled ($^{13}C(O)^{15}NH_2$) asparagine in the peptide substrate and unlabeled NH_3 would allow us to determine the active-site accessibility to exogenous NH_3 in a complementary manner. Experiments of this type are currently in progress.

Scheme I: Possible Intermediates (R-Asp*-X-Thr-NH_2) for Nucleophilic Activation

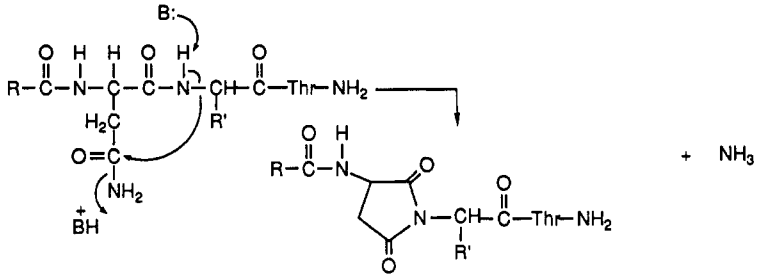
a. Enol Lactone intermediate



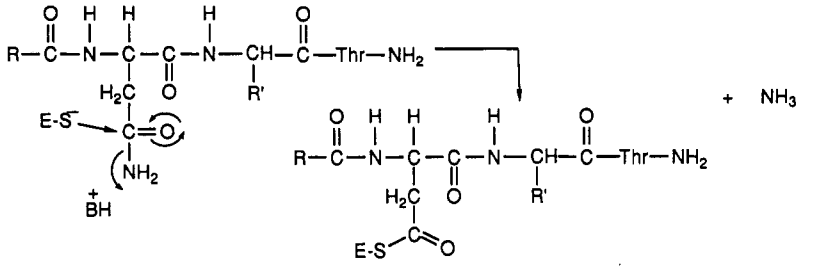
b. Ketene intermediate



c. Imide intermediate



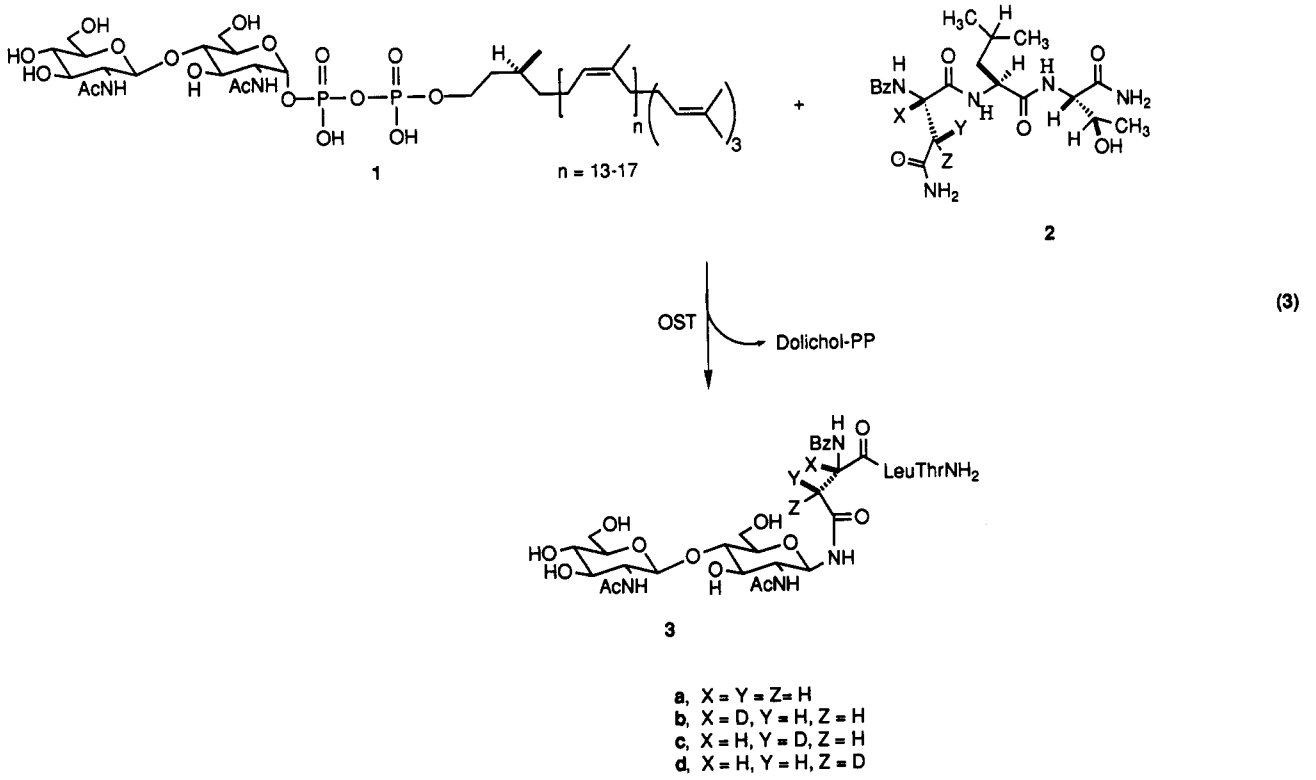
d. Thiol ester intermediate



would be to determine whether there is a kinetic isotope effect on the OST-catalyzed reaction (Sinnott, 1987). However, kinetic experiments of this type require homogeneous OST enzyme, which was not available until very recently (Kelleher et al., 1992).

Of the four proposed mechanisms shown in Scheme I, we have targeted the two possible nucleophilic activation mechanisms involving enol lactone (Ia) and ketene (Ib) intermediates. Enol lactones are well-studied intermediates in organic chemistry and biochemistry, e.g., β-ketoadipate pathway of microbial aromatic amino acid catabolism (Stanier & Ornston, 1973). Ketenes have been proposed as intermediates in both the nonenzymatic (Holmquist & Bruice, 1969) and enzyme-catalyzed (Maycock et al., 1975) reactions of esters via an E1cB mechanism. β-Fluoroasparagine-containing peptides are very poor OST substrates (Rathod et al., 1986; S. Banerjee, J. Lee, and J. K. Coward, unpublished data). One interpretation of these results suggests a possible role for one of the prochiral β-CH_2 hydrogens of asparagine (Scheme Ib). Fluoroglutamate-containing peptides have been used to study the mechanisms of γ-carboxyglutamate synthase, an enzyme which effects the abstraction of an apparently nonacidic proton during catalysis (Dubois et al., 1983, 1991). The imide intermediate (Scheme Ic) is considered unlikely on the basis of the regiospecificity of OST-catalyzed glycosylation (Clark et al., 1990) vs a lack of regiospecificity in asparagine deamidation (Stephenson & Clarke, 1989), a nonenzymatic reaction known to proceed through an imide intermediate. In addition, isoasparagine-containing peptides, which could form the same imide intermediate shown in Scheme Ic, are neither substrates nor inhibitors of the OST-catalyzed reaction (Clark et al., 1990). The thiol ester intermediate (Scheme Id) is reasonable, but some thiol reagents (ICH_2CO_2H, ICH_2CONH_2) have no effect on OST activity, whereas others (pCMB) inhibit both the microsomal and solubilized enzyme in a time-dependent manner (Y-L. Liu, L. Schretzman, and J. K. Coward, unpublished data).

By stereospecific isotopic labeling at either the α or β hydrogens of the asparagine residue of a tripeptide substrate and analysis for possible isotopic wash-out in the enzyme-catalyzed reaction, one can study the two mechanistic possibilities of interest (Scheme Ia,b). If the OST-catalyzed



(3)

reaction proceeds by either one of the two postulated mechanisms, the isotope in the tripeptide should be exchanged with a proton derived from solvent during the enzyme-catalyzed reaction (eq 1). By isolating the glycopeptide product from the enzyme-catalyzed reaction and analyzing for possible isotopic exchange, one can obtain supporting evidence for or against these proposed mechanisms of the reaction catalyzed by OST. We have carried out experiments using three deuterium-labeled peptides **2b–d** as substrates for the OST-catalyzed reactions (eq 3) and using ^{1}H NMR, have analyzed the glycopeptide products **3b–d** for possible isotopic wash-out during catalysis.

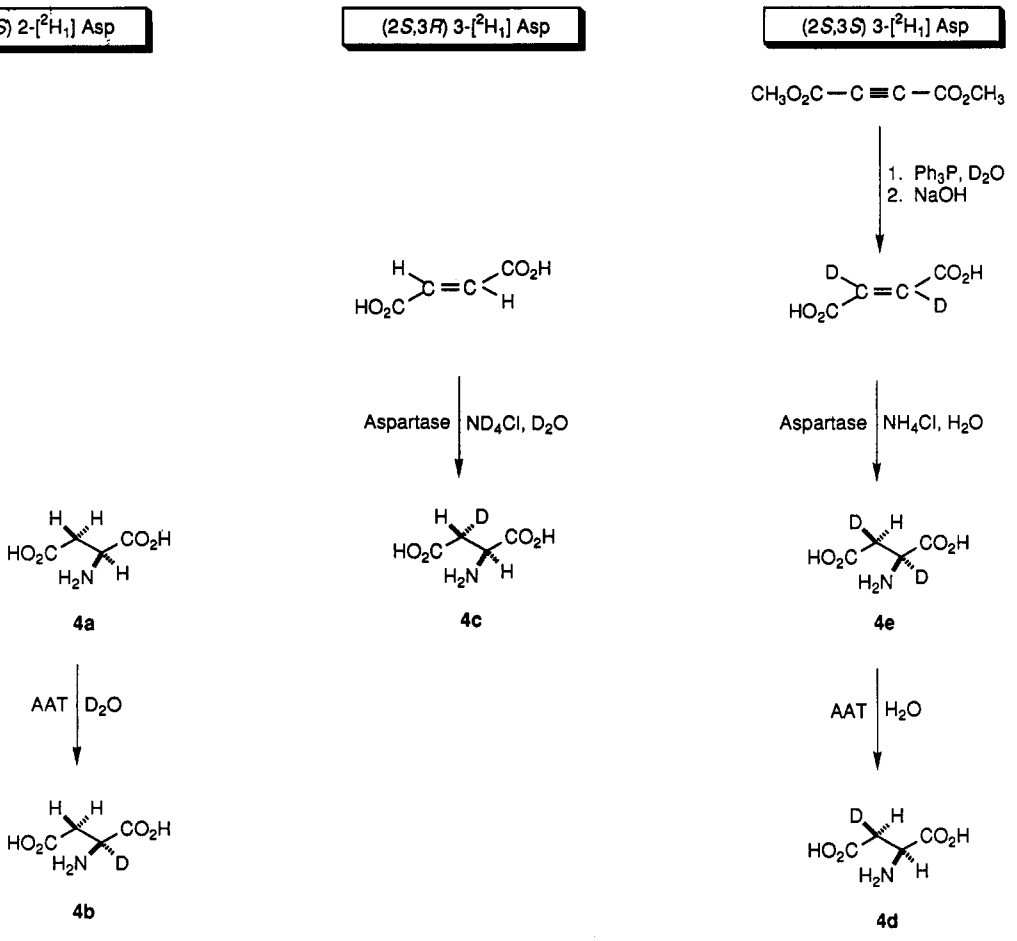
MATERIALS AND METHODS

General. Chemical shifts of ^{1}H NMR are reported in ppm downfield from TMS. The spectra shown in Figures 2 and 3 are referenced to DMSO (δ 2.49 ppm) or 2:1 D_2O/CD_3OD (δ 3.32 ppm). Mass spectra were obtained on a Finnigan 4500 GC/MS-EICI system. Unless otherwise indicated, reagents and solvents were obtained from commercial suppliers and were used without further purification. GDP-[^{3}H]Man and UDP-[^{3}H]GlcNAc were purchased from Du Pont NEN. Aspartase, aspartate aminotransferase, and pyridoxal phosphate were obtained from Sigma. Bio-Gel P-4 (–400 mesh) was purchased from Bio-Rad. Reversed-phase HPLC analyses were performed on an Altex liquid chromatography system (Vydac ODS column, 4.6 mm × 25 cm, 5 mm C18, 1.2 mL/min, 254 nm). Purification of products by preparative HPLC was performed on a Rainin liquid chromatography system (Dynamax 60A column, 100 mm × 25 cm, 5 mm C18, 5.64 mL/min, 254 nm). P_{40} yeast microsomes and [^{3}H]LOS were prepared as described by Clark et al. (1990). [^{3}H]LDS was prepared as described by Lee and Coward (1992). TLC of ^{3}H-labeled lipid intermediates (LOS, LDS) was performed on cellulose plates developed in a mobile phase of *n*-BuOH/EtOAc/H_2O (8:6:5.8). Radiographic bands were visualized on Kodak X-Omat AR film by autoradiography of the TLC plates after spraying with EN^{3}HANCE (Du Pont NEN). Film images were additionally enhanced by the use of an intensifying screen (Kodak). *N*-Bz-Asn-Leu-Thr-NH_2 (**2a**) was synthesized as described previously (Clark et al., 1990). Stereospecifically deuterated aspartic acids **4b–d** (Kim & Raushel, 1986; Gani & Young 1983), asparagines, and *N*-Boc asparagines **5b–d** were synthesized by modifications of literature procedures as described in the supplementary material.

Synthesis of N-Benzoyl-[2^{2}H]Asn-Leu-Thr-NH_2 (**2b**), *N-Benzoyl-(2S,3R)-[3-^{2}H]Asn-Leu-Thr-NH_2* (**2c**), *and N-Benzoyl-(2S,3S)-[3^{2}H]Asn-Leu-Thr-NH_2* (**2d**) (*Schemes II and III*). The deuterium-labeled aspartic acids **4b–d** were converted to the corresponding asparagines by esterification of the β-carboxylate and then aminolysis of the ester with concentrated NH_4OH (Gani et al., 1983). Protection of the N-terminus with Boc anhydride and coupling **5b–d** with a dipeptide (H-Leu-Thr-NH_2) resulted in an N-protected tripeptide. Removal of the Boc group and N-benzoylation of the N-terminus gave the desired deuterium-labeled tripeptides. The enrichment of deuterium at each position was determined by mass spectral analysis of the Boc-protected asparagines **5b–d** and measurement of the relative intensity of the M^+ and $(M + 1)^+$ peaks. All showed more than 98% ^{2}H enrichment. Synthetic details and spectral data are found in the supplementary material.

Oligosaccharyltransferase Assay. The OST assay using [^{3}H]LDS was performed as previously described (Sharma et al., 1981; Lee & Coward, 1992). The standard assay incubation was routinely carried out in a total volume of 150 μL in the following manner: [^{3}H]LDS (a mixture of [^{3}H]-$(GlcNAc)_2$-PP-dolichol and [^{3}H]GlcNAc-PP-dolichol, ca. 5000 dpm) was dried using a Speed Vac vacuum centrifuge, and the residue was dispersed in 150 μL of 50 mM Tris-HCl (pH 7.5) containing 1% (w/v) Triton-X-100, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM DTT, and 1–3 mg of P_{40} yeast microsome. For measurement of peptide acceptor capacity, a solution containing 7.2 mM tripeptide **2a** in DMSO was included in the assay mixture to give a final peptide concentration of 360

Scheme II



μM; final [DMSO]=5% (v/v). The reaction mixture was incubated at room temperature with vigorous shaking (250 rpm) for the periods indicated. The reaction was terminated by the addition of 3 mL of $CHCl_3$/MeOH (3:2) followed by a 30-s sonication. The sonicated reaction mixture was incubated on ice for 30 min. The soluble portion was then separated from the precipitated protein by centrifugation for 15 min at 2000*g*, and the supernatant was extracted with 0.4 mL of 4 mM $MgCl_2$ solution. After a thorough mixing, two layers were separated by centrifugation for 15 min at 2000*g*. The upper phase containing the water-soluble [^{3}H]glycopeptide was carefully removed, and radioactivity was determined in each layer.

Analysis of the Product Isolated from the OST Assay. Fractions containing [^{3}H]glycopeptide were concentrated to dryness using a Speed Vac vacuum centrifuge. The dry residue was resuspended in 250 μL of 0.1 M HOAc. To the [^{3}H]-glycopeptide solution were added 10 μL of 0.1 M mannose, 10 μL of 0.1 M GlcNAc, 10 μL of 0.2 M $(GlcNAc)_2$, and the chemically synthesized glycopeptide (*N*-benzoyl-Asn(GlcNAc)-Leu-Thr-NH_2) as markers for the Bio-Gel P-4 column (–400 mesh, 68.8 × 1.90 cm) (Clark et al., 1990). The solution was loaded onto the column and eluted with 0.1 M HOAc. The column was monitored with a flow rate of ca. 7 mL/h using a Haake Buchler polystaltic pump. Fractions (20 drops) were collected and analyzed by liquid scintillation counting, phenol/sulfuric acid assay, or Morgan–Elson assay (Clark et al., 1990).

Biosynthesis of Glycopeptide from the Chemically Synthesized Tripeptides and LDS Using the OST. Four sets of experiments were carried out exactly as previously described (Lee & Coward, 1992) using the tripeptide Bz-Asn-Leu-Thr-NH_2 (**2a**) and the isotopically labeled peptides **2b–d** (eq 3).

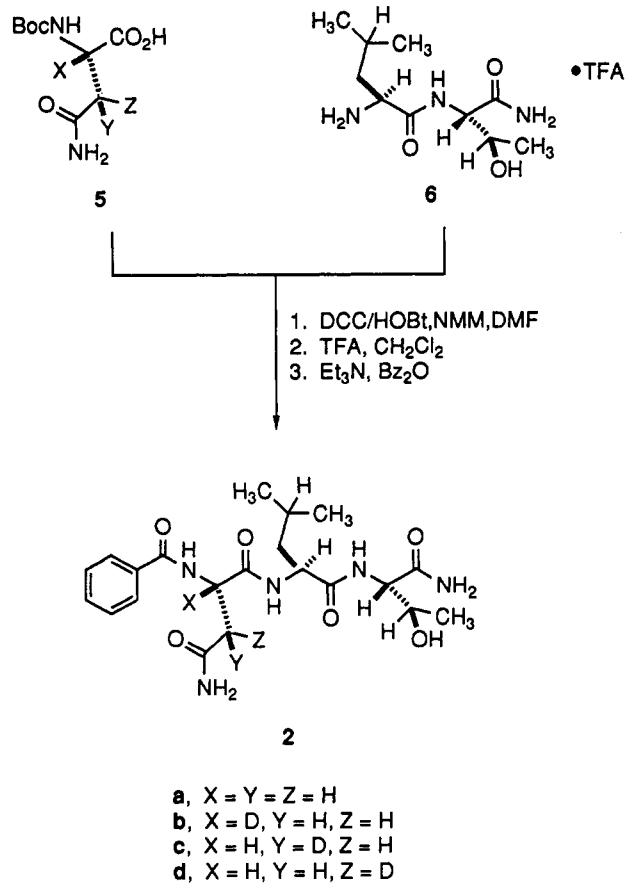
RESULTS AND DISCUSSION

Synthesis of Deuterium-Labeled Tripeptides: N-Benzoyl-[^{2}H]Asn-Leu-Thr-NH_2. Deuterium-labeled *N*-benzoyl tripeptides **2b–d** were synthesized in order to investigate two possible mechanisms of the reaction catalyzed by OST (Scheme 1a,b). The deuterium-labeled aspartic acids **4b–d** were synthesized from fumaric acid or aspartic acid, using L-aspartase or aspartate aminotransferase, respectively (Scheme II), as described briefly below. The labeled aspartic acids were converted to the corresponding asparagines and then coupled with the dipeptide, H-Leu-Thr-NH_2 (**6**), to obtain the desired tripeptides **2b–d** (Scheme III).

The synthesis of (2*S*)-[2-^{2}H]-*l*-aspartic acid (**4b**) was achieved using aspartate aminotransferase (AAT), PLP, and D_2O as described by Gani and Young (1983) (Scheme II). The reaction was stopped by heating the reaction mixture to denature the enzyme. After the denatured enzyme was removed, the filtrate was then acidified slowly to pH 3.0 with 1.0 N HCl, and white [2-^{2}H]-L-aspartic acid (**4b**) crystallized out of the solution in 65% yield. The isolated product did not contain any starting material, **4a**, as shown by ^{1}H NMR analysis.

The L-aspartase-mediated synthesis of (2*S*,3*R*)-[3-^{2}H]-L-aspartic acid (**4c**) from fumaric acid was accomplished in 38% yield as shown in Scheme II. The ^{1}H NMR spectra of the desired product showed a clean doublet for the single β hydrogen, coupling only to the α hydrogen (not shown). The typical NMR spectrum of the β-CH_2 region of aspartic acid shows a complex ABX system, indicating both geminal couplings of the β hydrogens and vicinal couplings with the α hydrogen. The α-CH region of the spectrum obtained with

Scheme III



4c showed a doublet of doublets, resulting from coupling with the NH and one β hydrogen.

Deuterium-labeled fumaric acid was synthesized accordingly to Richards and co-workers (1969): dimethyl [α,β-$^2H_2$]-fumarate was obtained in 65% yield and was then hydrolyzed (NaOH) to the diacid in 94% yield. Using L-aspartase, the [α,β^2H_2]fumaric acid was converted to (2*S*,3*S*)-[2,3-$^2H_2$]-*l*-aspartic acid in 35% yield (Scheme II). NH_3 and a proton were added stereospecifically across the double bond of the deuterium-labeled fumaric acid. Although this aspartic acid was labeled with deuterium at the β position, it was also labeled at the α position, so that it was necessary to exchange the α deuterium with a proton. The exchange was accomplished using a method similar to that which was used to label the α position of aspartic acid. The deuterium atom at the α position was exchanged with a proton from H_2O in the presence of PLP and AAT, thus giving the desired 2*S* isomer, **4d**.

The three stereospecifically deuterated aspartic acids **4b–d** were converted to their β-methyl esters by treatment with $CH_3OH/HCl(g)$, followed by ammonolysis to give the corresponding asparagines which were converted to the *N*-Boc derivatives, **5b–d**, by reaction with di-*tert*-butyl dicarbonate. These deuterium-labeled and N-protected asparagines were then coupled with H-Leu-Thr-NH_2 (**6**) (Scheme III). The Boc protecting groups were removed with CH_2Cl_2/TFA (1:1), and the N-termini of the deprotected tripeptides were then blocked with benzoyl groups by reaction with benzoic anhydride in the presence of Et_3N, dioxane, and water. Portions of the ^1H NMR spectra of the four benzoylated tripeptides **2a–d** are illustrated in Figure 2. In all cases, the spectral data confirm the stereospecific incorporation of deuterium at the α-methine (**2b**) and each of the prochiral β-methylene (**2c–d**) positions. Furthermore, mass spectral

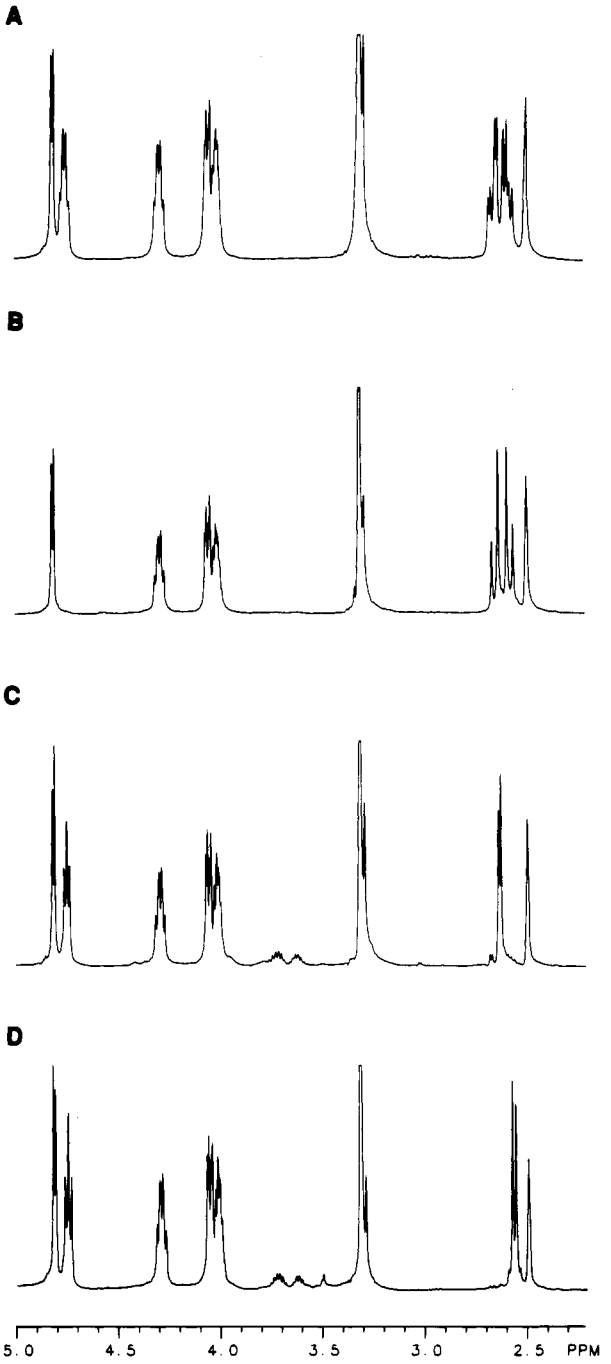


FIGURE 2: ^1H NMR spectra (DMSO-d_6) of the synthetic peptide substrates **2a–d**.

analyses of the N-protected asparagines **5b–d** showed that the enrichment of deuterium at each of the α and β positions was greater than 98%.

Substrate Specificity of Deuterium-Labeled Tripeptides. In a preliminary biochemical analysis, the deuterium-labeled tripeptides **2b–d** were tested as OST substrates. The tritium-labeled LOS ([^3H]LOS) was prepared according to Clark et al. (1990) and used as the other substrate. The OST assay was performed according to Clark et al. (1990), and the results of one such assay are shown in Table I. The column marked H_2O contains the biochemically synthesized glycopeptide and the column marked SDS contains the glycoprotein synthesized by the OST-catalyzed glycosylation of endogenous proteins in the crude P_{40} fraction. The control, in which no peptide was added, showed a net transfer of 2890 dpm (76% of the total OST product) to endogenous yeast microsomal protein

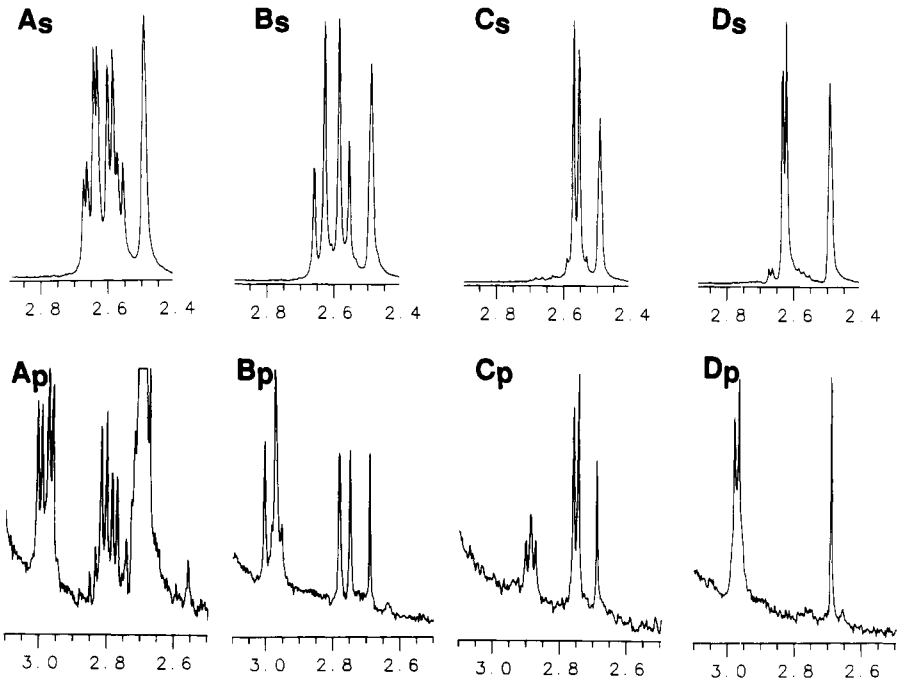


FIGURE 3: Portion of the ^{1}H NMR spectra (500 MHz) obtained for peptide substrates **2** (DMSO-d_6) and glycopeptide products **3** (D_2O/MeOD (2:1)). The spectra are arranged so that the biosynthetic glycopeptides **3a–d** (panels Ap–Dp) are placed below the peptide substrates **2a–d** (panels A_s–D_s) from which they were derived.

and 920 dpm (24%) to the H_2O layer; in the absence of peptide substrate, the radioactivity in the H_2O layer is believed to be due to free [^{3}H]oligosaccharide resulting from hydrolysis of [^{3}H]LOS under the assay conditions. The unlabeled tripeptide **2a** showed a net transfer of 4080 dpm (86%) to glycopeptide, confirming that the small peptide is an excellent oligosaccharide acceptor. As shown in the table, all deuterium-labeled tripeptides **2b–d** were equally effective oligosaccharide acceptors.

Large-Scale Biosynthesis of Glycopeptide Using Deuterium-Labeled Tripeptide and Chemically Synthesized LDS. To test for possible isotopic exchange during the enzyme-catalyzed reaction, the deuterium-labeled tripeptides and chemically synthesized LDS (Lee & Coward, 1992) were used as OST substrates in order to synthesize glycopeptides in quantities sufficient for ^{1}H NMR analysis. On the basis of prior unfavorable experience with large-scale OST reactions, we chose to synthesize the glycopeptides via multiple small-scale reactions and combine the products for the analysis. Time studies showed that the maximum amount of the glycopeptide product produced by the OST-catalyzed reaction was about 30 nmol, representing about 50% conversion to product over a period of 180 min.

On the basis of these results, 15 duplicate reactions were performed for each of the four peptides, **2a–d**, along with a control in which no peptide was added. The fractions containing the desired product were isolated as previously described (Lee & Coward, 1992), combined, and concentrated *in vacuo*. The crude residues were redissolved in 35% MeOH, and the desired glycopeptides **3a–d** were purified by HPLC (Lee & Coward, 1992). The fractions containing glycopeptide were combined and the solvents removed using a Speed Vac. The samples were then dissolved in a solution of D_2O/MeOD (2:1), and the solution was analyzed by NMR (500 MHz). The region of interest (Asn β-CH_2, δ 2.6–3.1) is shown in the lower portion of Figure 3,[3] The α-CH (δ 4.9–5.1) portions were partially obscured by the HOD peak. A singlet at ca. 2.7 ppm and a triplet at ca. 2.9–3.0 ppm were both observed

Table I: OST Assay[a] of the Deuterium-Labeled Tripeptides and [^{3}H]LOS

substrate	dpm[b]	
	H_2O	SDS
no peptide	920 (24%)[c]	2890 (76%)
1a	4080 (86%)	660 (14%)
1b	3860 (84%)	720 (16%)
1c	3420 (80%)	870 (20%)
1d	3890 (82%)	860 (18%)

[a] Assay as described by Clark and co-workers (1990) with 9000 dpm of [^{3}H]LOS and an incubation time of 10 min. [b] Each entry represents the average of duplicate assays. [c] Numbers in parentheses are expressed as percent of the total OST product (glycosylated peptide (H_2O fraction) + glycosylated protein (SDS fraction)).

in the spectrum of the material isolated from the control reaction lacking the peptide substrate (spectrum not shown). The singlet at ca. 2.7 ppm does not interfere with the peaks of interest. However, the triplet at ca. 2.9–3.0 is clearly seen in panel C_p and appears to be underneath the downfield doublet in panels B_p and D_p of Figure 3.

Panels A_s–D_s of Figure 3 show the β-CH_2 region of the tripeptide substrates **2a–d**. Panels A_p–D_p show the corresponding region of the glycopeptide products isolated from the OST-catalyzed reaction. If any of the deuterium atoms from the tripeptide substrates were exchanged with a proton from solvent during the enzyme-catalyzed reaction, one would expect to see a similar ABX-type pattern in the product isolated, as is observed in the product derived from the all-protio substrate, **2a** (panel A_p). In the other panels, B_p–D_p, the spectral patterns of the β region show a slight spreading of the multiplets, presumably due to a change in coupling

[3] Solubility differences dictated the solvents used for the peptide substrates (DMSO-d_6) vs the glycopeptide products (D_2O/CD_3OD, 2:1). As a result, the spectral regions shown in Figure 3 differ slightly between the peptides (δ 2.4–2.9) and the glycopeptides (δ 2.6–3.1). Complete ^{1}H and ^{13}C NMR spectral data for synthetic **3a** in DMSO-d_6 as well as a comparison of complete ^{1}H NMR spectra for synthetic vs biosynthetic **3a** in D_2O/CD_3OD (2:1) have been published (Lee & Coward, 1992).

constants in the products vs substrates. All glycopeptide products showed essentially the same type of pattern as the substrates from which they were derived, indicating that no deuterium was exchanged with a hydrogen atom during the enzyme-catalyzed reaction. These experiments have shown unambiguously that the deuterium atoms in the labeled tripeptides **2b–d** were retained in the products isolated. As is the case with all such experiments testing for isotopic wash-out during catalysis, one cannot rule out the possibility that one of the deuterium atoms was abstracted but returned by the enzyme to product without access to bulk solvent.

Recently, Imperiali and co-workers (1992a,b) suggested as interesting mechanistic possibility in the OST-catalyzed reaction. They proposed that the hydrogen of the asparagine side-chain amide could be abstracted by a base in the enzyme active site. The resulting tautomeric form of the amide could be stabilized by hydrogen bonds with the peptide backbone and the hydroxyl group of either threonine or serine. In addition, it was proposed that increased acidity of the side-chain NH_2 in peptides containing asparagine analogs may compensate for the absence of this stabilized Asx-turn structure. Our results do not address that mechanistic possibility. However, as noted in the introductory section, we have confirmed the findings of Rathod et al. (1986) that β-fluoroasparagine(FAsn)-containing peptides are very poor OST substrates. Although we have some concerns about the chemical stability of the FAsn-containing peptides under OST assay conditions (Banerjee et al., unpublished data; K. Patel and R. Borchardt, personal communication), any correlation between amide acidity and OST substrate activity must include the results with FAsn-containing peptides.

In conclusion, using enzymatic and chemical reactions deuterium was incorporated stereospecifically into the α and β positions of asparagine. The labeled asparagine was then incorporated into a tripeptide substrate. When the deuterium-labeled tripeptides were incubated with LDS in the presence of OST, none of the deuterium atoms were exchanged, providing strong evidence that the enzyme-catalyzed reaction does not go through either enol lactone or ketene intermediates. A major problem for studying this enzyme has been the lack of purified enzyme. Since OST is a membrane-bound enzyme, it has been difficult to purify the enzyme, and this problem has limited the kind of experiments one can design to elucidate the enzymatic mechanism. The recent purification of a mammalian OST (Kelleher et al., 1992) suggests an approach which may be effective in purifying OST from other species such as yeast.

ACKNOWLEDGMENT

We thank Dr. Chris Kojiro for his help with the NMR experiments and Mrs. Jane MacDonald for careful preparation of the manuscript.

SUPPLEMENTARY MATERIAL AVAILABLE

Complete experimental details on the synthesis of **2b–d** (9 pages). Ordering information is given on any current masthead page. A copy of this material will be provided by the authors upon request. This material is contained in many libraries on microfiche and immediately follows this article in the microfilm version of the journal.

REFERENCES

Buchanan, J. (1973) in *Advances in Enzymology* (Meister, A., Ed.) Vol. 39, pp 91–183, Wiley: New York.
Clark, R., Banerjee, S., & Coward, J. K. (1990) *J. Org. Chem. 55*, 6275–6285.
Dubois, J., Gaudry, M., Bory, S., Azerad, R., & Marquet, A. (1983) *J. Biol. Chem. 258*, 7897–7899.
Dubois, J., Dugave, C., Fourès, C., Kaminsky, M., Tabet, J.-C., Bory, S., Gaudry, M., & Marquet, A. (1991) *Biochemistry 30*, 10506–10512.
Gani, D., & Young, D. W. (1983) *J. Chem. Soc., Perkin Trans. 1*, 2393–2398.
Gani, D., Young, D. W., Carr, D. M., Poyser, J. P., & Sadler, I. H. (1983) *J. Chem. Soc., Perkin Trans. 1* 2811–2814.
Glabe, C. G., Hanover, J. A., & Lennarz, W. J. (1980) *J. Biol. Chem. 255*, 9236–9242.
Holmquist, B., & Bruice, T. C. (1969) *J. Am. Chem. Soc. 91*, 2993–3002, 3003–3009.
Imperiali, B., & Shannon, K. L. (1991) *Biochemistry 30*, 4374–4380.
Imperiali, B., Shannon, K. L., & Rickert, K. W. (1992a) *J. Am. Chem. Soc. 114*, 7942–7944.
Imperiali, B., Shannon, K. L., Unno, M., & Rickert, K. W. (1992b) *J. Am. Chem. Soc. 114*, 7944–7945.
Kelleher, D. J., Kreibich, G., & Gilmore, R. (1992) *Cell 69*, 55–65.
Kim, S. C., & Raushel, F. M. (1986) *Biochemistry 25*, 4744–4749.
Kirby, A. J. (1987) in *CRC Critical Reviews in Biochemistry* (Fasman, G. D., Ed.) Vol. 22, 283–315, CRC Press, Boca Raton, FL.
Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.
Kukuruzinska, M. A., Bergh, M. L. E., & Jackson, B. J. (1987) *Annu. Rev. Biochem. 56*, 915–944.
Lee, J., & Coward, J. K. (1992) *J. Org. Chem. 57*, 4126–4135.
Lennarz, W. J. (1987) *Biochemistry 26*, 7205–7210.
March, J. (1985) *Advanced Organic Chemistry*, p 359, Wiley, New York.
Maycock, A. L., Suva, R. H., & Abeles, R. H. (1975) *J. Am. Chem. Soc. 97*, 5613–5614.
Rathod, P. K., Tashjian, A. H., Jr., & Abeles, R. H. (1986) *J. Biol. Chem. 261*, 6461–6469.
Richards, E. M., Tebby, J. C., Ward, R. S., & Williams, D. H. (1969) *J. Chem. Commun. (C)* 1542–1544.
Schendel, F. J., Cheng, Y. S., Otvos, J. D., Wehrli, S., & Stubbe, J. (1988) *Biochemistry 27*, 2614–2623.
Sharma, C. B., Lehle, L., & Tanner, W., (1981) *Eur. J. Biochem. 116*, 101–108.
Sinnott, M. L. (1987) in *Enzyme Mechanisms* (Page, M. I., & Williams, A., Eds.) pp 259–297, Royal Society of Chemistry, London.
Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171–1202.
Stainer, R. Y., & Ornston, L. N. (1973) in *Advances in Microbial Physiology* (Rose, A. H., & Tempest, D. W., Eds.), Vol. 9, pp 89–151, Academic Press, London.
Stephenson, R. C., & Clarke, S. (1989) *J. Biol. Chem. 264*, 6164–6170.
Welply, J. K., Shenbagamurthi, P., Lennarz, W. J., & Naider, F. (1983) *J. Biol. Chem. 258*, 11856–11863.