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The Design, Synthesis, and Initial Evaluation of Benzophenone-containing Peptides as Potential Photoaffinity Labels of Oligosaccharyltransferase

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Abstract—The benzophenone photophore was incorporated into protected tripeptides and tetrapeptides as photoactivatable probes to study the multimeric enzyme oligosaccharyltransferase (OST). These peptides contain the -Asn-X-Thr- sequon which is required for OST-catalyzed *N*-glycosylation. Two tripeptides, Bz-Asn-Bpa-Thr-NH_2 (**3b**) and Bz-Asn-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2 (**4b**), were found to be good OST substrates. They were competitive inhibitors versus standard peptide substrate [^{14}C]Bz-Asn-Leu-Thr-NH_2 and their K_i values were determined to be $41 \pm 6\,\mu M$ and $21 \pm 6\,\mu M$, respectively, using synthetic $(GlcNAc)_2$-PP-dolichol.

Introduction

Oligosaccharyltransferase[1,2] (OST, EC 2.4.1.119) is the key enzyme in the *N*-linked glycoprotein synthesis. It transfers the oligosaccharide portion of $Glc_3Man_9GlcNAc_2$-PP-dolichol (Dol-PP-OS) to the side chain amide moiety of an asparagine residue that is part of the -Asn-X-Ser/Thr- sequon[3,4] in a growing polypeptide chain (Fig. 1). During the cotranslational process, the seemingly non-nucleophilic side chain carboxamido group effects a S_N2-like nucleophilic attack at the anomeric carbon of the first GlcNAc and displaces the dolichol pyrophosphate. Mechanistic studies of this intriguing reaction have been actively pursued during the past fifteen years. Several mechanisms have been proposed to address activation of the carboxamido moiety of asparagine during OST catalysis such as the active site base deprotonation mechanism proposed by Bause et al.[5] and the peptide 'Asx-turn' conformation activation mechanism proposed by Imperiali and co-workers.[1,6] Three nucleophilic activation mechanisms involving imide,[7] enol lactone,[8] and ketene[8] intermediates were investigated and found to be unlikely. Another proposed catalytic mechanism, similar to that of many glutamine-dependent amidotransferases, was recently studied using $^{13}C/^{15}N$-labeled peptide substrates.[9] A lack of 'NH_3' exchange suggested no similarity between the OST catalytic mechanism and that of the glutamine-dependent amidotransferases.

Based on the fact that methyl methanethiolsulfonate (MMTS)[10,11] and *p*-chloromercury benzoate (pCMB) (Liu, Y. L.; Schretzman, L.; Coward, J. K., unpublished results) inhibit OST-catalyzed glycosylation, it has been postulated that a crucial cysteine residue may be involved in catalysis. However, inhibition by nonspecific group reagents does not address the actual roles of this seemingly important cysteine residue in OST catalysis. Although Bause[12,13] has demonstrated that a heptapeptide containing -Asn-Gly-(epoxyethyl)Gly- sequon covalently labels OST, our recent work has shown that peptides with asparagine replaced by structurally similar amino acids could not act as effective affinity labels or competitive inhibitors of OST.[11] These results seemed to indicate that the important cysteine may not be located in the enzyme active site and it is less likely to play a major role in catalysis. This assumption is further supported by the fact that $(GlcNAc)_2$-PP-dolichol (Dol-PP-

Key words: Benzophenone; photophore; photoaffinity label; oligosaccharyltransferase.

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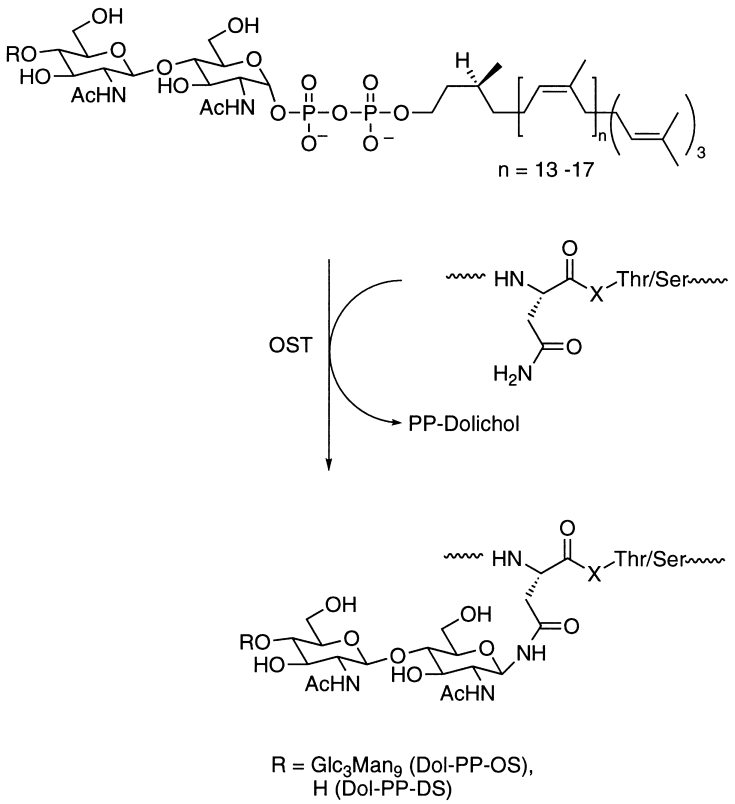
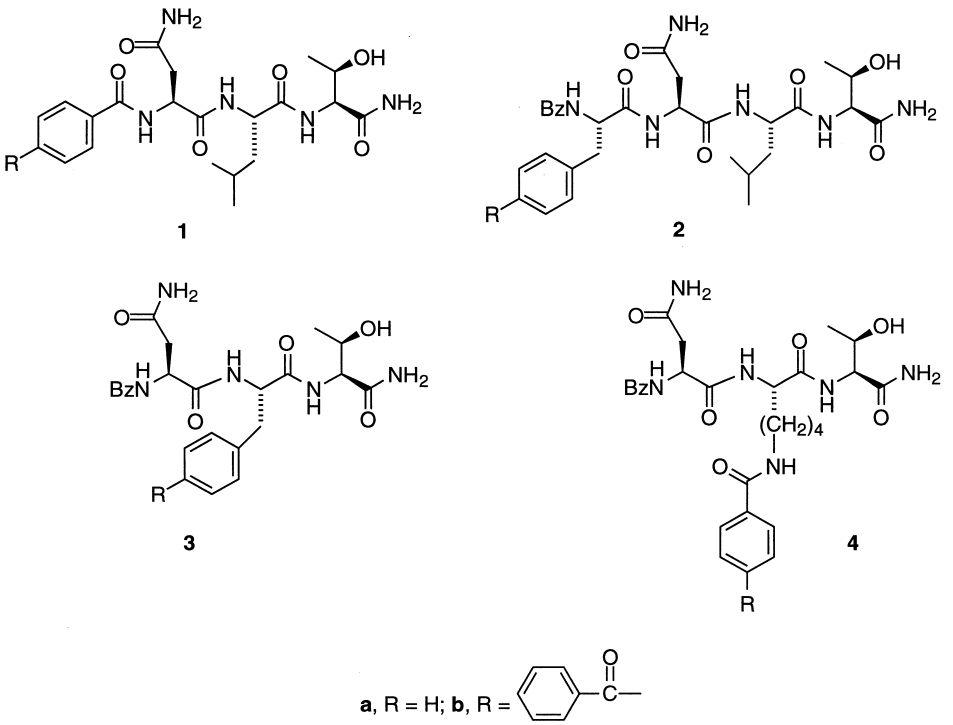


Figure 1. OST-catalyzed *N*-linked glycoprotein synthesis.

DS) protected OST from being inhibited by MMTS,[10,11] an indication that cysteine could be involved in glycolipid substrate binding. Therefore, mechanistic probes which target less specific groups on the protein need to be developed.

In the 1980s, Lennarz and co-workers developed ^{3}H- and ^{125}I-labeled, aryl azide-containing tripeptides as active site-directed photoaffinity labels of OST.[14–16] The photophore was tethered to the side chain ε-amino group of lysine in the -Asn-Lys-Thr- sequon. However, photoinhibition studies using microsomal OST enzyme showed that the protein covalently labeled was not membrane-bound but an enzyme located in the lumen of the endoplasmic reticulum.[17] Further research indicated that the labeled protein was protein disulfide isomerase[18] which was not required for *N*-linked glycoprotein synthesis. In the intervening years, OST has been successfully purified from five different sources,[2] suggesting that the photoaffinity label approach to study OST catalysis is now amenable to reinvestigation. OST is a complicated multisubunit enzyme with limited information available as to which subunits comprise its active site. Application of the photoaffinity label approach would enable us to identify the critical subunit(s) via the synthesis of alternate substrates that would carry photophores capable of labeling the active site when photoirradiated.

Application of the benzophenone photophore as a biochemical probe has increased dramatically during the past ten years.[19] Compared with an aryl azide, a benzophenone is chemically more stable, can be activated at longer wavelength (350–360 nm), and preferentially inserts into a C–H bond, even in aqueous solution or in the presence of other nucleophiles. If the benzophenone photophore is incorporated into peptides which contain the -Asn-X-Ser/Thr- sequon, these peptides, when photoactivated, should act as photoaffinity labels of OST. Four peptides have been designed for this purpose. They are (4-Bz)Bz-Asn-Leu-Thr-NH_2 (**1b**), Bz-Bpa-Asn-Leu-Thr-NH_2 (**2b**), Bz-Asn-Bpa-Thr-NH_2 (**3b**), and Bz-Asn-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2 (**4b**). The requirement of -Asn-X-Ser/Thr- sequon is satisfied in all four peptides. Nonphotolabile control peptides (**1**–**4a**), in which the benzophenone photophore of the four potential photoaffinity labels (**1**–**4b**) is replaced by a phenyl group, have also been synthesized.



Results and Discussion

Synthesis

Compound **1b** was synthesized by the coupling of H-Asn-Leu-Thr-NH_2·TFA with 4-benzoylbenzoic acid (**5b**) using EDC (Scheme 1). *N*-Boc-Phe (**6a**) or *N*-Boc-Bpa (**6b**) was coupled with H-Asn-Leu-Thr-NH_2·TFA to afford tetrapeptides **7a** and **7b** using standard solution phase carbodiimide coupling method. Deprotection at the N-terminal by TFA followed by the benzoylation gave the tetrapeptide **2**. Tripeptides **3a** and **3b** were synthesized in the similar fashion (Scheme 2). The synthesis of lysine-containing tripeptide **4** is more involved. Initially, N^{α}-Boc-N^{ε}-Cbz-Lys was treated with diazomethane to give the methyl ester. N^{α}-Boc-N^{ε}-Cbz-Lys-OMe was then treated under hydrogenolysis and the resulting free ε-amino group was coupled with 4-benzoylbenzoic acid (**5b**). The resulting lysine analogue was treated with TFA to remove the N^{α}-Boc group and the resulting free α-amine was coupled with *N*-Boc-Asn. Unfortunately, the resulting Boc-Asn-Lys[N^{ε}-(4-Bz)Bz]-OMe dipeptide was resistant to hydrolysis when treated with K_2CO_3 in aq DMF. The successful synthesis of **4** was accomplished using N^{α}-Boc-N^{ε}-Bz-Lys (**9a**)[20] and N^{α}-Boc-N^{ε}-[(4-Bz)Bz]-Lys (**9b**)[21] (Scheme 2). The synthesis procedure was similar to that of **3**. The synthesis of **9a** involved formation of the lysine-copper chelate[22] followed by the selective benzoylation of the lysine ε-amino group[23] (Scheme 3). Decomposition of the copper chelate complex **11** by thioacetamide followed by the protection of the α-amino group with Boc_2O afforded **9a**. The synthesis of **9b** started with N^{α}-Boc-N^{ε}-Cbz-Lys. 2-Trimethylsilylethyl (TMSE) ester formation[24] followed by hydrogenolysis gave N^{α}-Boc-Lys-OTMSE.[25] Coupling of N^{α}-Boc-Lys-OTMSE with 4-benzoylbenzoic acid followed by the deprotection of the TMSE ester afforded **9b**.

Biochemical studies

The four potential photoaffinity labels (**1**–**4b**) and the corresponding control peptides (**1**–**4a**) were first evaluated as peptide substrates for OST using microsomal enzyme and either [^{3}H]Dol-PP-OS or [^{3}H]Dol-PP-DS.[7,26] The results shown in Table 1 are independent of the saccharide donor; i.e. [^{3}H]Dol-PP-OS or [^{3}H]Dol-PP-DS lead to the same conclusions. The tetrapeptide **2b** is not a substrate while **1b** is a modest acceptor substrate. However, the other two tripeptides **3b** and **4b**, in which the middle amino acid is Bpa or Lys analogue, are good peptide substrates of OST. These two tripeptides were further evaluated as inhibitors (alternate substrates) versus the standard peptide substrate Bz-Asn-Leu-Thr-NH_2 (**1a**) using synthetic Dol-PP-DS.[26,27] Dixon plots[28] (1/V versus [I] at various substrate concentration) led to K_i values for **3b** and **4b** of $41 \pm 6\,\mu M$

and $21 \pm 6\,\mu M$, respectively (Fig. 2). These values are considerably lower than the K_m of Bz-Asn-Leu-Thr-NH_2 (282 μM) (Gibbs, B. S.; Coward, J. K., unpublished results), perhaps due to the hydrophobic environment at the glycosylation site at the rough endoplastic reticulum membrane.

Initial photochemical studies showed that **3b** and **4b** are photolabile and the absorbance at 262 nm decreases during photolysis. However, control peptides **3a** and **4a**, lacking the benzophenone photophore, did not show this characteristic. Further photochemical evaluations of **3b** and **4b** in the OST enzyme system and the synthesis of radiolabeled **3b** and **4b** are currently in progress.

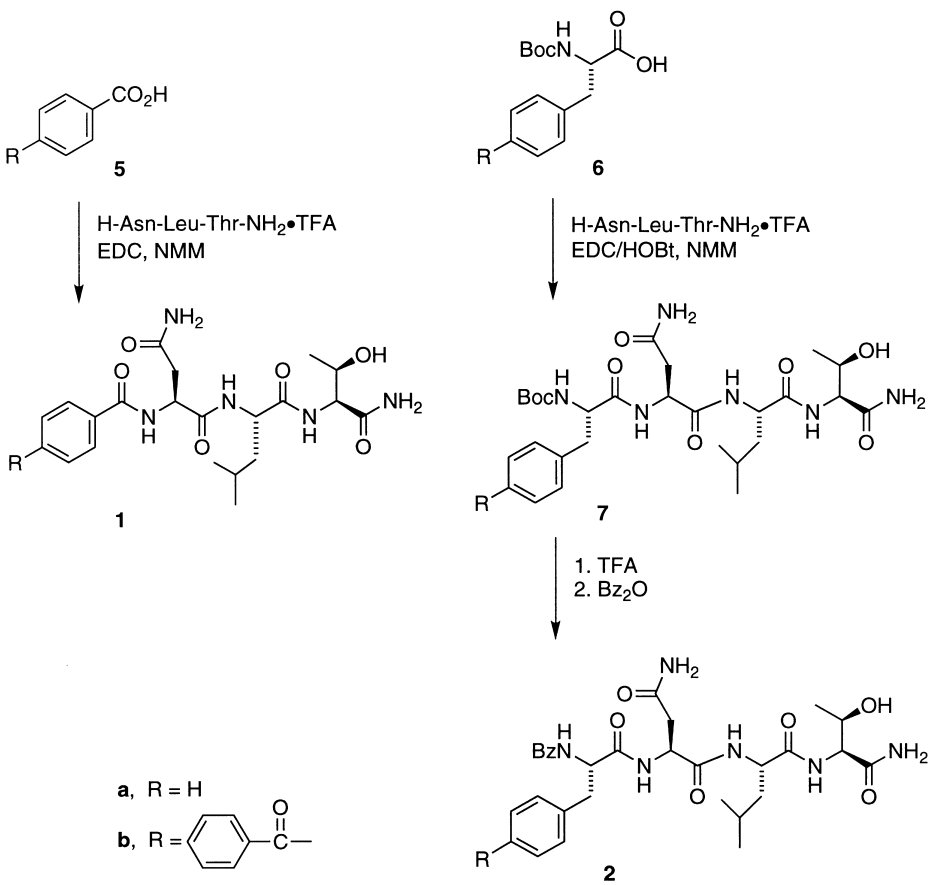
Conclusion

Two protected tripeptides, Bz-Asn-Bpa-Thr-NH_2 (**3b**) and Bz-Asn-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2 (**4b**), were found to be good OST substrates. They were also good competitive inhibitors (alternate substrates) versus standard substrate Bz-Asn-Leu-Thr-NH_2 (**1a**) and their K_i values were found to be $41 \pm 6\,\mu M$ and $21 \pm 6\,\mu M$, respectively. The photoaffinity approach is a useful tool that can be applied toward the understanding of the complicated OST enzyme. This approach to identifying the OST active site appears more promising following purification of this multimeric enzyme in recent years.

Experimental

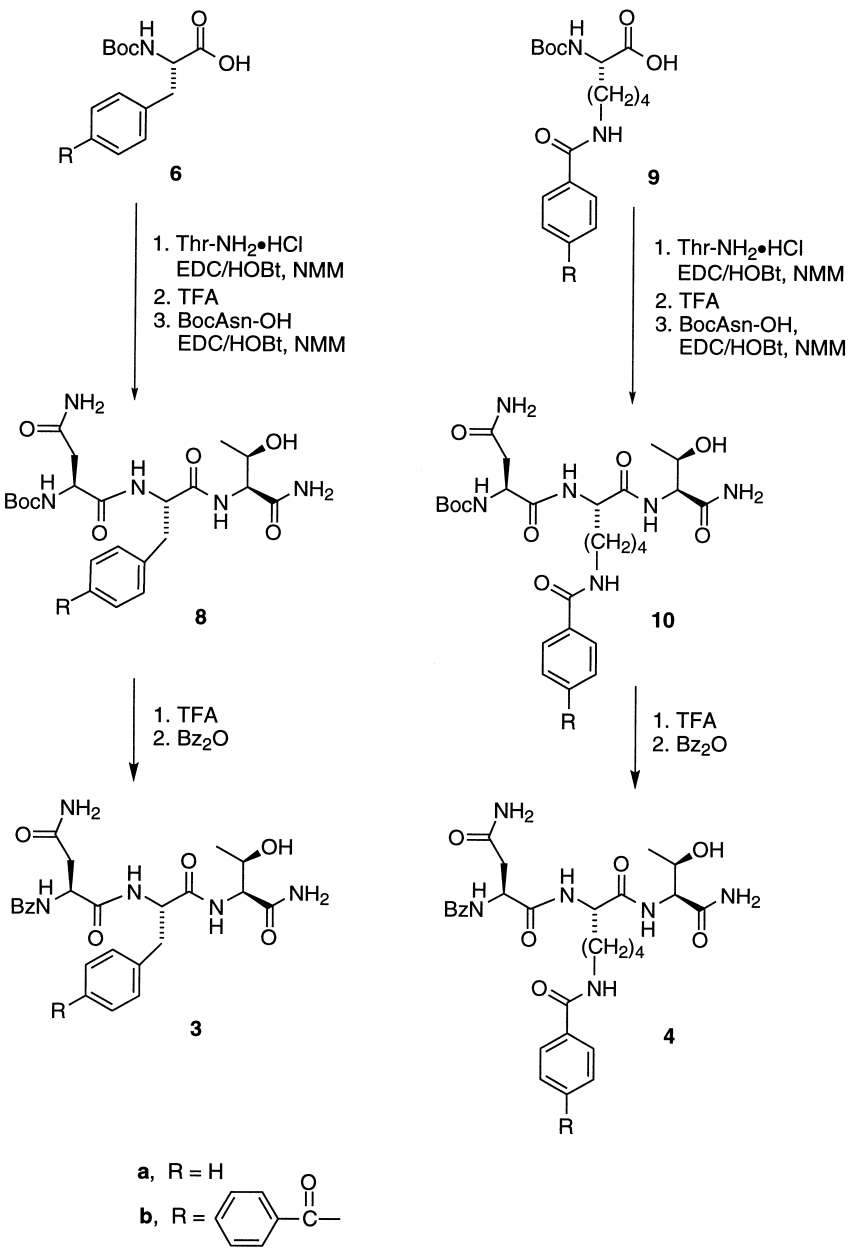
General

NMR Spectra were obtained with Bruker instruments operating at 360 MHz or 300 MHz. ^1H and ^{13}C NMR chemical shifts were reported in ppm downfield from TMS. Melting points were uncorrected. Infrared spectra were recorded as a thin film on sodium chloride plates or as a KBr pellet, and absorptions were reported in wavenumbers (cm^{-1}). MS, HRMS, and elemental analysis were performed in the Microanalytical Facility, Department of Chemistry, University of Michigan. TLC



Scheme 1.

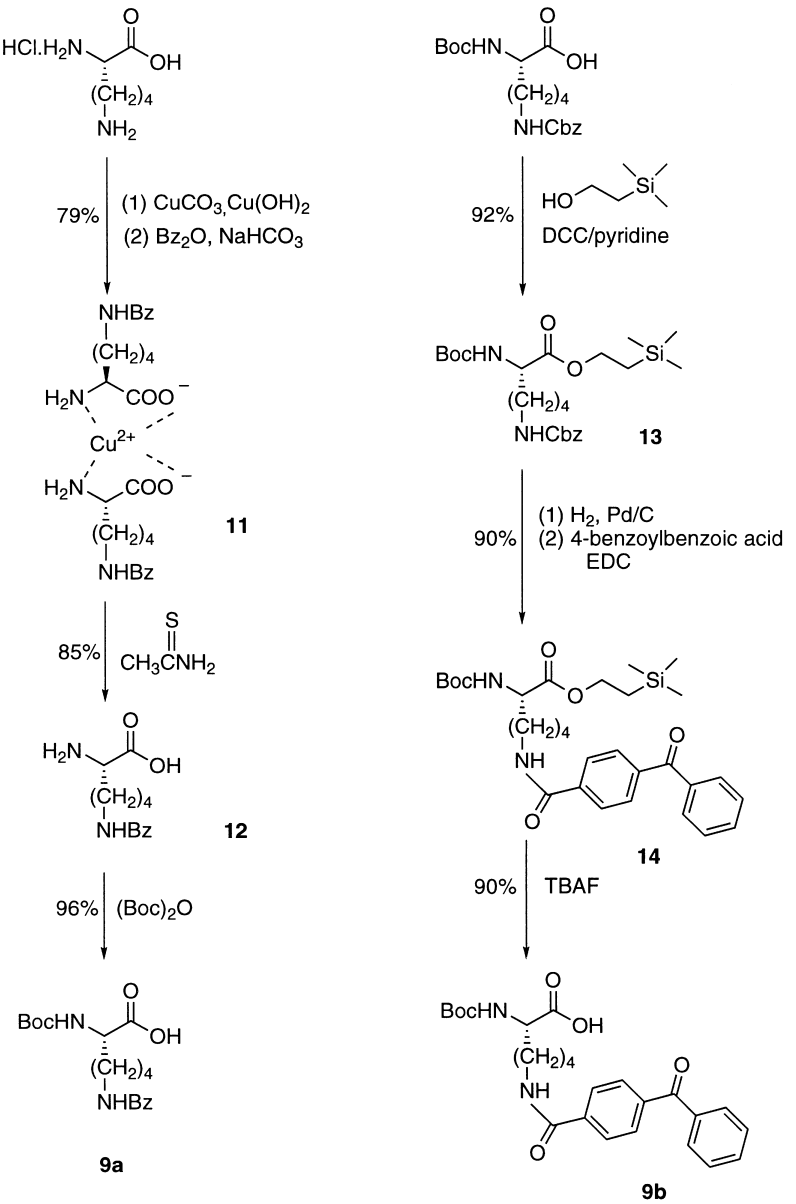
spots were visualized by UV, ninhydrin, or bromocresol green spray reagents. THF was freshly distilled from sodium benzophenone ketyl, while dichloromethane and *N*-methylmorpholine (NMM) were distilled from CaH_2. *N*-Boc-Bpa was bought from Bachem, *N*-Boc-Phe and 4-benzoylbenzoic acid were from Aldrich, and N^{α}-Boc-N^{ε}-Cbz-Lys was purchased from Sigma. All other reagents were purchased from major commercial sources and were used without further purification. H-Asn-Leu-Thr-NH_2·TFA and **1a** were synthesized as previously described.[7] $(GlcNAc)_2$-PP-dolichol was synthesized as described by Lee and Coward,[26] and its concentration was determined by high pH anion-exchange chromatography (HPAEC)[29] following hydrolysis to glucosamine. N^{ε}-Bz-L-Lysine (**12**)[23] and N^{α}-Boc-N^{ε}-Cbz-L-lysine 2-trimethylsilylethyl ester (**13**)[24] were prepared as described in the literature. P_{40} yeast microsomes and [^{3}H]$(GlcNAc)_2$-PP-dolichol were prepared as described by Clark et al.[7] and Lee and Coward,[26] respectively. Standard OST assay used to measure the



Scheme 2.

peptide acceptor ability was described previously.[7,26] Enzyme assays used for K_i determination were analyzed by reverse phase HPLC on a Waters liquid chromatography systems (6100A & 510 pumps), Rainin Microsorb–MV 5 μM C_{18}, 300 Å, 4.6×250 mm column, and monitored using a Waters 996 diode array spectrometer. Radioactivity was determined using a Packard 1600 TR liquid scintillation analyzer.

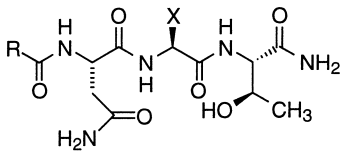
***N*-(4-Bz)Bz-Asn-Leu-Thr-NH_2 (1b).** 4-Benzoylbenzoic acid (**5b**, 45 mg, 0.20 mmol), EDC·HCl (42 mg, 0.22 mmol), and H-Asn-Leu-Thr-NH_2·TFA (0.20 mmol) were dissolved in 2 mL dry DMF at 0 °C while stirring and NMM (46 μL, 0.42 mmol) was added. The reaction mixture was stirred at 0 °C for 1 h and at rt for 16 h. The reaction mixture was concentrated in vacuo and the resulting brown residue was triturated with 5% citric acid solution. The white precipitate was collected, washed with cold water, and then crystallized from water/acetone/methanol (v/v/v, 1/1/1). 90 mg (87%) of a white solid was obtained: R_f 0.65 (MeOH/EtOAc, 1/1); mp 233–235 °C (dec); ^{1}H NMR (DMSO-d_6) δ 0.86 (dd,

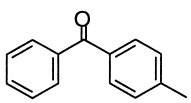
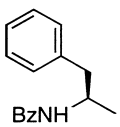
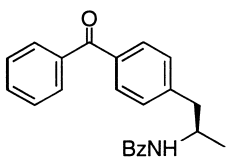
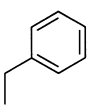
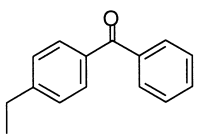
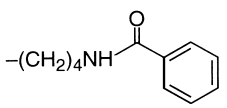
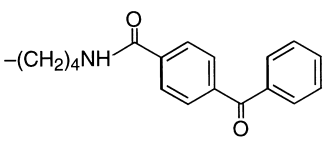


Scheme 3.

6H), 1.03 (d, 3H), 1.52 (m, 2H), 1.66 (m, 1H), 2.68 (m, 2H), 4.04 (m, 1H), 4.08 (m, 1H), 4.32 (m, 1H), 4.81 (m, 1H), 4.92 (d, 1H), 6.95–7.15 (m, 4H), 7.50–8.05 (m, 9H), 7.68 (d, 1H), 8.31 (d, 1H), 8.92 (d, 1H); ^{13}C NMR (DMSO-d_6) δ 19.9, 21.4, 23.0, 24.1, 36.8, 40.3, 51.0, 51.7, 58.3, 66.3, 127.2, 128.4, 129.1, 129.4, 132.8, 136.5, 137.2, 139.2, 165.4, 170.9, 171.6, 171.7, 172.0, 195.2; MS (FAB$^+$) *m/e* (rel. intensity) 554 (MH^+, 44), 209 (76), 86 (100); HRMS calcd for $C_{28}H_{36}N_5O_7$ (MH^+), 554.2615; found, 554.2587. Anal. calcd for $C_{28}H_{35}N_5O_7 \cdot 1.2H_2O$: C, 58.46; H, 6.55; N, 12.17. Found: C, 58.36; H, 6.35; N, 12.02.

Table 1. Evaluation of photophore-containing peptides as OST substrates[a]



Compd	R	X	Relative glycosylation activity[b] [^{3}H]LOS	[^{3}H]LDS
1a		$(CH_3)_2CHCH_2$-	1.00	1.00
1b		$(CH_3)_2CHCH_2$-	0.38	0.26
2a		$(CH_3)_2CHCH_2$-	0.24	0.23
2b		$(CH_3)_2CHCH_2$-	0	0.06
3a			1.04	1.11
3b			1.00	1.09
4a			1.23	1.30
4b			0.86	1.04

[a]Microsomal OST enzyme was used.
[b]Relative to glycosylation of Bz-Asn-Leu-Thr-NH_2 (1.00).

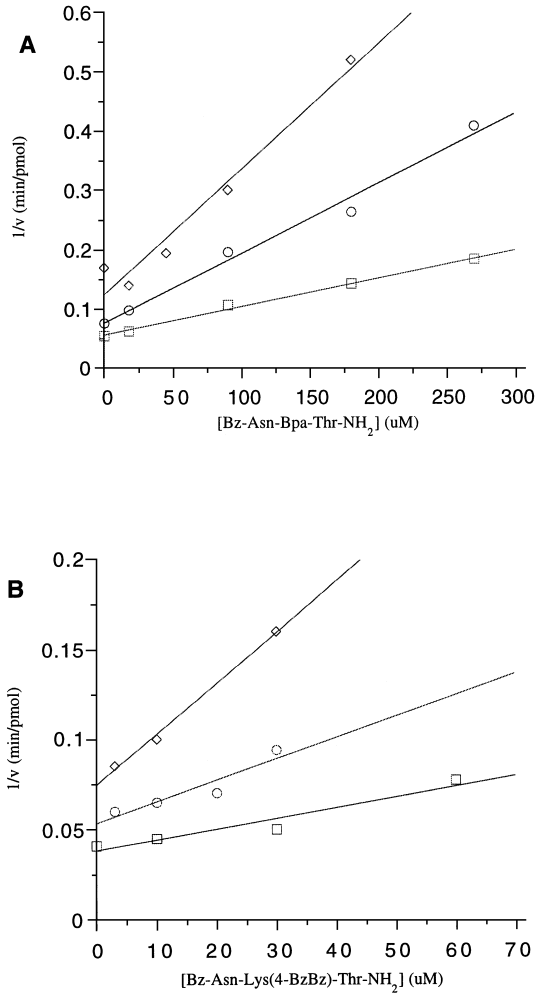


Figure 2. Dixon plot of OST inhibition by (A) compound **3b**, (B) compound **4b**. [[^{14}C]Bz-Asn-Leu-Thr-NH_2] = 90(◇), 180 (○), and 360 (□) μM.

***N*-Boc-Phe-Asn-Leu-Thr-NH_2 (7a).** N-Boc-Phe (**6a**, 89 mg, 0.34 mmol), EDC·HCl (78 mg, 0.40 mmol), and HOBt (68 mg, 0.51 mmol) were dissolved in 1 mL anhydrous DMF at −10 °C while stirring. NMM (44 μL, 0.41 mmol) was added followed by a precooled solution of H-Asn-Leu-Thr-NH_2·TFA (0.34 mmol) and NMM (37 μL, 0.34 mmol) in 1.5 mL anhydrous DMF. The reaction mixture was stirred at −10 °C for 1 h and at rt for 18 h. The reaction mixture was concentrated in vacuo and the yellow residue obtained was triturated with 5% citric acid solution. The white precipitate was collected and rinsed with water. The crude product was further triturated with saturated $NaHCO_3$ solution, rinsed with water and dried to give 165 mg (83%) of a white solid: R_f 0.59 (EtOAc/MeOH, 3/1); mp 220–222 °C; ^{1}H NMR (DMSO-d_6, CD_3OD) δ 0.80 (dd, 6H), 1.05 (d, 3H), 1.25 (s, 9H), 1.55 (m, 2H), 1.60 (m, 1H), 2.50–3.15 (m, 4H), 4.0–4.5 (m, 5H) 7.17 (m, 5H); ^{13}C NMR (DMSO-d_6, CD_3OD) δ 20.8, 22.0, 24.0, 25.9, 29.0, 37.6, 39.0, 41.4, 51.8, 54.0, 57.6, 60.7, 68.3, 80.7, 127.9, 129.6, 130.6, 139.0, 157.7, 173.4, 174.3, 174.77, 174.81, 175.2; MS (FAB$^+$) *m/e* (rel. intensity) 593 (MH^+, 28), 120 (72), 86 (100); HRMS calcd for $C_{28}H_{45}N_6O_8$ (MH^+), 593.3299; found, 593.3307.

***N*-Bz-Phe-Asn-Leu-Thr-NH_2 (2a).** *N*-Boc-Phe-Asn-Leu-Thr-NH_2 (**7a**, 70 mg, 0.12 mmol) was dissolved in 2 mL TFA/CH_2Cl_2 (v/v, 1/1) at rt and the mixture was allowed to set at rt for 30 min. The reaction mixture was concentrated in vacuo and the resulting clear oil was triturated with Et_2O. The precipitate was collected and rinsed with Et_2O and air dried to give 71 mg white solid: 99% yield; R_f 0.31 (EtOAc/MeOH, 1/1). This product, H-Phe-Asn-Leu-Thr-NH_2·TFA (66 mg, 0.11 mmol), was dissolved in 1.5 mL H_2O/dioxane/DMF (v/v/v, 1/1/1) mixed solvent. Et_3N (15 μL, 0.11 mmol) was added at 0 °C followed by Bz_2O (37 mg, 0.16 mmol) while stirring. The mixture was stirred for 5 min then Et_3N (8 μL, 0.06 mmol) was further added. The reaction mixture was stirred at rt for 12 h and then concentrated in vacuo. The resulting residue was dissolved in H_2O/THF (v/v, 1/1) and Dowex 50W-X8 (H^+) resin was added and the mixture was gently stirred. The filtrate was concentrated in vacuo and the resulting crude product was crystallized from H_2O/THF (v/v, 1/1), rinsed with Et_2O, and dried to give 57 mg (88%) of a white solid: R_f 0.69 (MeOH/EtOAc, 1/1); mp 247–249 °C; ^{1}H NMR (DMSO-d_6) δ 0.80 (dd, 6H), 1.01 (d, 3H), 1.51 (m, 2H), 1.60 (m, 1H), 2.55–3.15 (m, 4H), 4.02 (m, 1H), 4.04 (m, 1H), 4.28 (m, 1H), 4.57 (m, 1H), 4.69 (m, 1H), 6.95–7.80 (m, 14H), 7.62, 8.06, 8.51, 8.61 (d, 4H); ^{13}C NMR (DMSO-d_6) δ 20.1, 21.3, 23.2, 24.1, 36.7, 37.0, 40.2, 49.9, 51.5, 55.0, 58.5, 66.4, 126.3, 127.4, 128.1, 128.2, 129.2, 131.3, 134.0, 138.4, 166.5, 171.0, 171.5, 172.0, 172.3, 174.9; MS (FAB$^+$) *m/e* (rel intensity) 597 (MH^+, 34), 336 (40), 252 (42), 224 (41), 105 (100), 85 (68); HRMS calcd for $C_{30}H_{41}N_6O_7$ (MH^+), 597.3037; found, 597.3037. Anal. calcd for $C_{30}H_{40}N_6O_7 \cdot 0.5H_2O$: C, 59.50; H, 6.78; N, 13.88. Found: C, 59.58; H, 6.74; N, 13.40.

***N*-Boc-Bpa-Asn-Leu-Thr-NH_2 (7b).** N-Boc-Bpa (**6b**, 124 mg, 0.34 mmol), EDC·HCl (78 mg, 0.41 mmol), and HOBt (68 mg, 0.51 mmol) were dissolved in 1 mL dry DMF. NMM (44 μL, 0.41 mmol) was added at −10 °C and the mixture was stirred for 10 min under nitrogen. A precooled solution of H-Asn-Leu-Thr-NH_2·TFA, obtained by treatment of N-Boc-Asn-Leu-Thr-NH_2 (150 mg, 0.34 mmol) with TFA,[7] and NMM (37 μL, 0.34 mmol) in 1.5 mL dry DMF was added while stirring. The reaction mixture was stirred at −10 °C for 1 h and at rt for 18 h and then concentrated in vacuo. The resulting yellow residue was triturated with 5% citric

acid solution and the slightly yellowish precipitate obtained was further triturated with saturated $NaHCO_3$ solution. The precipitate was collected by filtration, rinsed with H_2O, and dried to give 178 mg (76%) of a white solid: R_f 0.54 (MeOH/EtOAc, 1/3); mp 209–211 °C; IR (KBr): 3416, 3311, 3072, 2973, 2931, 1666, 1525, 1277,702 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.87 (m, 6H), 1.10 (d, 3H), 1.34 (s, 9H), 1.62 (m, 2H), 1.73 (m, 1H), 2.55–3.20 (m, 4H), 4.10–4.75 (m, 5H), 6.85–7.05 (m, 4H), 7.35–8.40 (m, 13H); ^{13}C NMR (DMSO-d_6) δ 20.2, 21.4, 23.2, 24.2, 28.2, 37.0, 37.6, 40.3, 49.9, 51.7, 55.4, 56.7, 58.6, 66.4, 78.3, 128.6, 129.5, 132.6, 135.1, 137.3, 143.6, 155.3, 171.1, 171.4, 172.0, 172.3, 172.9, 195.6; MS (FAB$^+$) *m/e* (rel. intensity) 697 (MH^+, 28), 224 (30), 87 (42), 86 (100); HRMS calcd for $C_{35}H_{49}N_6O_9$ (MH^+), 697.3561; found, 697.3589.

***N*-Bz-Bpa-Asn-Leu-Thr-NH_2 (2b).** *N*-Boc-Bpa-Asn-Leu-Thr-NH_2 (**7b**, 80 mg, 0.11 mmol) was dissolved in 2 mL TFA/CH_2Cl_2 (v/v, 1/1) and the mixture was allowed to set at rt for 30 min. The clear solution was concentrated in vacuo and the resulting colorless oil was triturated with Et_2O. The white precipitate was collected and rinsed with Et_2O and air dried to give 81 mg white solid, 99% yield. R_f 0.36 (MeOH/EtOAc, 1/1). H-Bpa-Asn-Leu-Thr-NH_2·TFA (74 mg, 0.10 mmol) was dissolved in 1.5 mL H_2O/dioxane/DMF (v/v/v, 1/1/1) mixed solvent. Et_3N (14 μL, 0.10 mmol) was added at 0 °C followed by Bz_2O (35 mg, 0.16 mmol) while stirring. The reaction mixture was stirred for 5 min and Et_3N (7 μL, 0.05 mmol) was further added. The reaction mixture was stirred at rt for 12 h and then concentrated in vacuo. The resulting residue was dissolved in H_2O/THF (v/v, 1/1) and Dowex 50W X8 (H^+) resin was added and the mixture was gently stirred. The mixture was filtered and the filtrate was concentrated in vacuo. The resulting white residue was crystallized from H_2O/THF (v/v, 1/1). The crude product was rinsed with Et_2O and dried: 63 mg; 86% yield; R_f 0.72 (MeOH/EtOAc, 1/1); mp 244–246 °C; ^1H NMR (DMSO-d_6) δ 0.82 (m, 6H), 1.03 (d, 3H), 1.52 (m, 2H), 1.62 (m, 1H), 2.55–3.20 (m, 4H), 4.00–4.75 (m, 5H), 6.95–7.80 (m, 18H), 7.92, 8.11, 8.56, 8.67 (d, 4H); ^{13}C NMR (DMSO-d_6) δ 20.1, 21.3, 23.2, 24.1, 36.8, 37.1, 40.2, 49.9, 51.6, 54.5, 58.5, 66.4, 127.4, 128.2, 128.5, 129.4, 129.5, 129.6, 131.4, 132.5, 132.9, 133.9, 135.1, 137.2, 139.2, 143.7, 166.6, 171.0, 171.2, 171.9, 171.9, 172.2, 195.5; MS (FAB$^+$) *m/e* (rel intensity) 701 (MH^+, 27), 155 (33), 119 (52), 105 (100); HRMS calcd for $C_{37}H_{45}N_6O_8$ (MH^+), 701.3299; found, 701.3301. Anal. calcd for $C_{37}H_{44}N_6O_8·H_2O$: C, 61.83; H, 6.45; N, 11.69. Found: C, 61.81; H, 6.38; N, 11.39.

***N*-Boc-Asn-Phe-Thr-NH_2 (8a).** To a stirred solution of *N*-Boc-Phe (**6a**, 398 mg, 1.5 mmol) in 6 mL dry THF at −10 °C was added NMM (165 μL, 1.5 mmol) followed by iBCF (215 μL, 1.65 mmol). The mixture was stirred at −10 °C for 15 min and a precooled mixture of HCl·Thr-NH_2 (231 mg, 1.5 mmol) and NMM (165 μL, 1.5 mmol) in 4 mL dry DMF was added. The reaction mixture was stirred at −10 °C for 1 h and at rt for 10 h. Solvent was removed in vacuo and 6 mL 5% citric acid solution was added to triturate the slightly brown residue. The white precipitate was collected and rinsed with water and Et_2O. The crude product was further purified by Et_2O trituration to give 466 mg (85%) of *N*-Boc-Phe-Thr-NH_2 as a white solid: R_f 0.61 (MeOH/EtOAc, 1/1); mp 154–155 °C; IR (KBr): 3430, 3367, 3339, 3030, 2981, 2938, 1687, 1659, 1525, 1166 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.02 (d, 2H), 1.30 (s, 9H), 2.60–3.10 (m, 2H), 4.07 (m, 1H), 4.10 (m, 1H), 4.17 (m, 1H), 4.95 (d, 1H), 7.08–7.20 (m, 3H), 7.26 (m, 5H), 7.57 (d, 1H); ^{13}C NMR (DMSO-d_6) δ 19.9, 28.2, 36.9, 56.1, 57.8, 66.3, 78.4, 126.1, 128.0, 129.1, 138.1, 155.3, 171.6, 171.9.

N-Boc-Phe-Thr-NH_2 (292 mg, 0.80 mmol) was dissolved in 5 mL TFA/CH_2Cl_2 (v/v, 1/1) at rt and the mixture was stirred for 30 min. The solvent was removed in vacuo and the resulting clear oil was triturated with Et_2O. The white precipitate was collected, dried, and directly used for the following reaction. R_f 0.51 (MeOH/EtOAc, 1/1). *N*-Boc-Asn (186 mg, 0.80 mmol), DCC (198 mg, 0.96 mmol), and HOBt (162 mg, 1.2 mmol) were dissolved in 5 mL dry DMF at 0 °C while stirring. A precooled solution of H-Phe-Thr-NH_2·TFA (0.80 mmol) and NMM (88 μL, 0.80 mmol) in 4 mL dry DMF was added. The reaction mixture was stirred at 0 °C for 1 h and at rt for 16 h. The precipitate was filtered and the filtrate was concentrated in vacuo. The resulting yellow residue was triturated with EtOAc/Et_2O (v/v, 1/1). The precipitate collected was further triturated with EtOAc. The white precipitate was collected and air dried to give 310 mg (81%) of the desired product. R_f 0.70 (MeOH/EtOAc, 1/3); mp 207–209 °C; ^1H NMR (DMSO-d_6) δ 1.01 (d, 2H), 1.36 (s, 9H), 2.38 (m, 2H), 2.80–3.10 (m, 2H), 4.03 (m, 1H), 4.08 (m, 1H), 4.23 (m, 1H), 4.56 (m, 1H), 4.83 (d, 1H), 6.93 (s, 2H), 7.05 (d, 2H), 7.22 (m, 5H), 7.31 (d, 1H), 7.84 (d, 1H), 7.94 (d, 1H); ^{13}C NMR (DMSO-d_6) δ 19.9, 28.1, 33.3, 37.2, 51.3, 53.9, 58.2, 66.3, 78.2, 126.0, 127.8, 129.1, 137.4, 154.8, 170.6, 171.2, 171.5, 171.8.

***N*-Bz-Asn-Phe-Thr-NH_2 (3a).** *N*-Boc-Asn-Phe-Thr-NH_2 (**8a**, 184 mg, 0.384 mmol) was dissolved in 5 mL TFA/CH_2Cl_2 (v/v, 1/1) and the mixture was stirred for 30 min at rt. Solvents were removed in vacuo and the resulting clear oil was triturated with Et_2O. The white precipitate was collected, rinsed with Et_2O, dried, and used directly for the following reaction; R_f 0.32 (MeOH/EtOAc, 1/3). The product, H-Asn-Phe-Thr-NH_2·TFA, obtained was dissolved in 4 mL water and Et_3N (60 μL, 0.38 mmol) was added at 0 °C while stirring. 4 mL dioxane and Bz_2O (96 mg, 0.422 mmol) were added and the mixture

was stirred at 0 °C for 5 min. 30 μL Et_3N was further added. The reaction mixture was stirred at 0 °C for 1 h and at rt for 14 h and then concentrated in vacuo. The resulting residue was dissolved in minimum amount of hot H_2O/THF (v/v, 1/1). This solution was cooled, applied to a column packed with Dowex 50W-X8 resin, and then eluted with H_2O/THF (v/v, 1/1). Suitable fractions were combined and concentrated in vacuo. The white precipitate obtained was dissolved in hot THF and the solution was cooled to rt and anhydrous Et_2O was added. The white precipitate was collected and air dried to give 152 mg (82%) of the desired product: mp 250–252 °C; ^{1}H NMR (DMSO-d_6) δ 1.02 (d, 2H), 2.40–2.70 (m, 2H), 2.75–3.15 (m, 2H), 4.03 (m, 1H), 4.07 (m, 1H), 4.54 (m, 1H), 4.86 (m, 1H), 4.96 (d, 1H), 7.05–7.30 (m, 5H), 6.90–7.40 (m, 4H), 7.54 (d, 1H), 7.40–7.9 (m, 5H), 8.12 (d, 1H), 8.61 (d, 1H); ^{13}C NMR (DMSO-d_6) δ 20.0, 36.6, 36.9, 50.4, 54.1, 58.3, 66.3, 126.0, 127.3, 127.8, 128.0, 129.1, 131.2, 133.7, 137.5, 166.1, 170.6, 170.8, 171.6, 171.8; MS (CI) *m*/*e* (rel. intensity) 484 (MH^+, 38), 219 (43), 122 (100); HRMS calcd for $C_{24}H_{30}N_5O_6$ (MH^+), 484.2196; found, 484.2198. Anal. calcd for $C_{24}H_{29}N_5O_6 \cdot 0.5H_2O$: C, 58.53; H, 6.14; N, 14.22. Found: C, 58.58; H, 6.40; N, 14.02.

***N*-Boc-Asn-Bpa-Thr-NH_2 (8b).** *N*-Boc-Bpa (**6b**, 296 mg, 0.80 mmol), EDC·HCl (161 mg, 0.84 mmol), and HOBt (162 mg, 1.20 mmol) were dissolved in 3 mL dry THF at 0 °C while stirring. NMM (92 μL, 0.84 mmol) was added and the mixture was stirred at 0 °C under nitrogen for 10 min. A precooled mixture of HCl·Thr-NH_2 (124 mg, 0.80 mmol) and NMM (88 μL, 0.80 mmol) in 3 mL dry DMF was added and the mixture was stirred at 0 °C for 1 h and at rt for 1 day. However, TLC still showed unreacted starting material. Thirty-eight milligrams of EDC·HCl (0.20 mmol) and NMM (22 μL, 0.20 mmol) was further added at 0 °C and the reaction mixture was stirred at rt for another day. The reaction mixture was concentrated in vacuo and the yellowish residue was partitioned between EtOAc and 5% citric acid solution. The organic layer was separated and washed with saturated $NaHCO_3$ solution, dried, and concentrated in vacuo. The resulting syrup was dissolved in minimum amount of MeOH and hexane was slowly added. The precipitate was collected and dried to give 277 mg (74%) of *N*-Boc-Bpa-Thr-NH_2 as a white solid: R_f 0.64 (MeOH/EtOAc/AcOH, 4/4/1); mp 143–145 °C; ^{1}H NMR (CD_3OD) δ 1.17 (d, 3H), 1.30 (s, 9H), 2.96–3.31 (m, 2H), 4.23 (m, 1H), 4.28 (m, 1H), 4.43 (m, 1H), 7.44–7.78 (m, 9H); ^{13}C NMR (DMSO-d_6/CD_3OD) δ 20.7, 29.1, 38.6, 57.4, 59.7, 68.1, 80.7, 129.8, 130.9, 131.1, 131.3, 133.9, 137.2, 139.1, 144.8, 157.6, 173.9, 174.6, 197.7.

N-Boc-Bpa-Thr-NH_2 (200 mg, 0.43 mmol) was dissolved in 6 mL TFA/CH_2Cl_2 (v/v, 1/1) and the mixture was allowed to set at rt for 30 min. The mixture was concentrated in vacuo and the colorless syrup was triturated with Et_2O. The white precipitate was collected and rinsed with Et_2O and then dissolved in 2 mL dry DMF. NMM (47 μL, 0.43 mmol) was added and the mixture was cooled to 0 °C. *N*-Boc-Asn (99 mg, 0.43 mmol), EDC·HCl (86 mg, 0.45 mmol), and HOBt (86 mg, 0.64 mmol) were dissolved in 2 mL dry THF at 0 °C under nitrogen. NMM (49 μL, 0.45 mmol) was added and the mixture was stirred for 10 min at 0 °C. The precooled solution of TFA·Bpa-Thr-NH_2 (0.43 mmol) and NMM (47 μL, 0.43 mmol) in 2 mL dry DMF was added and the reaction mixture was stirred at 0 °C for 1 h and at rt for 15 h. The reaction mixture was concentrated in vacuo and the brown residue was triturated with 4 mL 5% citric acid. The precipitate collected was further triturated with saturated $NaHCO_3$ solution. The crude product collected was crystallized from MeOH/H_2O to give 190 mg (76%) of a white solid: R_f 0.56 (MeOH/EtOAc/Et_3N, 3/3/1); mp 204–207 °C; ^{1}H NMR (CD_3OD) δ 1.17 (d, 3H), 1.36 (s, 9H), 2.38 (m, 2H), 2.96–3.30 (m, 2H), 4.18 (m, 1H), 4.27 (m, 1H), 4.41 (m, 1H), 4.94 (m, 1H), 7.44–7.75 (m, 9H); ^{13}C NMR (DMSO-d_6/CD_3OD) δ 20.1, 28.2, 37.3, 40.2, 51.4, 53.6, 58.4, 66.5, 78.3, 128.6, 129.5, 129.6, 129.7, 132.5, 135.0, 137.4, 143.2, 155.1, 170.6, 171.5, 171.8, 172.1, 195.6; MS (FAB^+) *m*/*e* (rel. intensity) 584 (MH^+, 60), 484 (100), 224 (66), 79 (66); HRMS calcd for $C_{29}H_{38}N_5O_8$ (MH^+), 584.2720; found, 584.2719.

***N*-Bz-Asn-Bpa-Thr-NH_2 (3b).** *N*-Boc-Asn-Bpa-Thr-NH_2 (**8b**, 150 mg, 0.26 mmol) was dissolved in 5 mL TFA/CH_2Cl_2 (v/v, 1/1) and the mixture was allowed to set at rt for 30 min. The mixture was concentrated in vacuo and the colorless oil was triturated with Et_2O. The white precipitate was collected by filtration and rinsed with Et_2O and then dissolved in 9 mL H_2O/dioxane/DMF (v/v/v, 1/1/1). Et_3N (36 μL, 0.26 mmol) and Bz_2O (64 mg, 0.28 mmol) were added and the mixture was stirred for 5 min. Et_3N (20 μL, 0.14 mmol) was further added and the reaction mixture was stirred at rt for 12 h. The mixture was concentrated in vacuo and the white residue was dissolved in warm H_2O/THF (v/v, 1/1). Dowex 50W X8 resin was added to the cooled mixture and the mixture was stirred slightly. The filtrate was concentrated in vacuo and the white residue was crystallized from H_2O/THF. The crude product was further recrystallized from MeOH/THF to give 122 mg white solid, 81% yield: R_f 0.65 (MeOH/EtOAc, 1/1); mp 260–261 °C; ^{1}H NMR (DMSO-d_6/CD_3OD) δ 1.14 (d, 3H), 2.55–2.85 (m, 2H), 3.00–3.35 (m, 2H), 4.14 (m, 1H), 4.21 (m, 1H), 4.69 (m, 1H), 4.89 (m, 1H), 7.30–7.80 (m, 14H); ^{13}C NMR (DMSO-d_6/CD_3OD) δ 20.1, 36.5, 37.0, 50.5, 53.8, 58.5, 66.5, 127.5, 128.2, 128.5, 129.5, 131.5, 132.5, 133.8, 134.9, 137.3, 143.2, 166.3, 170.7, 171.1, 172.0, 172.1, 195.5; MS (FAB^+) *m*/*e* (rel. intensity) 588 (MH^+, 9), 233 (20), 157 (33), 105 (22), 79 (100); HRMS calcd

for $C_{31}H_{34}N_5O_7$ (MH^+), 588.2458; found, 588.2459. Anal. calcd for $C_{31}H_{33}N_5O_7$: C, 63.36; H, 5.66; N, 11.92. Found: C, 63.36; H, 5.75; N, 11.86.

N^{α}-Boc-N^{ε}-Bz-L-lysine (9a). N^{ε}-Bz-L-lysine (**12**, 1.0 g, 4.0 mmol) was dissolved in 20 mL NaOH solution (0.20 M, 4.0 mmol) at 0 °C and 20 mL dioxane was added. $(Boc)_2O$ (960 mg, 4.4 mmol) was added and the reaction mixture was stirred at rt overnight. The mixture was adjusted by NaOH solution to pH 9 and was washed with Et_2O to remove unreacted $(Boc)_2O$. The aqueous layer was cooled to 0 °C and 2 N HCl was added slowly while stirring to adjust pH to 3. The acidic aqueous layer was washed with EtOAc (3×) and the organic layers were combined, dried, and concentrated in vacuo to give 1.35 g white foam in 96% yield: R_f 0.51 (EtOAc/MeOH, 1/1); ^{1}H NMR ($CDCl_3/CD_3OD$) δ 1.35–1.50 (m, 2H), 1.41 (s, 9H), 1.55–1.90 (m, 4H), 3.41 (t, 2H), 4.22 (m, 1H), 7.35–7.80 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 22.5, 28.1, 28.6, 31.8, 39.7, 53.1, 79.7, 127.1, 128.3, 131.4, 134.0, 155.8, 168.5, 175.2; MS (FAB^+) *m/e* (rel intensity) 351 (MH^+, 11), 295 (24), 251 (79), 105 (100); HRMS calcd for $C_{18}H_{27}N_2O_5$ (MH^+), 351.1920; found, 351.1902.

***N*-Boc-Asn-Lys(N^{ε}-Bz)-Thr-NH_2 (10a).** N^{α}-Boc-Lys(N^{ε}-Bz)-OH (**9a**, 526 mg, 1.50 mmol), HOBt (304 mg, 2.25 mmol), and H-Thr-NH_2·HCl (232 mg, 1.50 mmol) were dissolved in 10 mL dry DMF and then cooled to 0 °C while stirring. NMM (338 μL, 3.08 mmol) was added followed by EDC·HCl (303 mg, 1.58 mmol). The reaction mixture was stirred at 0 °C for 1 h and at rt for 18 h and then concentrated in vacuo. The resulting brown residue was partitioned between 30 mL EtOAc and 10 mL 5% citric acid solution. The organic layer was further washed with saturated $NaHCO_3$ solution, dried, and concentrated in vacuo. The white residue obtained was crystallized from EtOAc/MeOH to give 532 mg (79%) of a white solid: R_f 0.69 (EtOAc/MeOH, 1/1); mp 142–144 °C; ^{1}H NMR (CD_3OD) δ 1.08 (d, 3H), 1.37 (s, 9H), 1.42 (m, 2H), 1.59 (m, 2H), 1.60–1.85 (m, 2H), 3.32 (m, 2H), 3.98 (m, 1H), 4.22 (m, 2H), 7.35–7.80 (m, 5H); ^{13}C NMR (CD_3OD) δ 20.4, 24.4, 28.9, 30.2, 32.3, 40.8, 56.9, 59.6, 68.0, 81.1, 128.4, 129.6, 132.7, 135.8, 158.5, 170.3, 175.2, 175.6; MS (FAB^+) *m/e* (rel intensity) 451 (MH^+, 25), 351 (58), 188 (29), 105 (46), 79 (100); HRMS calcd for $C_{22}H_{35}N_4O_6$ (MH^+), 451.2556; found, 451.2544.

N-Boc-Asn (139 mg, 0.60 mmol), HOBt (122 mg, 0.90 mmol), and H-Lys(N^{ε}-Bz)-Thr-NH_2·TFA (from TFA-mediated N^{α}-deprotection of 270 mg (0.60 mmol) N^{α}-Boc-(N^{ε}-Bz)Lys-Thr-NH_2) were dissolved in 5 mL dry DMF and the mixture was cooled to 0 °C while stirring. NMM (135 μL, 1.23 mmol) was added followed by EDC·HCl (121 mg, 0.63 mmol). The reaction mixture was stirred at 0 °C for 1 h and at rt for 1 day and then concentrated in vacuo. The resulting residue was partitioned between EtOAc and 5% citric acid solution. The organic layer was separated and washed with saturated $NaHCO_3$ solution and then concentrated in vacuo. The resulting white residue was triturated with H_2O/MeOH (v/v, 2/1) at 0 °C, collected, and dried to give 273 mg (81%) of a white solid. The crude product was further crystallized from MeOH/Et_2O. R_f 0.58 (EtOAc/MeOH, 3/1); mp 196–198 °C; ^{1}H NMR (CD_3OD/DMSO-d_6) δ 1.02 (d, 3H), 1.31 (s, 9H), 1.42 (m, 2H), 1.50 (m, 2H), 1.60–1.85 (m, 2H), 2.45–2.65 (m, 2H), 3.24 (t, 2H), 4.04 (m, 1H), 4.07 (m, 1H), 4.21 (m, 1H), 4.30–4.40 (covered by HDO, 1H), 7.35–7.80 (m, 5H); ^{13}C NMR (DMSO-d_6) δ 20.1, 22.7, 28.2, 28.8, 31.5, 37.1, 39.1, 51.4, 53.1, 58.4, 66.4, 78.3, 127.2, 128.2, 131.0, 134.7, 155.1, 166.1, 171.7, 171.7, 171.9, 172.3.

***N*-Bz-Asn-Lys(N^{ε}-Bz)-Thr-NH_2 (4a).** *N*-Boc-Asn-Lys(N^{ε}-Bz)-Thr-NH_2 (**10a**, 169 mg, 0.30 mmol) was dissolved in 4 mL TFA/CH_2Cl_2 (v/v, 1/1) at rt and was allowed to set for 30 min. The clear solution was concentrated in vacuo and the colorless oil was triturated with Et_2O. The white precipitate was collected, rinsed with Et_2O, dried, and then dissolved in 6 mL DMF/H_2O (v/v, 2/1) mixed solvent. The mixture was cooled to 0 °C and Et_3N (42 μL, 0.30 mmol) was added followed by Bz_2O (71 mg, 0.33 mmol). The reaction mixture was stirred for 5 min and Et_3N (21 μL, 0.15 mmol) was further added. The reaction mixture was stirred at rt overnight and then concentrated in vacuo. The resulting white residue was dissolved in 20 mL THF/H_2O (v/v, 1/1) and 5 mL bed volume of Dowex 50W-X8 resin (H^+ form) was added and the mixture was gently stirred and then filtered. The filtrate was concentrated in vacuo and the resulting white residue was crystallized from THF/H_2O (v/v, 1/1) and then triturated with Et_2O. The white precipitate was collected, washed with Et_2O, and dried to give 138 mg (81%) of a white powder. R_f 0.40 (EtOAc/MeOH, 3/1); mp 266–268 °C; ^{1}H NMR (CD_3OD/DMSO-d_6) δ 0.99 (d, 3H), 1.32 (m, 2H), 1.47 (m, 2H), 1.55–1.80 (m, 2H), 2.62 (m, 2H), 3.19 (m, 2H), 3.99 (m, 1H), 4.03 (m, 1H), 4.22 (m, 1H), 4.74 (m, 1H), 7.35–7.85 (m, 5H); ^{13}C NMR (DMSO-d_6) δ 20.1, 22.8, 28.9, 31.2, 37.0, 39.1, 50.8, 53.2, 58.2, 66.4, 127.2, 127.5, 128.3, 131.0, 131.5, 134.0, 134.7, 166.1, 166.4, 171.4, 171.6, 171.9, 172.2; MS (FAB^+) *m/e* (rel intensity) 569 (MH^+, 28), 105 (73), 79 (100); HRMS calcd for $C_{28}H_{37}N_6O_7$ (MH^+), 569.2724; found, 569.2725. Anal. calcd for $C_{28}H_{36}N_6O_7$: C, 59.14; H, 6.38; N, 14.78. Found: C, 59.31; H, 6.05; N, 14.63.

N^{α}-Boc-N^{ε}-(4-Bz)Bz-L-Lysine (9b). N^{α}-Boc-N^{ε}-Cbz-L-lysine-OTMSE (**13**, 1.76 g, 3.66 mmol) was dissolved in 12 mL MeOH and 120 mg Pd/C catalyst was added. The reaction mixture was shaken in a Parr shaker under H_2

pressure of 40 psi for 15 h. The mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo to give 1.24 g (98% yield) *N*-Boc-L-Lysine 2-trimethylsilylethyl ester as a clear oil. R_f 0.13 (EtOAc/MeOH, 3/1); ^{1}H NMR ($CDCl_3$) δ 0.03 (s, 9H), 0.98 (t, 2H), 1.42 (s, 9H), 1.50–1.95 (m, 6H), 2.79 (m, 2H), 4.19 (m, 3H), 5.19 (br, 1H), 5.36 (br, 2H); ^{13}C NMR ($CDCl_3$) δ -1.8, 17.0, 22.3, 28.0, 30.0, 31.8, 40.2, 53.1, 63.1, 79.1, 155.2, 172.5.

N-Boc-L-lysine-OTMSE (1.24 g, 3.58 mmol) was dissolved in 20 mL dry THF and 4-benzoylbenzoic acid (810 mg, 3.58 mmol) and NMM (433 μL, 3.94 mmol) were added. The mixture was cooled to 0 °C and EDC·HCl (755 mg, 3.94 mmol) was added and the mixture was stirred at 0 °C for 1 h and at rt for 20 h. The reaction mixture was concentrated in vacuo and the resulting brown residue was partitioned between EtOAc and 5% citric acid solution. The organic layer was further washed with saturated $NaHCO_3$ solution and brine, dried by anhydrous $MgSO_4$, and concentrated in vacuo. A slightly yellowish oil (1.83 g) was obtained with 92% yield: R_f 0.68 (EtOAc/MeOH, 3/1); ^{1}H NMR ($CDCl_3$) δ 0.04 (s, 9H), 1.01 (t, 2H), 1.42 (s, 9H), 1.45–2.00 (m, 6H), 3.49 (m, 2H), 4.22 (t, 2H), 4.27 (m, 1H), 5.14 (d, 1H), 6.49 (m, 1H), 7.45–7.90 (m, 9H); ^{13}C NMR ($CDCl_3$) δ -1.7, 17.1, 22.5, 28.1, 28.7, 31.8, 39.5, 53.3, 63.3, 79.3, 127.1, 128.2, 129.5, 129.6, 129.8, 132.7, 136.7, 137.8, 139.4, 155.5, 166.8, 172.7, 195.8.

N^{α}-Boc-N^{ε}-(4-Bz)Bz-L-lysine-OTMSE (**14**, 1.80 g, 3.24 mmol) was dissolved in 4 mL THF and 1.0 M tetrabutylammonium fluoride (9.7 mL, 9.7 mmol) was added and the reaction mixture was stirred at rt overnight. The mixture was concentrated in vacuo and then partitioned between EtOAc and 5% citric acid solution. The organic layer was then washed with saturated $NaHCO_3$ solution and the resulting aqueous layer was cooled to 0 °C, adjusted to pH 3 by cold saturated $KHSO_4$ solution under vigorous stirring. The acidic aqueous layer was washed with EtOAc (3×) and the organic layers were combined, dried, and concentrated in vacuo to give 1.32 g slightly yellowish oil with 90% yield: R_f 0.39 (EtOAc/MeOH, 3/1); ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.45–2.00 (m, 6H), 3.48 (m, 2H), 4.29 (m, 1H), 5.32 (d, 1H), 6.88 (m, 1H), 7.45–7.95 (m, 9H); ^{13}C NMR ($CDCl_3$) δ 19.4, 23.5, 28.1, 31.7, 39.8, 53.2, 60.3, 79.7, 127.2, 128.3, 129.7, 129.8, 132.8, 136.6, 137.5, 139.5, 155.9, 167.3, 175.4, 195.8; MS (FAB^+) 455 (MH^+, 4), 355 (74), 242 (100), 209 (76), 142 (36); HRMS calcd for $C_{25}H_{31}N_2O_6$ (MH^+), 455.2182; found, 455.2170.

***N*-Boc-Asn-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2 (10b).** N^{α}-Boc-Lys[N^{ε}-(4-Bz)Bz]-OH (**9b**, 455 mg, 1.0 mmol) and HOBt (203 mg, 1.5 mmol) were dissolved in 8 mL dry DMF and the mixture was cooled to 0 °C. NMM (231 μL, 2.1 mmol), EDC·HCl (211 mg, 1.1 mmol), and HCl·Thr-NH_2 (155 mg, 1.0 mmol) were added. The reaction mixture was stirred at 0 °C for 1 h and at rt for 18 h and then concentrated in vacuo. The resulting brown residue was partitioned between EtOAc and 5% citric acid solution. The organic layer was further washed with saturated $NaHCO_3$ solution, dried, and concentrated in vacuo. The resulting white residue was crystallized from MeOH/EtOAc to give 392 mg (71%) of N^{α}-Boc-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2 as a white solid: R_f 0.64 (EtOAc/MeOH, 3/1); mp 139–141 °C; ^{1}H NMR (CD_3OD/DMSO-d_6) δ 1.17 (d, 3H), 1.46 (s, 9H), 1.45–1.95 (m, 6H), 3.44 (t, 2H), 4.24 (m, 1H), 4.41 (m, 1H), 4.55 (m, 1H), 7.55–8.05 (m, 9H); ^{13}C NMR (DMSO-d_6) δ 20.0, 23.1, 28.2, 28.8, 31.0, 55.0, 57.7, 66.3, 78.5, 127.4, 128.7, 129.5, 129.7, 133.0, 136.7, 138.1, 139.51 155.8, 165.5, 172.2, 172.3, 195.5; MS (FAB^+) *m*/*e* (rel. intensity) 555 (MH^+, 12), 455 (37), 209 (29), 155 (52), 119 (81), 85 (100); HRMS calcd for $C_{29}H_{38}N_4O_7$ (MH^+), 555.2819; found, 555.2795.

N-Boc-Asn (128 mg, 0.55 mmol) and HOBt (111 mg, 0.83 mmol) were dissolved in 2 mL dry DMF and then cooled to 0 °C while stirring. EDC·HCl (117 mg, 0.61 mmol) and NMM (67 μL, 0.61 mmol) were added and the mixture was stirred at 0 °C under nitrogen for 5 min. A precooled solution of H-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2·TFA (from TFA-mediated N^{α}-deprotection of 305 mg (0.55 mmol) N^{α}-Boc-N^{ε}-(4-Bz)Bz-Lys-Thr-NH_2) and NMM (61 μL, 0.55 mmol) in 2.5 mL dry DMF was added and the reaction mixture was stirred at 0 °C for 1 h and at rt for 18 h. The mixture was concentrated in vacuo and the brown residue was triturated with 4 mL 5% citric acid solution. The white precipitate was collected and further triturated with saturated $NaHCO_3$ solution. The white precipitate was collected by filtration and washed with water. The crude product was crystallized from MeOH/H_2O to give 315 mg white solid with 86% yield: R_f 0.66 (EtOAc/MeOH, 1/1); mp 197–199 °C; ^{1}H NMR (CD_3OD/DMSO-d_6) δ 1.15 (d, 3H), 1.41 (s, 9H), 1.50 (m, 2H), 1.66 (m, 2H), 1.75–2.05 (m, 2H), 2.55–2.80 (m, 2H), 3.40 (t, 2H), 4.18 (m, 1H), 4.21 (m, 1H), 4.32 (m, 1H), 4.41 (m, 1H), 7.53–7.98 (m, 9H); ^{13}C NMR (DMSO-d_6) δ 20.2, 22.9, 28.3, 29.0, 31.6, 37.3, 39.4, 51.5, 53.3, 58.6, 66.6, 78.7, 127.6, 128.9, 129.8, 130.0, 133.2, 137.0, 138.3, 139.4, 155.5, 165.8, 172.0, 172.1, 172.4, 172.7, 195.8; MS (FAB^+) *m*/*e* (rel. intensity) 669 (MH^+, 16), 569 (28), 209 (34), 85 (32), 79 (100); HRMS calcd for $C_{33}H_{45}N_6O_9$ (MH^+), 669.3248; found, 669.3269.

***N*-Bz-Asn-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2 (4b).** *N*-Boc-Asn-Lys[N^{ε}-(4-Bz)Bz]-Thr-NH_2 (**10b**, 150 mg, 0.22 mmol) was dissolved in 4 mL TFA/CH_2Cl_2 (v/v, 1/1) and was allowed to set at rt for 30 min. The clear solution was concentrated in vacuo and the resulting colorless oil was

triturated with Et_2O. The white precipitate was collected and rinsed with Et_2O and then dissolved in 6 mL DMF/H_2O (v/v, 2/1). Et_3N (31 μL, 0.22 mmol) was added followed by Bz_2O (56 mg, 0.25 mmol). The reaction mixture was stirred for 5 min and Et_3N (15 μL, 0.11 mmol) was further added and the mixture was stirred at rt overnight. The reaction mixture was concentrated in vacuo and the residue was triturated with Et_2O. The white precipitate was collected and redissolved in hot THF/H_2O (v/v, 1/1). The solution was cooled to rt and 5 mL bed volume of Dowex 50W-X8 (H^+) resin was added and the mixture was gently stirred. The mixture was filtered and the filtrate was concentrated in vacuo. The resulting white residue was crystallized from H_2O/THF (v/v, 1/1). 132 mg white solid was obtained, 88% yield. The product was further triturated with Et_2O to give the pure tripeptide product. R_f 0.64 (EtOAc/MeOH, 1/1); mp 244–246 °C; ^{1}H NMR (CD_3OD/DMSO-d_6) δ 1.03 (d, 3H), 1.39 (m, 2H), 1.53 (m, 2H), 1.60–1.80 (m, 2H), 2.65 (m, 2H), 3.27 (m, 2H), 4.05 (m, 2H), 4.27 (m, 1H), 4.79 (m, 1H), 7.45–8.00 (m, 9H); ^{13}C NMR (DMSO-d_6) δ 20.2, 22.9, 28.8, 31.3, 37.0, 39.3, 50.9, 53.3, 58.3, 66.5, 127.5, 127.6, 128.4, 128.8, 129.6, 129.8, 131.6, 133.1, 134.0, 136.8, 138.1, 139.1, 165.5, 166.5, 171.6, 171.8, 172.0, 172.3, 195.6; MS (FAB$^+$) *m/e* (rel. intensity) 673 (MH^+, 14), 292 (17), 209 (30), 105 (60), 79 (100); HRMS calcd for $C_{35}H_{41}N_6O_8$ (MH^+), 673.2986; found, 673.2991. Anal. calcd for $C_{35}H_{40}N_6O_8{\cdot}0.5H_2O$: C, 61.66; H, 6.06; N, 12.33. Found: C, 61.67; H, 6.42; N, 12.24.

Standard OST assay. The synthetic peptides **1**–**4** were tested as OST substrates using either [^{3}H]Dol-PP-OS or [^{3}H]Dol-PP-DS as previously described.[7,26] The OST assay procedure using [^{3}H]Dol-PP-DS was carried out as follows: The assay mixture contained ca. 6000 dpm [^{3}H]Dol-PP-DS, 50 mM Tris, pH 7.5, 1% Triton X-100, 1 mM $MnCl_2$, 360 μM tested peptide in DMSO (final [DMSO] = 5% v/v), and 600 μg of P_{40} yeast microsome[7] in a total volume of 100 μL. The assay mixture was shaken at 250 rpm for 2 h at rt, then quenched by the addition of 3 mL cold $CHCl_3$/MeOH (v/v, 3/2). The mixture was allowed to set on ice for 30 min and the layers separated by centrifugation at 1000 *g* for 15 min. The supernatant was removed and extracted with 1 mL of 4 mM $MgCl_2$. The biphasic mixture was thoroughly agitated (Vortex) and then centrifuged at 1000 *g* for another 15 min. The upper aq layer containing the water soluble ^{3}H-labeled glycopeptide was carefully removed. A significant increase in ^{3}H radioactivity observed in the aq layer compared with that observed in the aq layer from a control assay not containing the peptide substrate provided evidence for the formation of ^{3}H-labeled glycopeptide.[26] The relative glycosylation activity of each tested peptide was determined by dividing the net increase of ^{3}H radioactivity in the aq layer observed in the presence of the tested peptide by that observed in the standard assay using Bz-Asn-Leu-Thr-NH_2. Each assay was run in duplicate and the result given (Table 1) is the average of the duplicate assays. For each inhibitor evaluated, the percentage inhibition was determined as follows. Activity in either the absence or presence of an inhibitor was obtained by determining the ^{3}H (%) in the aqueous phase due to formation of the glycopeptide product.[26]

***K*$_i$ determination of 3b and 4b.** Each OST assay mixture contained 200 μM chemically synthesized $(GlcNAc)_2$-PP-dolichol, 50 mM Tris, pH 7.5, 1% Triton X-100, 1 mM $MnCl_2$, 90, 180, or 360 μM [^{14}C]Bz-Asn-Leu-Thr-NH_2, 0, 18, 45, 90, 180, or 270 μM Bz-Asn-Bpa-Thr-NH_2 (**3b**) or Bz-Asn-[N^{ε}-(4-Bz)Bz]Lys-Thr-NH_2 (**4b**) in DMSO (final [DMSO] = 10% v/v), and 400 μg of P_{40} yeast microsome in a total volume of 100 μL. The assay was worked up as described above and the aqueous layer which contained the biosynthetic glycopeptide was analyzed by reverse phase HPLC. A gradient profile of 10–25% MeOH in 20 min followed with 25% MeOH isocratic condition was employed. The unreacted [^{14}C]Bz-Asn-Leu-Thr-NH_2 (t_R = 69 min) and the glycopeptide product [^{14}C]Bz-Asn$(GlcNAc)_2$-Leu-Thr-NH_2 (t_R = 76.5 min) were separated and quantitated using a liquid scintillation counter. The rate of ^{14}C-labeled glycopeptide formation was determined and the value of K_i was obtained by a Dixon plot of these data (Fig. 2).

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