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Enzymatic Synthesis of N- and O-Linked Glycopeptides

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Abstract: This paper describes the study of kinetically controlled enzymatic coupling of *N*- and *O*-glycopeptide fragments using subtilisin BPN′ and two of its variants developed for use in high concentrations of dimethylformamide and in aqueous solution, respectively. Glycosyl amino acids were exploited as the P_1, P_2, P_3, P_1', P_2', or P_3' residue in the enzymatic coupling. Glycosyltransferase-mediated glycosylation of the glycopeptide fragments obtained prior to or after enzymatic peptide bond formation is demonstrated.

Proteases have proven to be useful catalysts for the stereoselective and racemization-free coupling of peptide fragments.[1] A logical extension of this strategy is their application to the synthesis of glycopeptides. Although glycopeptides[2] have been synthesized chemically by peptide chain elongation[3] starting from appropriate glycosyl amino acid derivatives and by chemical[4] and enzymatic[5] glycosylation of glycopeptides, enzymatic coupling of glycopeptide fragments has not yet been reported. We envisioned that the enzymatic synthesis may not require protection of amino acid side chain functions and sugar hydroxy groups due to the high stereo- and regioselectivity of most proteases under common reaction conditions. Thus, the glycopeptide building blocks obtained in this manner may be further converted to oligosaccharyl peptides with glycosyltransferases[6] without additional protection steps (Figure 1).

Reported here is the study of the coupling of *N*- and *O*-glycosyl amino acids and glycopeptide fragments catalyzed by the serine protease subtilisin BPN′, its stable variant 8397,[7] and a thiosubtilisin[8] derived from 8397. The 8397 variant was designed for synthesis in anhydrous or high concentrations of dimethylformamide (DMF),[7] and the thiosubtilisin variant was developed

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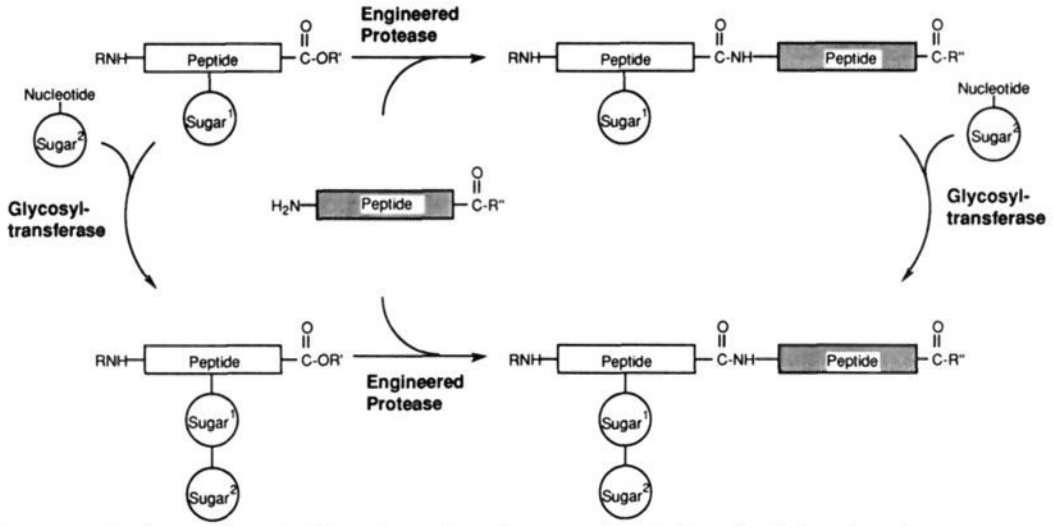

Figure 1. New strategy for glycopeptide synthesis based on engineered proteases (peptide ligases) and glycosyltransferases.

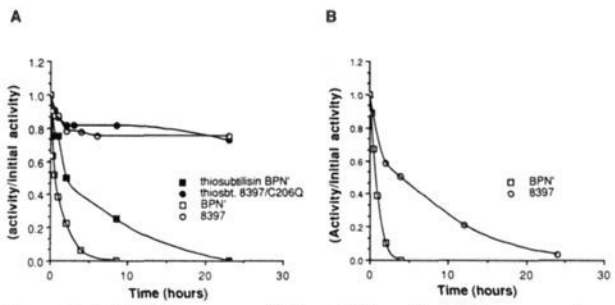

Figure 2. A: Stability courses of thiosubtilisin and subtilisin preparations at 50 °C in buffer (10 mM Tris/5 mM dithiothreitol, pH 8, for thiosubtilisins; 50 mM MES, pH 6.0, for subtilisins). B: Stability courses of subtilisin BPN′ and 8397 in buffer (50 mM MES, pH 6.0) in the presence of 50% DMF at 50 °C. Stability courses were generated by measuring activities toward the substrate succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide as a function of time.

for peptide synthesis in aqueous solution.[8] Though subtilisin 8397 was developed for use in anhydrous DMF ($t_{1/2}$ = 14 days at 25 °C compared to 30 min for the wild-type enzyme),[7a] it is also more stable than the wild-type enzyme in aqueous solution and in 50% DMF at 50 °C (Figure 2). The thiosubtilisin variant is also much more stable than the wild-type thiosubtilisin in aqueous solution at 50 °C. Figure 3 shows the energy diagrams for reactions catalyzed by subtilisin BPN′ and the thiosubtilisin, and the tetrahedral intermediates for the deacylation via hydrolysis and aminolysis. It is obvious that, on the basis of the substrates studied, the acyl thiosubtilisin favors aminolysis over hydrolysis in aqueous solution by a factor of ~2 kcal/mol compared to acyl

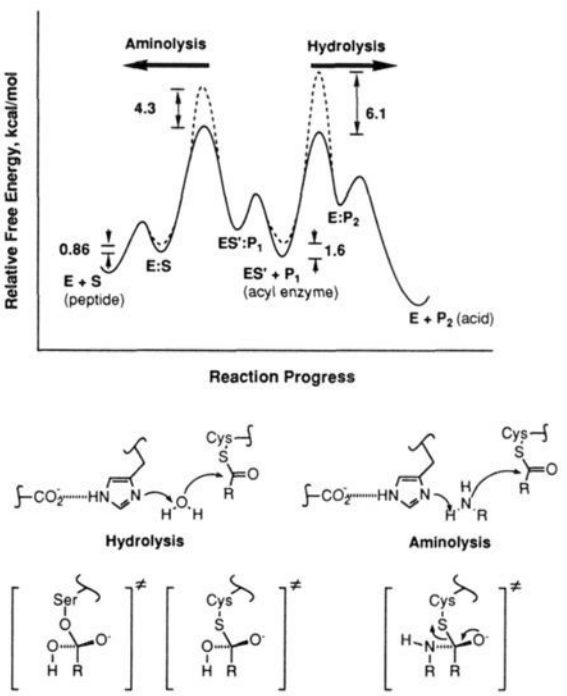

Figure 3. A, top: Free energy diagrams for subtilisin reactions generated as described previously (Zhong, Z.; Wong, C.-H. *Biomed. Biochim. Acta* **1991**, *50*, S9. Zhong, Z.; Bibbs, J.; Yuan, W.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 2259) using the amide substrate succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide and the ester substrate succinyl-Ala-Ala-Pro-Phe thiobenzyl ester. Dotted line indicates the difference in free energies observed with thiosubtilisin BPN′. Essentially the same results were observed for the thiosubtilisin variant. The numbers indicated are in kcal/mol. B: Mechanisms of hydrolysis and aminolysis for subtilisin and thiosubtilisin.

(8) Prepared from 8397 (see ref 7) via the C206Q (Cys206 → Gln) mutation followed by conversion of the active-site Ser to Cys. This variant is about 5000 times more stable and 7000 times more selective than the wild type for aminolysis than hydrolysis in aqueous solution under the conditions described previously.[1c] Subtilisin 8397 contains the following changes from the wild-type enzyme BPN′: Met50 → Phe, Gly169 → Ala, Asn76 → Asp, Gln206 → Cys, and Asn218 → Ser. Conversion of the active-site Ser residue to Cys to subtilisin BPN′ was reported by Bender and Koshland (Polgar, L.; Bender, M. L. *J. Am. Chem. Soc.* **1966**, *88*, 3153. Neet, K.; Koshland, D. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *56*, 1606) based on the chemistry developed by Zioudrou et al. (Zioudrou, C.; Wilchek, M.; Patchornik, A. *Biochemistry* **1965**, *4*, 1811). The thiosubtilisin was shown to be an effective catalyst for aminolysis of esters (Philipp, M.; Bender, M. L. *Mol. Cell. Biochem.* **1983**, *51*, 5), and further application of the enzyme to peptide segment coupling was reported by Kaiser (Nakatsuka, T.; Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 3808). Mutagenesis of the thiosubtilisin, especially via Pro225 → Ala, to improve the aminolysis reaction was reported by Wells (Abrahmsen, L.; Tom, J.; Burnier, J.; Butcher, K. A.; Kossiakoff, A.; Wells, J. A. *Biochemistry* **1991**, *30*, 4151). Conversion of the active-site Ser to selenocysteine was shown to further increase the selectivity for aminolysis (Wu, Z. P.; Hilvert, D. *J. Am. Chem. Soc.* **1989**, *111*, 4513). A detailed study of our thiosubtilisin variant will be published separately.

subtilisin BPN′. This enhancement of aminolysis seems to be both enzymatic and chemical as the acyl thiosubtilisin has a higher affinity for and is also more reactive toward the amine nucleophile vs water. Although the degree of selectivity for aminolysis may depend on the substrates (the donor and acceptor), it appears to be general that thiosubtilisin can be used as catalyst for aminolysis in aqueous solution. As the reactivity of thiosubtilisin is much weaker than that of subtilisin, a moderately active ester is often used in the enzymatic aminolysis.[8] Using a normal ester (such as a methyl ester) as substrate requires high temperature, which tends to inactivate the enzyme. The thermally stable thiosubtilisin variant developed in this study, however, effectively accepts peptide

methyl esters for aminolysis at high temperatures (see below for synthetic applications).

Owing to its abundance in biological systems,[9] *N*-acetylglucosamine β-linked to the side chain of asparagine was chosen as a central structure unit for *N*-glycopeptide synthesis. *O*-glycopeptide synthesis was performed using substrates containing either xylosyl serine as a characteristic element of the proteoglycans of the extracellular matrix and of the connective tissue,[10] or α-mannosyl threonine as a typical core unit of *O*-glycoproteins found in yeast.[11] A kinetically controlled approach[1] was used to transfer the acyl moiety of a peptide or glycopeptide donor to various amino acid, peptide, and glycopeptide derivatives. Reactions were performed either in aqueous solution or in aqueous DMF. For reactions in the presence of high concentrations of DMF, the wild-type subtilisin BPN′ was replaced by subtilisin BPN′ 8397. Since some glycopeptide donors containing the peracetylated or unprotected *N*-acetylglucosamine moiety were found to be insoluble in water as well as in many aqueous and organic solvent mixtures, the *N*-Boc protecting group was replaced with the more water soluble *N*-maleyl group (donors **1**–**4**).

We have found that N-protected dipeptide esters with either a peracetylated or an unprotected βGlcNAc-moiety in the P_2 position[12] are suitable substrates for subtilisin BPN′ (Table I). No coupling was observed, however, when the glycosyl amino acid residue was in the P_1 position. Under our reaction conditions, **3** is reacted approximately 2 orders of magnitude faster than **2**. Deprotection of the sugar moiety results in a significant decrease of the reaction rate, though the yield increases. Using the donor substrates **2**, **3**, and **4**, no acyl transfer to acceptors containing leucine in the P_1' position was observed (not shown). No limitations regarding the size of the acceptor molecule are observed when the glycosyl moiety is shifted from P_2 to P_3 (e.g. **5**). The donor substrate **5** used in these reactions contains the Asn-(βGlcNAc)-X-Ser motif, which represents the connective region of many *N*-glycoproteins. N-protected *O*-glycosyl amino acid and *O*-glycopeptide esters (**6**β, **7**β, **8**) containing a peracetylated or unprotected β-xylosyl residue in the P_1 position are accepted by the enzyme. Compound **23** represents the highly conserved O-glycosylation site of mammalian proteoglycans.[13] The reaction proceeds faster when the β-xylose moiety is unprotected. We found that the β anomer was a better substrate for subtilisin than the α anomer when the xylose moiety was attached to the P_1 position. For example, the fully protected β anomer **6**β was coupled with Gly-NH_2 by using subtilisin 8397. No coupling reaction was detected with the α anomer **6**α under the same conditions. For the deacetylated β anomer **7**β, the reaction was carried out with a much smaller amount of subtilisin 8397 in 70% DMF in 43% yield. In contrast, no desired product could be isolated when the deacetylated α anomer was used. α-*O*-glycosyl amino acids are, however, accepted by the S_2 subsite of subtilisin 8397, as indicated by the synthesis of **24** and **25**, respectively. The synthesis of yeast *O*-glycopeptide fragments is thus possible. Glycosyl amino acids can also be placed in the P_2' or P_3' position, but not the P_1' position (**10**–**13**) in the enzymatic coupling.

To demonstrate the synthetic utility of the thiosubtilisin variant in aqueous solution, **8** was reacted with Gly-Ala-NH_2 at 50 °C to give **23** in 55% yield (Figure 4). For comparison, when subtilisin BPN′ was used as a catalyst, the product was detected by HPLC in less than 20% yield under indentical conditions. To combine

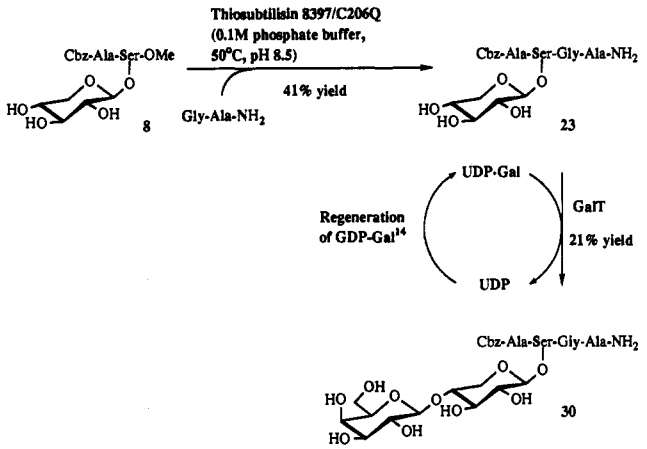

Figure 4. Schematic synthesis of glycopeptide **30** based on peptide ligation in aqueous solution at 50 °C catalyzed by the thiosubtilisin variant followed by glycosylation catalyzed by β-1,4-galactosyltransferase.

the use of glycosyltransferases and proteases in the synthesis of glycopeptides, the acyl donors **14** and **15** were prepared by galactosyltransferase-catalyzed galactosylation of **8** and **5**, respectively. The galactosylation involved regeneration of UDP-Gal.[14] Though **8** is acceptable as an acyl donor for the enzyme and the product **23** can be galactosylated to give **30**, compound **14** is not. On the other hand, **15** (prepared from **5** via enzymatic galactosylation) is a good substrate for the protease (Figure 5), and the glycopeptide **29** has been prepared in 53% yield.

In summary, the presented experiments demonstrate a new strategy for the synthesis of glycopeptides. Both *N*- and *O*-glycopeptide fragments can be coupled in aqueous or organic solvents with nucleophilic amino acids, peptides, and their glycosylated derivatives based on subtilisin BPN′ and its variants designed to improve the stability and selectivity. As demonstrated, certain glycopeptides are accepted by subtilisins, and the glycopeptides obtained can be further elongated along the peptide backbone or glycosylated with glycosyltransferases. While subtilisin 8397 is useful in polar organic solvents such as DMF, the thermostable thiosubtilisin variant may find a general use in aqueous solution for glycopeptide synthesis, as many glycopeptides are only soluble in aqueous solution. Work is in progress to determine the stereoselectivity and kinetics of the glycopeptide coupling and to extend the strategy to the synthesis of more complex bioactive glycopeptides.

Experimental Section

General Methods. Subtilisin BPN′ was obtained from Sigma (lot 22H0142). Subtilisin 8397 was prepared as described previously.[15] All reagents and solvents used were of the highest available purity. HPLC was performed using a Gilson gradient system equipped with a semi-preparative C8-column (Dynamx 60-A, Rainin). Silica gel 60 (Merck) was used for silica gel chromatography. NMR spectra were recorded on Bruker AM-300 and AM-500 instruments, respectively. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrophotometer under fast-atom bombardment (FAB) conditions.

Substrate Synthesis. The chemical synthesis of *N*- and *O*-glycopeptide substrates followed established literature procedures.[2,16,17] All substrates were characterized by ^{1}H-NMR and HRMS.

Mixed Anhydride Peptide Coupling. The carboxyl component (1 equiv) is dissolved in DMF (0.1 M). At −15 °C, 1 equiv of organic base (NMM or DIEA) and 1.1 equiv of isobutyl chloroformate are added while stirring.

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Table I. Subtilisin-Catalyzed Coupling of *O*- and *N*-Glycopeptide Fragments[a]

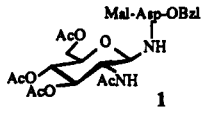
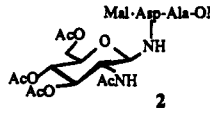
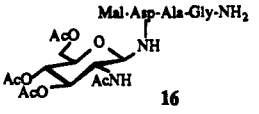
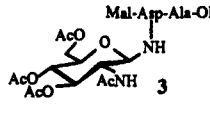
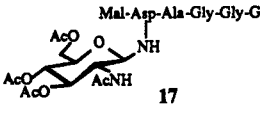
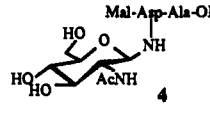
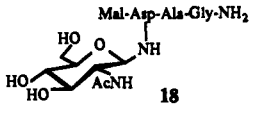
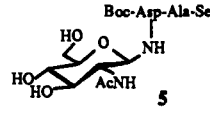
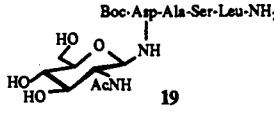
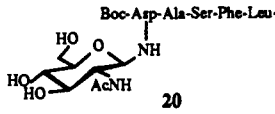
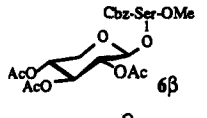
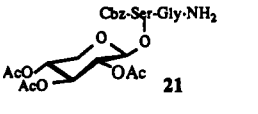
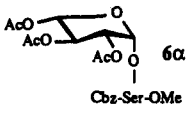
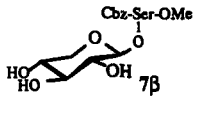
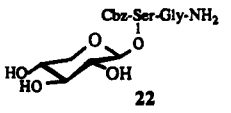
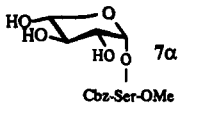
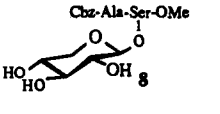
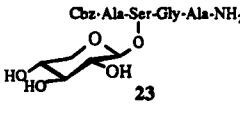
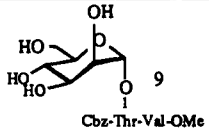
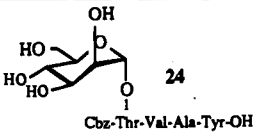
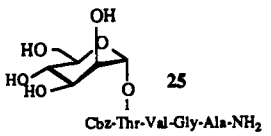
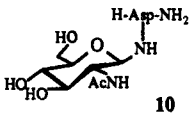
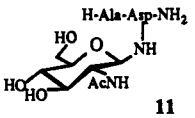
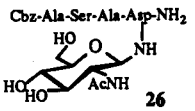
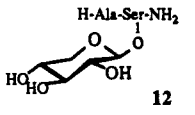
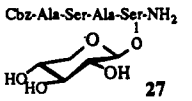
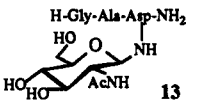
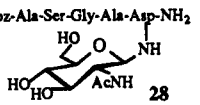
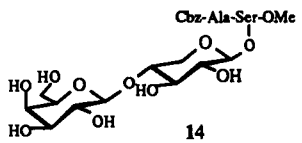
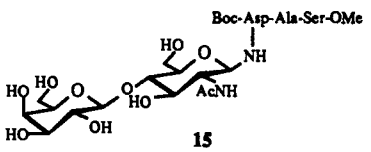
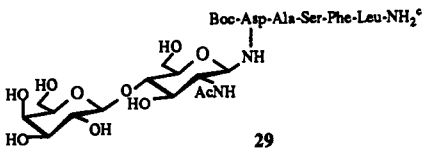
acyl donor	acyl acceptor	enzyme[b]	product	yield (%)
 Mal-Asp-OBzl **1**	$Gly\text{-}NH_2$	E_1	no coupling	
 Mal-Asp-Ala-OMe **2**	$Gly\text{-}NH_2$	E_1	 Mal-Asp-Ala-Gly-NH_2 **16**	48
 Mal-Asp-Ala-OBzl **3**	$Gly\text{-}NH_2$	E_1	**16**	61
3	Gly-Gly-Gly-OH	E_1	 Mal-Asp-Ala-Gly-Gly-Gly-OH **17**	50
 Mal-Asp-Ala-OMe **4**	$Gly\text{-}NH_2$	E_1	 Mal-Asp-Ala-Gly-NH_2 **18**	64
 Boc-Asp-Ala-Ser-OMe **5**	$Leu\text{-}NH_2$	E_1	 Boc-Asp-Ala-Ser-Leu-NH_2 **19**	42
5	Phe-Leu-NH_2	E_2	 Boc-Asp-Ala-Ser-Phe-Leu-NH_2[c] **20**	53
 Cbz-Ser-OMe **6β**	$Gly\text{-}NH_2$	E_2	 Cbz-Ser-Gly-NH_2 **21**	30
 **6α** Cbz-Ser-OMe	$Gly\text{-}NH_2$	E_2	no coupling	
 Cbz-Ser-OMe **7β**	$Gly\text{-}NH_2$	E_2	 Cbz-Ser-Gly-NH_2 **22**	43
 **7α** Cbz-Ser-OMe	$Gly\text{-}NH_2$	E_2	no coupling	
 Cbz-Ala-Ser-OMe **8**	Gly-Ala-NH_2	E_2	 Cbz-Ala-Ser-Gly-Ala-NH_2 **23**	52
8	Gly-Ala-NH_2	E_3	**23**[d]	55
8	Gly-Ala-NH_2	E_3	**23**[e]	41
8	Gly-Ala-NH_2	E_1	**23**[f]	20

Table I (Continued)

acyl donor	acyl acceptor	enzyme[b]	product	yield (%)
 9 Cbz-Thr-Val-OMe	Ala-Tyr-OH	E_2	 24 Cbz-Thr-Val-Ala-Tyr-OH	48
9	Gly-Ala-NH_2	E_2	 25 Cbz-Thr-Val-Gly-Ala-NH_2	52
Cbz-Ala-Ser-OMe	 10	E_2	no coupling	
Cbz-Ala-Ser-OMe	 11	E_2	 26	30
Cbz-Ala-Ser-OMe	 12	E_2	 27	25
Cbz-Ala-Ser-OMe	 13	E_2	 28	45
 14	Gly-Ala-NH_2	E_2	no coupling	
 15	Phe-Leu-NH_2	E_2	 29	53

[a] For details see Experimental Section. [b] E_1, Wild-type subtilsin BPN′; E_2, Stable subtilisin 8397 variant; E_3, Stable thiosubtilsin variant. [c] The reaction was performed in DMF/water (30/70 v/v) at pH 9 using 0.25 M acyl donor and 0.6 M acyl acceptor in the presence of subtilisin BPN′ 8397 (1 mg/mL). [d] The reaction was performed in 1 mL of 100 mM sodium phosphate buffer containing 20% (v/v) CH_3CN at pH 9 using 0.066 M acyl donor and 0.37 M acyl acceptor in the presence of thiosubtilisin 8397/C206Q (5 mg/mL) at 50 °C. The product was purified by silica gel flash chromatography. [e] The reaction was carried out in 150 μL of 100 mM sodium phosphate buffer at pH 8.5 using 0.066 M acyl donor and 0.56 M acyl acceptor in the presence of thiosubtilisin 8397/C206Q (5 mg/mL) at 50 °C for 6 h. The product was separated by HPLC. [f] Subtilisin BPN′ (0.01 mg/mL) was used to replace thiosubtilisin 8397/C206Q. Other conditions were the same as that in footnote *e*. When subtilisin BPN′ was used at the concentration of 5 mg/mL, 15% of prioduct **23** was detected in 20 s by HPLC, which was then rapidly hydrolyzed to Cbz-Ala-(βXyl)-Ser-OH.

After 8 min, the nucleophile (1 equiv in a small amount of DMF) is added. The reaction mixture is allowed to warm to room temperature and 1 drop of acetic acid is added. After removal of the DMF *in vacuo*, the residue is dissolved in methylene chloride and washed with 0.1 M HCl (3×), water, saturated sodium bicarbonate (3×), and water. After evaporation of the solvent, the residue is dried *in vacuo* and crystallized (usually from ethyl acetate/hexane).

Amidation Reactions. The compound is dissolved in a small amount of methanol (0.1–0.2 M) and saturated with gaseous ammonia at −15 °C for 1 h. The reaction mixture is then kept at −10 °C until both amidation of the carboxy terminus and deacetylation of the sugar moiety are completed. The product is obtained after evaporation *in vacuo*.

Removal of the *N*-Boc Protecting Group. At 0 °C the *N*-Boc-protected compound is dissolved in a 25% solution of TFA in methylene chloride (1 M). After completion of the reaction (TLC), the methylene chloride and TFA are evaporated *in vacuo*. The product is either used directly or after precipitation with ether.

Removal of the *N*-Cbz Protecting Group. *N*-Cbz-protected compound is dissolved in methanol (0.5 M) and hydrogenated in the presence of palladium on charcoal. After completion of the reaction (TLC), the suspension is filtered on a Dowex-1 column (Cl^--form) to give the hydrochloride salt after evaporation *in vacuo*.

Deacylation of Protected *N*- and *O*-Glycopeptides. 1% MeONa in dry methanol was added to a solution of an acylated *N*- or *O*-glycopeptide in dry methanol at 0 °C until the pH reached 10. The solution was stirred at 0 °C for 1.5 h, and then Dowex H^+ was added to the reaction to bring it to pH 4.0. After filtration, the solvent was removed *in vacuo* to afford the deacylated product.

Maleylation. To a solution of 1 mmol of N-deprotected derivative (salt form) in 1 mL of DMF were added maleic anhydride (98 mg, 1 mmol) in ethyl acetate and DIEA (0.385 mL, 2 mmol) at 4 °C. After the reaction mixture was stirred for 1 h, the solvent was evaporated. The residue was dissolved in methanol and treated with Dowex-50W (H^+ form) to give the pure products.

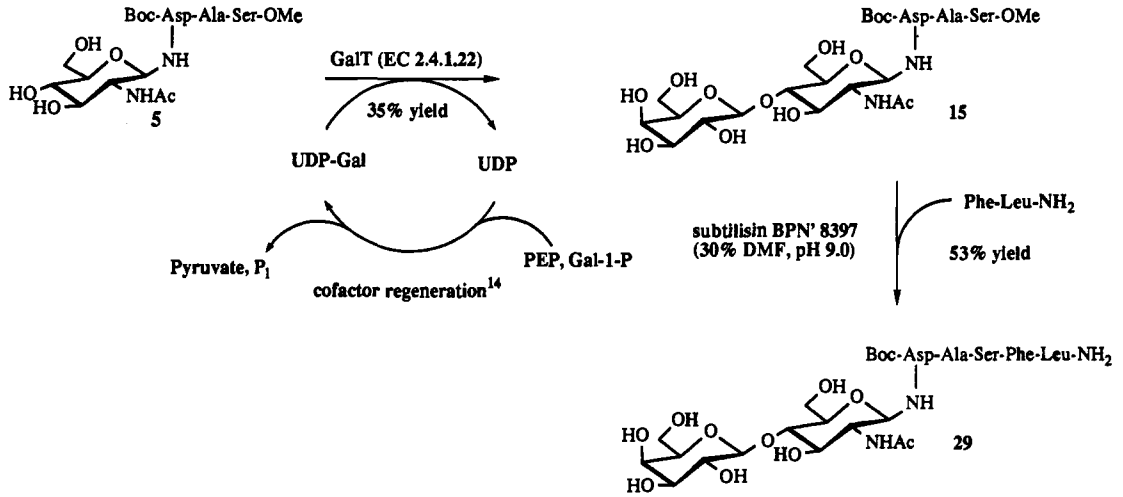


Figure 5. Schematic synthesis of glycopeptide **29** based on enzymatic glycosylation of glycopeptide **5** followed by peptide ligation catalyzed by subtilisin 8397 in aqueous DMF.

Boc-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl. To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*O*-glycopyranosylamine (obtained following established procedures[18,19]) (3 g, 8.7 mmol) and 2.81 g (8.7 mmol) of Boc-Asp-OBzl in 50 mL of methylene chloride was added 3.15 g (12.6 mmol) of EEDQ. After 24 h, the reaction mixture was washed with 0.5 M HCl (3×), saturated $NaHCO_3$ (3×), and water. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The product was obtained after washing the resulting oil with ether and chromatographed on Kiesel gel 60 (eluent ethyl acetate): yield 4.2 g (76%); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.42 (s, 9H), 1.86 (s, 3H), 2.05 (s, 3H), 2.06 (s, 3H), 2.09 (s, 3H), 2.68 (dd, 1H, *J* = 16.2, 4.2 Hz), 2.87 (dd, 1H, *J* = 16.6, 4.6 Hz), 3.70–3.79 (m, 1H), 4.02–4.12 (m, 2H), 4.29 (dd, 1H, *J* = 12.5, 4.3 Hz), 4.55–4.62 (m, 1H), 4.93–5.15 (m, 4H), 5.22 (d, 1H, *J* = 11.63 Hz), 5.74 (d, 1H, *J* = 9.1 Hz), 5.91 (d, 1H, *J* = 8.1 Hz), 7.22 (d, 1H, *J* = 8.1 Hz), 7.30–7.40 (m, 5H).

Mal-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl (1). Using the general procedures, the above compound was obtained, N-deprotected, and subsequently maleylated to afford **1** in 68% overall yield: ^{1}H-NMR (CD_3OD) δ 1.73 (s, 3H), 1.86 (s, 3H), 1.89 (s, 3H), 1.90 (s, 3H), 2.66–2.78 (m, 2H), 3.68–3.74 (m, 1H), 3.90 (t, 1H, *J* = 10.0 Hz), 3.95 (dd, 1H, *J* = 2.0, 10.0 Hz), 4.14 (dd, 1H, *J* = 4.5, 12.5 Hz), 4.82 (t, 1H, *J* = 6.0 Hz), 4.88 (t, 1H, *J* = 4.5 Hz), 5.00–5.12 (m, 4H), 6.16 (d, 1H, *J* = 12.5 Hz), 6.34 (dd, 1H, *J* = 12.5 Hz), 7.17–7.28 (m, 5H); HRMS calculated for $C_{29}H_{35}N_3O_{14}$ + Cs^+ 782.1173, found 782.1173.

Boc-Asn(tri-*O*-acetyl-βGlcNAc)-OH. Boc-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl (1 g, 1.6 mmol) was hydrogenated in 20 mL of anhydrous methanol in the presence of 100 mg of palladium on charcoal. After 3 h the reaction mixture was filtrated and evaporated *in vacuo*: yield 860 mg (97%); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.45 (s, 9H), 1.97 (s, 3H), 2.03 (s, 3H), 2.05 (s, 3H), 2.09 (s, 3H), 2.75 (dd, 1H, *J* = 16.3, 4.7 Hz), 2.91 (dd, 1H, *J* = 16.5, 4.3 Hz), 3.69–3.77 (m, 1H), 4.09 (dd, 1H, *J* = 12.5, 1.8 Hz), 4.18 (q, 1H, *J* = 9.8 Hz), 4.30 (dd, 1H, *J* = 12.5, 4.2 Hz), 4.47–4.58 (m, 1H), 5.10 (t, 1H, 9.4 Hz), 5.15–5.26 (m, 2H), 5.93 (d, 1H, *J* = 8.5 Hz), 6.97 (d, 1H, 8.95 Hz), 7.53 (d, 1H, *J* = 8.4 Hz); MS calculated for $C_{23}H_{35}N_3O_{13}$ – H^+ 560, found 560.

Boc-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OMe. Boc-Asn(tri-*O*-acetyl-βGlcNAc)-OH (2.4 g, 4.27 mmol), 0.7 g (5 mmol) of H-Ala-NH_2·HCl, 1.03 g (5 mmol) of DCC, and 0.945 g (5 mmol) of HOBt were dissolved in 20 mL of DMF and stirred at 0 °C for 3 h and at 25 °C for 12 h after addition of 1.39 mL (10 mmol) of TEA. The reaction mixture was filtered off after addition of 50 mL of water and extracted using 3 × 20 mL of ethyl acetate. The product was obtained after evaporation of the solvent *in vacuo* and crystallization of the residue from ethyl acetate: yield 1.63 g (59%); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.40 (d, 3H), 1.45 (s, 9H), 2.01 (s, 3H), 2.03 (s, 3H), 2.07 (s, 3H), 2.09 (s, 3H), 2.55 (dd, 1H), 2.77 (dd, 1H), 3.7–3.76 (m, 1H), 3.7 (s, 3H), 4.03–4.20 (m, 2H), 4.28 (dd, 1H), 4.43–4.54 (m, 2H), 5.01–5.18 (m, 3H), 6.07–6.19 (m, 2H), 7.21 (d, 1H), 7.38 (d, 1H); HRMS calculated for $C_{27}H_{42}N_4O_{14}$ + H^+ 647.2776, found 647.2789.

Boc-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OBzl. The synthesis was performed using the mixed anhydride standard procedure with a yield of 76%: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.39–1.46 (m, 12H), 1.98 (s, 3H), 2.03 (s, 6H), 2.07 (s, 3H), 2.53 (dd, 1H, *J* = 4.5, 16.2 Hz), 2.70 (dd, 1H, *J* = 4.8, 16.2 Hz), 3.77 (m, 1H), 4.08 (dd, 1H, *J* = 1.8, 12.5 Hz), 4.10–4.21 (m, 1H), 4.29 (dd, 1H, *J* = 4.0, 12.5 Hz), 4.50–4.62 (m, 2H), 5.07–5.23 (m, 5H), 6.24 (d, 1H, *J* = 8.4 Hz), 6.45 (d, 1H, *J* = 8.4 Hz), 7.30–7.40 (m, 5H), 7.45–7.55 (m, 2H); MS calculated for $C_{33}H_{46}N_4O_{14}$ + Cs^+ 855, found 855.

H-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OMe·TFA. Using the standard procedure Boc-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OMe was deprotected in 95% yield after crystallization from methanol/ether: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.40 (d, 3H), 1.98 (s, 3H), 2.05 (s, 6H), 2.09 (s, 3H), 2.95–3.13 (m, 2H), 3.71 (s, 3H), 3.80–3.90 (m, 1H), 4.05–4.20 (m, 3H), 4.45–4.56 (m, 2H), 5.08 (t, 1H), 5.23 (t, 1H), 5.32 (t, 1H), 7.44 (d, 1H), 7.76 (d, 1H), 7.94 (d, 2H); HRMS calculated for $C_{22}H_{34}N_4O_{14}$ + Cs^+ 679.1228, found 679.1228.

H-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OBzl·TFA. Using the standard procedure this compound was prepared similarly: ^{1}H-NMR (CD_3OD, 300 MHz) δ 1.40 (d, 1H, *J* = 7.4 Hz), 1.88 (s, 3H), 1.97 (s, 3H), 1.99 (s, 3H), 2.00 (s, 3H), 2.72 (dd, 1H, *J* = 8.8, 17.5 Hz), 2.91 (dd, 1H, *J* = 4.2, 17.5 Hz), 3.80–3.88 (m, 1H), 4.00 (t, 1H, *J* = 10.19 Hz), 4.08 (dd, 1H, *J* = 2.1, 11.4 Hz), 4.21 (d, 1H, *J* = 4.3 Hz), 4.24 (dd, 1H, *J* = 3.0, 3.8 Hz), 4.50 (q, 1H, *J* = 5.4 Hz), 4.99 (t, 1H, *J* = 9.8 Hz), 5.16 (d, 2H, *J* = 6.4 Hz), 5.20–5.33 (m, 2H), 7.30–7.38 (m, 5H); HRMS calculated for $C_{28}H_{38}N_4O_{12}$ + Cs^+ 755.1541, found 755.1541.

Mal-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OMe (2). The compound was prepared in 83% yield by maleylation of H-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OMe using the standard procedure: ^{1}H-NMR (DMSO-d_6) δ 1.28 (d, 3H, *J* = 7.3 Hz), 1.73 (s, 3H), 1.89 (s, 3H), 1.94 (s, 3H), 1.98 (s, 3H), 2.44 (dd, 1 H, *J* = 8.8, 16.4 Hz), 2.66 (dd, 1H, *J* = 3.9, 16.4 Hz), 3.60 (s, 3H), 3.77–3.97 (m, 3H), 4.12–4.20 (m, 1H), 4.20–4.30 (m, 1H), 4.69–4.76 (m, 1H), 4.80 (t, 1H, *J* = 9.8 Hz), 5.06 (t, 1H, *J* = 9.7 Hz), 5.15 (t, 1H, *J* = 9.6 Hz), 6.22 (d, 1H, *J* = 12.4 Hz), 6.37 (d, 1H, *J* = 12.4 Hz), 7.90 (d, 1H, *J* = 9.3 Hz), 8.40 (d, 1H, *J* = 6.9 Hz), 8.59 (d, 1H, *J* = 9.1 Hz), 9.15 (d, 1H); HRMS calculated for $C_{26}H_{36}N_4O_{15}$ + H^+ 645.2255, found 645.2230.

Mal-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-OBzl (3). Using the same procedure, this compound was obtained in 91% yield: ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.32 (d, 3H, *J* = 7.3 Hz), 1.78 (s, 3H), 1.87 (s, 3H), 1.90 (s, 3H), 1.91 (s, 3H), 2.59 (dd, 1H, *J* = 7.6, 16.2 Hz), 2.70 (dd, 1H, *J* = 4.6, 16.2 Hz), 3.69–3.76 (m, 1H), 3.86–4.01 (m, 2H), 4.13 (dd, 1H, *J* = 4.4, 12.3 Hz), 4.28–4.37 (m, 1H), 4.72–4.83 (m, 1H), 4.88 (t, 1H, *J* = 9.7 Hz), 5.02–5.16 (m, 4H), 6.11 (d, 1H, *J* = 12.2 Hz), 6.41 (d, 1H, *J* = 12.2 Hz), 7.27–7.39 (m, 5H), 7.1–8.6 (partially exchanged amide protons); HRMS calculated for $C_{32}H_{40}N_4O_{15}$ + H^+ 721.2568, found 721.2568.

Mal-Asn(βGlcNAc)-Ala-OMe (4). Deacetylation of **2** catalyzed by NaOMe yielded this material in 74% yield: ^{1}H-NMR (D_2O, 300 MHz) δ 1.24 (d, 3H, *J* = 7.31 Hz), 1.82 (s, 3H), 2.61 (dd, 1H, *J* = 7.2, 16.3 Hz), 2.69 (dd, 1H, *J* = 5.5, 16.3 Hz), 3.25–3.38 (m, 2H), 3.42–3.50 (m, 1H), 3.56 (s, 3H), 3.54–3.59 (m, 1H), 3.59–3.73 (m, 2H), 4.24 (q, 1H, *J* = 7.3 Hz), 4.59–4.71 (m, 1H), 4.88 (d, 1H, *J* = 9.7 Hz), 6.14 (d, 1H, *J* = 12.1 Hz), 6.37 (d, 1H, *J* = 12.1 Hz).

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(19) Bolton, C. H.; Jeanloz, R. W. *J. Org. Chem.* **1963**, *28*, 3228.

Boc-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-Ser(OBzl)-OMe. Using the mixed anhydride procedure, Boc-Asn(tri-*O*-acetyl-βGlcNAc)-OH and H-Ala-Ser(OBzl)-OMe were coupled in 80% yield: ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 1.18 (d, 3H, J = 6.9 Hz), 1.34 (s, 9H), 1.74 (s, 3H), 1.89 (s, 3H), 1.94 (s, 3H), 1.97 (s, 3H), 2.34 (dd, 1H), 2.54 (dd, 1H), 3.57–3.63 (m, 1H), 3.61 (s, 3H), 3.68–3.95 (m, 4H), 4.11–4.26 (m, 2H), 4.33–4.40 (m, 1H), 4.42–4.58 (m, 2H), 4.46–4.55 (m, 1H), 4.79 (t, 1H, J = 9.8 Hz), 5.10 (t, 1H, J = 10.0 Hz), 5.15 (t, 1H, J = 9.52 Hz), 6.88 (d, 1H, NH), 7.25–7.37 (m, 5H), 7.77 (d, 1H, NH), 7.89 (d, 1H, NH), 8.43 (d, 1H, NH), 8.50 (d, 1H, NH); HRMS calculated for $C_{37}H_{53}N_5O_{16}$ + Cs^+ 956.2542, found 956.2588.

Boc-Asn(βGlcNAc)-Ala-Ser(OBzl)-OMe. In a methanolic solution at 70 °C, crude Boc-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-Ser(OBzl)-OMe was quantitatively deacetylated within 20 min. The pure product was obtained in 90% yield by crystallization from methanol/ether: ^{1}H-NMR (DMSO-d_6) δ 1.18 (d, 3H), 1.35 (s, 9H), 1.80 (s, 3H), 2.42 (dd, 1H), 2.50 (dd, 1H), 3.40–3.65 (m, 2H), 3.61 (s, 3H), 3.74 (dd, 1H), 4.16–4.25 (m, 1H), 4.32–4.41 (m, 1H), 4.42–4.58 (m, 4H), 4.75–4.84 (m, 1H), 4.92–5.04 (m, 1H), 6.77 (d, 1H), 7.22–7.87 (m, 5H), 7.77 (d, 2H), 8.12 (d, 1H), 8.43 (d, 1H); HRMS calculated for $C_{31}H_{47}N_5O_{13}$ + Cs^+ 830.2225, found 830.2239.

Boc-Asn(βGlcNAc)-Ala-Ser-OMe (5). The above compound (50 mg, 0.072 mmol) in 5 mL of methanol was hydrogenated in the presence of Pd/C for 5 days at 50 atm to yield 42 mg (92%) of product after filtration and evaporation of the solvent: ^{1}H-NMR (D_2O, 500 MHz) δ 1.22–1.28 (m, 12H), 1.86 (s, 3H), 2.49 (dd, 1H, J = 7.9, 15.5 Hz), 2.64 (dd, 1H, J = 4.6, 16.0 Hz), 3.26–3.48 (m, 3H), 3.60 (s, 3H), 3.54–3.76 (m, 4H), 3.80 (dd, 1H, J = 4.8, 11.8 Hz), 4.20–4.27 (m, 1H), 4.27–4.33 (m, 1H), 4.87–4.93 (m, 1H), 4.90 (d, 1H, J = 9.8 Hz); HRMS calculated for $C_{24}H_{41}N_5O_{13}$ + Na^+ 630.2599, found 630.2610.

Cbz-Ser(tri-*O*-acetyl-βXyl)-OMe (6β) and Cbz-Ser(tri-*O*-acetyl-αXyl)-OMe (6α). To a solution of 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl bromide (2.67 g, 7.87 mmol) in dry dichloromethane (30 mL) was added at 0 °C Cbz-Ser-OMe (2.19 g, 8.66 mmol), 1,1,3,3-tetramethylurea (2.83 mL, 23.63 mmol), and silver triflate (4.45 g, 17.32 mmol). The suspension was stirred in the dark for 12 h at 0 °C and then filtered through a bed of Celite. The filtrate was washed twice with water and twice with saturated sodium bicarbonate. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*, and the residue was chromatographed on silica gel (eluted with EtOAc/hexanes (1/1 to 2/1) to give the α anomer as a colorless liquid (137 mg, 4%) (R_f = 0.9, EtOAc/hexane = 2/1) and the β anomer as a colorless liquid (567 mg, 15%) (R_f = 0.8, EtOAc/hexane = 2/1).

6β: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.99–2.03 (m, 9H), 3.32 (dd, 1H, J = 4.0, 8.0 Hz), 3.72 (s, 3H), 3.76 (dd, 1H, J = 3.3, 7.0 Hz), 4.00 (dd, 1H, J = 4.7, 7.2 Hz), 4.18 (dd, 1H, J = 2.8, 7.4 Hz), 4.45 (d, 1H, J = 6.2 Hz), 4.81 (m, 2H), 5.05 (t, 1H, J = 7.9 Hz), 5.09 (d, 2H, J = 3.7), 5.61 (d, 1H, J = 8.3 Hz), 7.28–7.34 (m, 5H); ^{13}C {^{1}H} NMR ($CDCl_3$) δ 20.44, 20.49, 20.58, 52.55, 54.01, 61.25, 66.96, 68.19, 68.79, 69.82, 70.28, 100.25, 128.00, 128.09, 128.39, 135.95, 169.27, 169.68, 169.78, 169.94.

6α: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 2.01 (m, 14H), 3.52 (t, 1H, J = 10.5 Hz), 3.73 (m, 4H), 3.91 (s, 2H), 4.51 (d, 1H, J = 7.5 Hz), 4.70 (d, 1H, J = 7.0 Hz), 4.90 (d, 1H, J = 5.5 Hz), 4.94 (s, 1H), 5.11 (dd, 2H, J = 4.0, 12.0 Hz), 5.36 (t, 1H, J = 10.0 Hz), 5.77 (d, 1H, J = 8.0 Hz), 7.31–7.35 (m, 5H). ^{13}C {^{1}H} NMR ($CDCl_3$) δ 20.46, 20.56, 20.61, 52.49, 54.12, 58.44, 67.08, 68.68, 68.94, 69.17, 70.74, 96.32, 128.06, 128.13, 128.42, 135.97, 155.83, 196.80, 169.90, 170.09, 170.16.

Cbz-Ser(βXyl)-OMe (7β). Synthesized by deacetylation from 6β: yield 93%; ^{1}H-NMR (500 MHz, CD_3OD) δ 3.05–3.11 (m, 2H), 3.22 (t, 1H, J = 9.0 Hz), 3.39 (m, 1H), 3.65 (s, 3H), 3.75 (dd, 1H, J = 5.5, 11.5 Hz), 4.10 (d, 1H, J = 7.5 Hz), 4.20 (dd, 1H, J = 3.5, 10.0 Hz), 4.39 (t, 1H, J = 3.0 Hz), 5.03 (s, 2H), 7.20–7.30 (m, 5H); HRMS calculated for $C_{17}H_{23}NO_9$ + Na^+ 408.1271, found 408.1275.

Cbz-Ser(αXyl)-OMe (7α). Synthesized by deacetylation of 6α: yield 92%; ^{1}H-NMR (500 MHz, CD_3OD) δ 3.21 (t, J = 2.0 Hz), 3.23 (d, J = 3.5 Hz), 3.26 (d, J = 3.5 Hz), 3.34 (d, J = 2.5 Hz), 3.42–3.46 (m), 3.65 (s), 3.66 (s), 3.77–3.82 (m), 4.38 (t, J = 3.5 Hz), 4.61 (d, J = 4.0 Hz), 5.01 (s), 7.20–7.26 (m); ^{13}C-NMR (CD_3OD) δ 55.92, 63.39, 67.82, 68.96, 71.33, 73.45, 74.88, 128.86, 129.48; HRMS calculated for $C_{17}H_{23}NO_9Cs^+$ (M + Cs^+) 518.0427, found 518.0427.

Cbz-Ala-Ser(tri-*O*-acetyl-βXyl)-OMe. To a solution of 2,3,4-triacetyl-α-D-xylopyranosyl bromide (0.66 g, 1.94 mmol) in dry CH_2Cl_2 (20 mL) was added Cbz-Ala-Ser-OMe (0.60 g, 1.85 mmol). The reaction was cooled to −40 °C in an acetonitrile/dry ice bath. Then silver trifluoromethanesulphonate (0.713 g, 2.78 mmol) was added, and the reaction was stirred at −40 °C for 2 h. Then dry pyridine (0.5 mL, 5.55 mmol) was added, and the reaction was allowed to warm up to room temperature. The mixture was then filtered through a bed of Celite. The filtrate was washed with water twice and with saturated sodium bicarbonate twice; then the organic layer was dried ($MgSO_4$) and concentrated *in vacuo*, and the residue was chromatographed by eluting (EtOAc/hexanes 3/1) on silica gel to yield 415 mg (44%) of the desired product as a colorless liquid: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.31 (d, 3H, J = 5.0 Hz), 3.40 (t, 1H, J = 4.8 Hz), 3.81 (d, 1H, J = 9.5 Hz), 4.03 (s, 3H), 4.04 (dd, 1H, J = 4.8, 5.0 Hz), 4.20 (m, 1H), 4.28 (m, 1H), 4.71 (d, 1H, J = 9.4 Hz), 4.83 (t, 1H, J = 7.0 Hz), 4.88 (dd, 1H, J = 2.9, 4.9 Hz), 5.15 (m, 3H), 5.43 (d, 1H, J = 6.4 Hz), 6.81 (d, 1H, J = 4.3 Hz); ^{13}C {^{1}H} NMR ($CDCl_3$) δ 14.03, 20.66, 20.73, 20.77, 52.75, 61.52, 66.96, 68.12, 68.42, 68.51, 70.40, 70.48, 100.40, 128.00, 128.11, 128.53, 169.80, 172.20; HRMS calculated for $C_{26}H_{34}N_2O_{13}$ + Cs^+ 715.1115, found 715.1116.

Cbz-Ala-Ser(βXyl)-OMe (8). Synthesized by deacylation of Cbz-Ala-Ser(tri-*O*-acetyl-βXyl)-OMe: yield 91%; ^{1}H-NMR (500 MHz, D_2O) δ 1.27 (d, J = 7.0 Hz), 3.04–3.10 (m, 3H), 3.20–3.21 (m, 3H), 3.35–3.36 (m, 2H), 3.62 (s), 3.65–3.68 (m, 1H), 3.72–3.75 (m, 2H), 4.12 (d, 1H, J = 7.0 Hz), 4.16–4.18 (m, 1H), 4.57 (d, 1H, J = 8.0 Hz), 5.68 (s, 3H), 7.17–7.26 (m, 5H); ^{13}C-NMR (D_2O) δ 18.30, 51.75, 52.99, 53.94, 66.99, 67.62, 70.15, 71.06, 74.90, 77.72, 105.16, 128.83, 128.99, 129.47, 138.16, 158.19, 171.63, 175.70; HRMS calculated for $C_{20}H_{28}N_2O_{10}$ + Cs^+ 589.0798, found 589.0795.

Cbz-Thr(tetra-*O*-acetyl-αMan)-Val-OMe. To a solution of 2,3,4,6-tri-*O*-acetyl-D-mannopyranosyl bromide (2.40 g, 5.84 mmol) in dry dichloromethane (30 mL) was added at −20 °C Cbz-Thr-Val-OMe (2.00 g, 5.67 mmol) and silver triflate (2.92 g, 11.4 mmol). The suspension was stirred at −20 °C for 4 h and then filtered through a bed of Celite. The filtrate was washed twice with water and twice with saturated sodium bicarbonate. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*, and the residue was chromatographed with silica gel (eluted with EtOAc/hexanes 2/1) to give the desired product as a white solid (2.8 g, 72% yield): ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.86 (dd, 6H, J = 4.5, 7.0 Hz), 1.20 (d, 3H, J = 1.0 Hz), 1.91 (s, 3H), 1.98 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.12 (m, 1H), 3.67 (s, 3H), 4.05–4.07 (m, 2H), 4.18–4.20 (m, 1H), 4.33 (t, 2H, J = 3.5 Hz), 4.44–4.45 (m, 1H), 5.00 (s, 1H), 5.08 (s, 2H), 5.16–5.24 (m, 3H), 5.81 (d, 1H, NH, J = 7.5 Hz), 6.82 (d, 1H, NH, J = 8.5 Hz), 7.25–7.32 (m, 5H); ^{13}C {^{1}H} NMR ($CDCl_3$) δ 16.53, 17.74, 18.71, 20.40, 20.53, 20.58, 30.65, 51.82, 57.31, 58.15, 62.35, 66.69, 67.10, 68.78, 68.83, 60.05, 76.18, 98.85, 127.40, 128.40, 135.81, 156.12, 168.71, 169.40, 170.43, 171.75; HRMS calculated for $C_{32}H_{44}N_2O_{15}$ + Cs^+ 829.1796, found 829.1796.

Cbz-Thr(αMan)-Val-OMe (9). Synthesized by deacetylation of Cbz-Thr(tetra-*O*-acetyl-αMan)-Val-OMe: yield 87%; ^{1}H-NMR (500 MHz, D_2O) δ 0.85 (dd, 6H, J = 4.0, 7.0 Hz), 1.21 (d, 3H, J = 6.5 Hz), 2.04–2.07 (m, 1H), 3.26–3.54 (m, 2H), 3.62 (s, 3H), 3.64–3.68 (m, 2H), 3.69–3.74 (m, 2H), 4.10 (m, 1H), 4.24–4.29 (m, 2H), 5.02 (s, 2H), 7.19 (d, 1H, NH, J = 3.0 Hz), 7.20–7.28 (m, 5H), 8.10 (d, 1H, NH, J = 8.5 Hz); ^{13}C {^{1}H} NMR (D_2O) δ 18.52, 18.91, 19.36, 31.70, 48.66, 48.82, 49.00, 49.17, 49.33, 52.61, 59.19, 60.45, 62.80, 67.83, 68.50, 71.94, 72.26, 74.89, 77.13, 103.01, 128.82, 128.98, 129.41, 137.92, 158.50, 172.58, 173.26; HRMS calculated for $C_{24}H_{36}N_2O_{11}$ + Cs^+ 661.1373, found 661.1392.

Boc-Asn(βGlcNAc)-NH_2. The material was obtained in 90% yield by amidation and simultaneous O-deacetylation under standard amidation conditions: ^{1}H-NMR (D_2O, 500 MHz) δ 1.26 (s, 9H), 1.82 (s, 3H), 2.51 (m, 1H), 2.62 (dd, 1H, J = 4.5, 16.0 Hz), 3.27–3.37 (m, 2H), 3.44 (t, 1H, J = 9.8 Hz), 3.58 (dd, 1H, J = 7.5, 4.5 Hz), 3.64 (t, 1H, J = 10.0 Hz), 3.68–3.73 (m, 1H), 4.20–4.30 (m, 1H), 4.91 (d, 1H, J = 10.0 Hz); HRMS calculated for $C_{17}H_{30}N_4O_9$ + Cs^+ 567.1067, found 567.1067.

H-Asn(βGlcNAc)-NH_2-TFA (10). The above compound was quantitatively N-deprotected using TFA in CH_2Cl_2: ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.84 (1.87) (s, 3H), 2.60–2.72 (m, 1H), 2.76–2.87 (m, 1H), 3.35–3.42 (m, 1H), 3.56 (dd, 1H, J = 12.0, 5.5 Hz), 3.62–3.69 (m, 1H), 3.72–3.77 (m, 1H), 4.01–4.09 (m, 1H), 4.87 (d, 1H, J = 10.2 Hz); HRMS calculated for $C_{12}H_{22}N_4O_7$ + Cs^+ 467.0543, found 467.0543.

H-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl-TFA. This compound was obtained using the same standard procedure: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.83 (s, 3H), 2.00 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 2.93–3.10 (m, 2H), 3.80–3.90 (m, 1H), 4.03–4.13 (m, 2H), 4.21 (dd, 1H), 4.33–4.41 (m, 1H), 5.07 (t, 1H), 5.13–5.23 (m, 3H), 7.05 (d, 1H), 7.25–7.37 (m, 5H), 7.80 (d, 1H).

Boc-Ala-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl. Mixed anhydride coupling of Boc-Ala-OH to the previous compound proceeded in 75% yield: ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.14 (d, 3H, J = 7.5 Hz), 1.32 (s, 9H),

1.74 (s, 3H), 1.81 (s, 3H), 1.90 (s, 3H), 1.91 (s, 3H), 2.65–2.73 (m, 2H), 3.68–3.73 (m, 1H), 3.88–3.95 (m, 1H), 3.96 (dd, 1H, J = 2.3, 17.3 Hz), 4.14 (dd, 1H, J = 4.5, 12.5 Hz), 4.70 (t, 1H, J = 5.5 Hz), 4.73–4.78 (m, 1H), 4.88 (t, 1H, J = 9.8 Hz), 4.98–5.10 (m, 4H), 7.15–7.27 (m, 5H).

Boc-Ala-Asn(βGlcNAc)-NH$_2$. Boc-Ala-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl was amidated and O-deacetylated using the standard amidation procedure: ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.20 (d, 3H, J = 7.0 Hz), 1.35 (s, 9H), 1.88 (s, 3H), 2.51–2.59 (m, 1H), 2.62–2.72 (m, 1H), 3.32–3.41 (m, 1H), 3.52–3.58 (m, 1H), 3.63 (t, 1H, J = 10.0 Hz), 3.73 (d, 1H, J = 10.5 Hz), 3.86 (q, 1H, J = 7.5 Hz), 4.59 (t, 1H, J = 5.5 Hz), 4.84 (d, 1H, J = 9.5 Hz); HRMS calculated for $C_{20}H_{35}N_5O_{10}$ + Cs^+ 638.1438, found 638.1443.

H-Ala-Asn(βGlcNAc)-NH$_2$·TFA (11). The above compound was quantitatively N-deprotected using TFA in CH_2Cl_2: ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.4 (d, 3H, J = 7.0 Hz), 1.86 (s, 3H), 2.51 (dd, 1H, J = 7.5, 16.4 Hz), 2.63 (dd, 1H, J = 5.5, 17.0 Hz), 3.33–3.40 (m, 1H), 3.52–3.60 (m, 1H), 3.62–3.68 (m, 1H), 3.69–3.75 (m, 1H), 3.84 (q, 1H, J = 7.0 Hz), 4.57–4.62 (m, 1H), 4.86 (d, 1H, J = 6.8 Hz); HRMS calculated for $C_{15}H_{27}N_5O_8$ + Cs^+ 538.0914, found 538.0911.

Cbz-Ala-Ser(βXyl)-NH$_2$. Cbz-Ala-Ser(tri-*O*-acetyl-βXyl)-OMe was routinely amidated and O-deacetylated to afford 85% product. ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.25 (d, 3H, J = 7.5 Hz), 3.03–3.10 (m, 1H), 3.32–3.45 (m, 1H), 3.60–3.71 (m, 1H), 3.75 (q, 1H, J = 5.5 Hz), 4.00–4.08 (m, 1H), 4.12 (d, 1H, J = 7.5 Hz), 4.41 (t, 1H, J = 4.5 Hz), 5.00 (s, 2H), 7.17–7.30 (m, 5H); HRMS calculated for $C_{19}H_{27}N_3O_9$ + Cs^+ 574.0802, found 574.0802.

H-Ala-Ser(βXyl)-NH$_2$·HCl (12). Using the general procedure for N-Cbz removal, the desired product was obtained in 90% yield: ^{1}H-NMR (D_2O, 500 MHz) δ 1.40 (d, 3H, J = 7.0 Hz), 3.09–3.19 (m, 2H), 3.27 (t, 1H, J = 4.3 Hz), 3.41–3.49 (m, 1H), 3.70–3.83 (m, 2H), 3.97 (q, 1H, J = 7.0 Hz), 4.02 (dd, 1H, J = 5.3, 10.8 Hz), 4.25 (d, 1H, J = 8.0 Hz), 4.45–4.50 (m, 1H); HRMS calculated for $C_{11}H_{21}N_3O_7$ + Cs^+ 440.0434, found 440.0430.

Cbz-Gly-Ala-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl. The compound was obtained by removal of the *N*-Boc protection from Boc-Ala-Asn(tri-*O*-acetyl-βGlcNAc)-OBzl by TFA in CH_2Cl_2 and subsequent coupling to Cbz-Gly-OH via mixed anhydride in 73% yield: ^{1}H-NMR (DMSO-d_6, 500 MHz) δ 1.12 (d, 3H, J = 7.0 Hz), 1.70 (s, 3H), 1.90 (s, 3H), 1.95 (s, 3H), 1.97 (s, 3H), 2.45–2.53 (m, 1H), 2.69 (dd, 1H, J = 6.5, 16.5 Hz), 3.61 (d, 2H, J = 5.5 Hz), 3.78–3.83 (m, 1H), 3.83–3.94 (m, 2H), 4.16 (dd, 1H, J = 3.8, 7.2 Hz), 4.27–4.33 (m, 1H), 4.67 (q, 1H, J = 7.5 Hz), 4.81 (t, 1H, J = 9.7 Hz), 4.98–5.13 (m, 5–6H), 5.16 (t, 1H, J = 9.5 Hz), 7.28–7.37 (m, 10H), 7.42 (t, 1H, J = 6.0 Hz), 7.90 (d, 1H, J = 9.0 Hz), 7.98 (d, 1H, J = 8.0 Hz), 8.40 (d, 1H, J = 8.0 Hz), 8.71 (d, 1H, J = 9.5 Hz); MS calculated for $C_{38}H_{47}N_5O_{15}$ + CS^+ 946, found 946.

Cbz-Gly-Ala-Asn(βGlcNAc)-NH$_2$. Amidation of Cbz-Gly-Ala-Asn-(tri-*O*-acetyl-βGlcNAc)-OBzl under standard conditions afforded 90% product: ^{1}H-NMR (D_2O, 500 MHz) δ 1.33 (d, 3H, J = 7.0 Hz), 1.95 (s, 3H), 2.62–2.72 (m, 1H), 2.73–2.82 (m, 1H), 3.40–3.50 (m, 2H), 3.51–3.60 (m, 1H), 3.69 (dd, 1H, J = 4.5, 12.5 Hz), 3.74–3.85 (m, 5H), 4.10–4.35 (m, 1H), 4.55–4.65 (m, 1H), 5.00 (d, 1H, J = 9.5 Hz), 5.11 (s, 2H), 7.32–7.44 (m, 5H).

H-Gly-Ala-Asn(βGlcNAc)-NH$_2$·HCl (13). The *N*-Cbz group of Cbz-Gly-Ala-Asn(βGlcNAc)-NH$_2$ was quantitatively removed under standard conditions: ^{1}H-NMR (D_2O, 500 MHz) δ 1.25 (d, 3H, J = 7.0 Hz), 1.85 (s, 3H), 2.50–2.65 (m, 2H), 3.48–3.74 (m, 6H), 3.21 (s, 2H), 3.26–3.47 (m, 1H), 4.18–4.23 (m, 1H), 4.45–4.55 (m, 1H); HRMS calculated for $C_{17}H_{30}N_6O_9$ + Cs^+ 595.1129, found 595.1129.

Cbz-Ala-Ser(Galβ1,4Xylβ)-OMe (14). To 5 mL of an aqueous solution (containing 100 mM Hepes, 4 mM KCl, 4 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM DTT) were added 115 mg of PEP, 195 mg of Gal-1-P, 150 mg of Cbz-Ala-Ser(β-Xyl)-OMe, 20 mg of UDP, and 15 mg of Glc-1-P. The pH of the solution was adjusted to 7.5. After addition of 2 mg of α-lactabumin, 200 units of pyruvate kinase (EC 2.7.1.40), 10 units of UDP-glucose pyrophosphorylase (EC 2.7.7.9), 10 units of Gal-1-P uridyltransferase (EC 2.7.7.10), 10 units of inorganic pyrophosphatase (EC 3.6.1.1), and 5 units of bovine galactosyltransferase (EC 2.4.1.22) were added and the reaction mixture was incubated at 25 °C for 48 h. After lyophilization of the reaction mixture, the product (45 mg, 32% yield) was separated by chromatography on silica gel using chloroform/methanol/water (7/3/2.5) as eluent: ^{1}H-NMR (500 MHz, D_2O) δ 1.18 (s, 3H), 3.16 (d, 1H), 3.30 (t, 1H, J = 9.0 Hz), 3.42 (t, 1H, J = 9.5 Hz), 3.48 (d, 1H, J = 3.1 Hz), 3.53 (d, 1H, J = 4.2 Hz), 3.56 (s, 1H), 3.58 (m, 3H), 3.60 (s, 3H), 3.61 (m, 3H), 3.71 (s, 1H), 3.74 (d, 1H, J = 10.0 Hz), 3.86 (m, 1H), 4.24 (m, 2H), 4.53 (s, 1H), 4.94 (s, 2H), 7.18–7.24 (m, 5H); ^{13}C {^{1}H} NMR (D_2O) δ 10.91, 16.90, 30.50, 50.89, 53.27, 61.27, 63.19, 67.19, 68.79, 69.11, 70.78, 72.78, 72.82, 73.92, 75.51, 76.60, 101.90, 103.20, 127.84, 127.45, 128.50, 129.02, 136.51, 157.80, 171.71, 176.12; HRMS calculated for $C_{26}H_{38}N_2O_{15}$ + Cs^+ 751.1327, found 751.1301.

Boc-Asn(βLacNAc)-Ala-Ser-OMe (15). To 2 mL of an aqueous solution (containing 100 mM Hepes, 4 mM KCl, 4 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM DTT) were added 60 mM PEP, 30 mM Gal-1-P, 3 mM Glc-1-P, 3 mM UDP, and 0.06 mmol of **5**, and the pH was adjusted to 7.5. After addition of 200 units of pyruvate kinase, 10 units of UDP-glucose pyrophosphorylase, 10 units of Gal-1-P uridyltransferase, 10 units of inorganic pyrophosphatase, and 1 units of bovine galactosyltransferase, the reaction mixture was incubated at 25 °C for 6 h. After lyophilization of the reaction mixture, the product was separated by chromatography on silica gel using chloroform/methanol/water (6/3/0.5) as eluent. **15** was obtained in 35% yield: ^{1}H-NMR (500 MHz, D_2O) δ 1.23–1.28 (m, 12H), 1.86 (s, 3H), 2.49 (dd, 1H, J = 8.5, 15.5 Hz), 2.64 (dd, 1H, J = 4.5, 15.5 Hz), 3.37 (dd, 1H, 7.5, 9.5 Hz), 3.50 (dd, 2H, J = 3.5, 10.0 Hz), 3.53–3.64 (m, 8H), 3.64–3.78 (m, 5H), 3.80 (dd, 1H, J = 4.5, 11.5 Hz), 4.22 (q, 1H, J = 7.0 Hz), 4.31 (d, 1H, J = 7.5 Hz), 4.40 (t, 1H, J = 4.5 Hz), 4.93 (d, 1H, J = 9.5 Hz); HRMS calculated for $C_{30}H_{51}N_5O_{18}$ + Cs^+ 902.2283, found 902.2291.

Subtilisin-Catalyzed Reactions. The subtilisin-catalyzed coupling of *N*- and *O*-glycopeptide fragments was performed as follows:

1. Use of Glycopeptide Fragments as Acyl Donor. (a) *N*-Glycopeptide Coupling. An acyl acceptor (as HCl or trifluoroacetate salt) (0.1 mmol) and 0.02–0.04 mmol of acyl donor were dissolved in 100 μL of 0.75 M KOH. Reactions were started by addition of 0.2–1 mg of subtilisin and monitored by HPLC. After lyophilization of the reaction mixture, the products were separated by RP-HPLC or flash silica gel chromatography using chloroform/methanol/water mixtures as eluents. Alternatively, the reaction was performed in DMF/water (30/70 v/v) at pH 9.0 using 0.04 mmol of an acyl donor and 0.1 mmol of an acyl acceptor, respectively.

(b) *O*-Glycopeptide Coupling. An acyl acceptor (1 mmol) and 0.3 mmol of an acyl donor were dissolved in 2 mL of DMF/water (70/30 v/v). After adjustment of the pH to 8.5–9.0 using TEA, the reactions were started by addition of 5 mg of subtilisin BPN′ 8397 and monitored by TLC. After completion, the solvent was evaporated and the products were separated by HPLC or silica gel flash chromatography using methylene chloride/methanol mixtures as eluent.

2. Use of Glycopeptides as Acyl Acceptors. In a typical experiment, 100 μmol of acyl donor substrate and 100 μmol of acyl acceptor were dissolved in 100 μL of DMF/1.5 M carbonate buffer (1/1 v/v) and the pH was adjusted to 8.5–9.0 using 3 M NaOH. The reaction was started by addition of 0.1–1 mg of enzyme and monitored by RP-HPLC. After completion, the solvent was evaporated. The residue was dissolved in methanol, and the product was separated by HPLC after centrifugation.

Mal-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-Gly-NH$_2$ (16). ^{1}H-NMR (DMSO-d_6, 500 MHz) δ 1.23 (d, 3H, J = 7.5 Hz), 1.73 (s, 3H), 1.89 (s, 3H), 1.95 (s, 3H), 1.98 (s, 3H), 2.71 (dd, 1H, J = 5.6, 16.1 Hz), 3.54 (dd, 1H, J = 5.5, 16.6 H), 3.63 (dd, 1H, J = 6.0, 16.6 Hz), 3.77–3.82 (m, 1H), 3.86 (q, 1H, J = 10.0 Hz), 3.91–3.96 (m, 1H), 4.14–4.20 (m, 2H), 4.64–4.70 (m, 1H), 4.80 (t, 1H, J = 9.5 Hz), 5.08 (t, 1H, J = 9.9 Hz), 5.15 (t, 1H, J = 9.8 Hz), 6.1–8.7 (partially exchanged amide protons); HRMS calculated for $C_{27}H_{38}N_6O_{15}$ + Na^+ 709.2293, found 709.2261.

Mal-Asn(tri-*O*-acetyl-βGlcNAc)-Ala-Gly-Gly-Gly-OH (17). ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.32 (d, 3H, J = 7.2 Hz), 1.78 (s, 3H), 1.87 (s, 3H), 1.90 (s, 3H), 1.92 (s, 3H), 2.66 (dd, 1H, J = 6.3, 16.0 Hz), 2.78 (dd, 1H, J = 6.2, 16.3 Hz), 3.70–3.92 (m, 8H), 3.97 (dd, 1H, J = 2.8, 12.6 Hz), 4.11–4.20 (m, 2H), 4.82–4.89 (m, 1H), 5.06–5.13 (m, 2H), 6.12 (d, 2H, J = 12.2 Hz), 6.44 (d, 1H, J = 12.2 Hz), downfield 8.0 (partially exchanged amide protons); HRMS calculated for $C_{31}H_{43}N_7O_{18}$ + H^+ 802.2743, found 802.2713.

Mal-Asn(βGlcNAc)-Ala-Gly-NH$_2$ (18). ^{1}H-NMR (CD_3OD, 500 MHz) δ 1.32 (d, 1H, J = 7.0 Hz), 1.85 (s, 3H), 2.61 (dd, 1H, J = 5.8, 16.3 Hz), 2.73 (dd, 1H, J = 6.5, 16.5 Hz), 3.15–3.24 (m, 2H), 3.33 (dd, 1H, J = 8.0, 10.0 Hz), 3.53 (dd, 1H, J = 5.5, 12.0 Hz), 3.62 (t, 1H, J = 9.8 Hz), 3.73 (d, 2H, J = 6.5 Hz), 3.73–3.79 (m, 1H), 4.12–4.18 (m, 1H), 4.70 (t, 1H, J = 6.3 Hz), 4.83 (d, 1H, J = 10.0 Hz), 6.12 (d, 1H, J = 12.0 Hz), 6.44 (d, 1H, J = 12.0 Hz); HRMS calculated for $C_{21}H_{32}N_6O_{12}$ + H^+ 561.2156, found 561.2155.

Boc-Asn(βGlcNAc)-Ala-Ser-Leu-NH$_2$ (19). ^{1}H-NMR (CD_3OD, 500 MHz) δ 0.78 (d, 3H, J = 5.5 Hz), 0.86 (d, 3H, J = 5.5 Hz), 1.30 (d, 3H, J = 7.0 Hz), 1.33 (s, 9H), 1.50–1.63 (m, 3H), 1.87 (s, 3H), 2.59 (dd, 1H, J = 5.3, 15.9 Hz), 2.71 (dd, 1H, J = 6.5, 15.8 Hz), 3.16–3.25 (m, 1H), 3.34 (dd, 1H, J = 8.0, 10.0 Hz), 3.54 (dd, 1H, J = 4.8 Hz, 11.8 Hz), 3.61 (t, 1H, J = 5.0 Hz), 3.66–3.80 (m, 3–4H), 4.07–4.14 (m, 1H),

4.18–4.27 (m, 3H), 4.83–4.89 (m, 1H), downfield 7.0 (partially exchanged amide protons); MS calculated for $C_{29}H_{51}N_7O_{13}$ + H^+ 706.3623, found 706.3621.

Boc-Asn(βGlcNAc)-Ala-Ser-Phe-Leu-NH$_2$ (20). ^{1}H-NMR (CD_3OD, 500 MHz) δ 0.78 (d, 3H, *J* = 6.0 Hz), 0.82 (d, 3H, *J* = 6.0 Hz), 1.29 (d, 3H, *J* = 7.0 Hz), 1.31 (s, 3H), 2.61 (dd, 1H, *J* = 4.7, 16.6 Hz), 2.70 (dd, 1H, *J* = 6.5, 16.5 Hz), 2.95 (dd, 1H, *J* = 9.0, 14.0 Hz), 3.12 (dd, 1H, *J* = 5.0, 14.0 Hz), 3.21–3.27 (m, 2H), 3.34 (dd, 1H, *J* = 8.0, 10.0 Hz), 3.57 (dd, 1H, *J* = 5.0, 12.0 Hz), 3.60–3.70 (m, 3H), 3.71–3.76 (m, 1H), 4.05–4.12 (m, 1H), 4.12–4.22 (m, 2H), 4.25–4.30 (m, 1H), 4.40–4.45 (m, 1H), 7.12–7.20 (m, 5H), downfield 7.0 (partially exchanged amide protons); HRMS calculated for $C_{38}H_{60}N_8O_{14}$ + Cs^+ 985.3283, found 985.3283.

Cbz-Ser(tri-*O*-acetyl-βXyl)-Gly-NH$_2$ (21). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.37 (t, 1H), 3.77 (d, 1H), 3.86 (d, 1H), 3.89 (d, 1H), 4.03–4.06 (m, 1H), 4.11 (s, 2H), 4.43 (s, 1H), 4.51 (d, 1H), 4.87 (t, 1H, *J* = 5.0 Hz), 4.94 (d, 1H), 5.18 (t, 1H, *J* = 9.0 Hz), 5.66 (s, 1H), 5.78 (s, 1H), 7.26–7.36 (m, 5H); ^{13}C-NMR ($CDCl_3$) δ 20.63, 20.68, 36.48, 42.86, 54.48, 62.41, 67.40, 68.62, 69.60, 70.81, 71.16, 101.65, 128.18, 128.40, 128.60, 169.86, 169.96; HRMS calculated for $C_{24}H_{31}N_3O_{12}Cs^+$ 686.0962, found 686.0969.

Cbz-Ser(βXyl)-Gly-NH$_2$ (22). ^{1}H-NMR (500 MHz, D_2O) δ 3.01–3.09 (m, 3H), 3.22 (t, 1H, *J* = 9.2 Hz), 3.36–3.40 (m, 1H), 3.70–3.73 (m, 4H), 3.96–3.99 (m, 1H), 4.18 (d, 1H, *J* = 8.0 Hz), 4.22 (m, 1H), 4.96 (d, 2H, *J* = 4.5 Hz), 7.20–7.26 (m, 5H); ^{13}C NMR (CD_3OD) δ 52.98, 55.66, 67.03, 67.77, 70.48, 71.01, 74.75, 77.56, 105.12, 128.90, 129.02, 129.54, 138.10, 158.63, 172.30; HRMS calculated for $C_{18}H_{25}N_3O_9Na^+$ 450.1488, found 450.1488.

Cbz-Ala-Ser(βXyl)-Gly-Ala-NH$_2$ (23). ^{1}H-NMR (500 MHz, D_2O) δ 1.23 (d, 3H, *J* = 3.0 Hz), 1.27 (t, 3H, *J* = 3.5 Hz), 3.11–3.06 (m, 3H), 3.24 (t, 1H, *J* = 9.2 Hz), 3.43–3.38 (m, 1H), 3.73 (s, 2H), 3.75–3.79 (m, 2H), 3.83 (d, 1H, *J* = 1.5 Hz), 3.86 (d, 1H, *J* = 1.2 Hz), 3.95–4.00 (m, 2H), 4.11 (dd, 1H, *J* = 7.2, 7.3 Hz), 4.29 (d, 1H, *J* = 7.7 Hz), 4.41 (t, 1H, *J* = 4.5 Hz), 4.95 (d, 2H, *J* = 5.3 Hz), 7.20–7.27 (m, 5H); ^{13}C-NMR (D_2O) δ 21.81, 24.56, 25.85, 31.44, 32.24, 33.15, 33.38, 35.80, 47.26, 49.23, 50.54, 51.18, 54.82, 57.59, 85.17, 109.68, 110.47, 110.86, 118.41, 140.03, 152.90, 153.81, 158.29, 159.73; HRMS calculated for $C_{24}H_{35}N_5O_{11}Cs^+$ 702.1387, found 702.1399.

Cbz-Thr-(αMan)-Val-Ala-Tyr-OH (24). ^{1}H-NMR (500 MHz, D_2O) δ 0.61 (d, 3H, *J* = 7 Hz), 0.66 (d, 3H, *J* = 6.5 Hz), 1.08 (d, 3H, *J* = 6.0 Hz), 1.12 (d, 3H, *J* = 7.0 Hz), 1.74 (m, 1H), 2.80 (dd, 1H), 2.92 (dd, 1H), 3.41 (t, 1H), 3.54 (m, 4H), 3.66 (d, 1H), 3.89 (d, 1H), 4.05–4.12 (m, 3H), 4.40 (m, 2H), 4.97 (d, 2H, *J* = 5.5 Hz), 6.63 (d, 2H, *J* = 8.0 Hz), 6.95 (d, 2H, *J* = 8.0 Hz), 7.20–7.24 (m, 5H); ^{13}C-NMR (D_2O) δ 16.81, 17.84, 17.90, 30.51, 35.91, 49.40, 54.20, 59.30, 59.84, 61.10, 67.00, 67.62, 70.41, 70.51, 73.30, 76.10, 101.40, 115.60, 127.80, 128.30, 128.60, 129.01, 130.7, 136.50, 154.60, 172.20, 172.41, 174.48, 174.84; HRMS calculated for $C_{35}H_{48}N_4O_{14}Cs^+$ (M + Cs^+) 881.2221, found 881.2225.

Cbz-Thr(αMan)-Val-Gly-Ala-NH$_2$ (25). ^{1}H-NMR (500 MHz, CD_3OD) δ 0.87 (d, 3H, *J* = 3.0 Hz), 1.18 (d, 3H, *J* = 6.0 Hz), 1.28 (d, 3H, *J* = 7.0 Hz), 1.97 (m, 1H), 3.21 (q, 1H, *J* = 1.5 Hz), 3.51 (m, 2H), 3.61–3.65 (m, 3H), 3.74 (m, 1H), 3.83 (d, 2H, *J* = 3.5 Hz), 4.02 (d, 1H, *J* = 7.0 Hz), 4.20 (s, 2H), 5.02 (s, 3H), 7.20–7.31 (m, 5H); HRMS calculated for $C_{28}H_{43}N_5O_{12}Cs^+$ 774.1963, found 774.1958.

Cbz-Ala-Ser-Ala-Asn(βGlcNAc)-NH$_2$ (26). ^{1}H-NMR (D_2O, 500 MHz) δ 1.15–1.24 (m, 6H), 1.83 (s, 3H), 2.48–2.70 (m, 2H), 3.27–3.36 (m, 2H), 3.40–3.47 (m, 1H), 3.52–3.60 (m, 1H), 3.62–3.77 (m, 4H), 3.95–4.03 (m, 1H), 4.06–4.15 (m, 1H), 4.23–4.30 (m, 1H), 4.48–4.53 (m, 1H), 4.87 (d, 1H, *J* = 9.5 Hz), 4.98 (s, 2H), 7.15–7.30 (m, 5H); HRMS calculated for $C_{29}H_{43}N_7O_{13}$ + Cs^+ 830.1973, found 830.1977.

Cbz-Ala-Ser-Ala-Ser(βXyl)-NH$_2$ (27). ^{1}H-NMR (D_2O, 500 MHz) δ 1.25 (d, 3H, *J* = 7.0 Hz), 1.28 (d, 3H, *J* = 7.5 Hz), 3.03–3.10 (m, 2H), 3.21–3.24 (m, 2H), 3.32–3.38 (m, 1H), 3.60 (dd, 1H, *J* = 4.0, 10.0 Hz), 3.66 (q, 1H, *J* = 5.5 Hz), 3.74 (dd, 1H, *J* = 5.0, 11.5 Hz), 3.79 (dd, 1H, *J* = 5.0, 10.5 Hz), 4.04 (q, 1H, *J* = 7.0 Hz), 4.12 (d, 2H, *J* = 7.5 Hz), 4.20 (q, 1H, *J* = 7.5 Hz), 4.29 (t, 1H, *J* = 5.3 Hz), 4.42 (t, 1H, *J* = 4.5 Hz), 4.97–5.01 (m, 2H), 7.15–7.28 (m, 5H); HRMS calculated for $C_{25}H_{37}N_5O_{12}$ + Cs^+ 732.1493, found 732.1532.

Cbz-Ala-Ser-Gly-Ala-Asn(βGlcNAc)-NH$_2$ (28). ^{1}H-NMR (D_2O, 500 MHz) δ 1.15–1.23 (m, 6H), 1.83 (s, 3H), 2.50–2.70 (m, 2H), 3.27–3.36 (m, 2H), 3.40–3.46 (m, 1H), 3.56 (dd, 1H, *J* = 4.5, 12.5 Hz), 3.61–3.80 (m, 5H), 3.97–4.07 (m, 1H), 4.08–4.17 (m, 1H), 4.24–4.30 (m, 1H), 4.43–4.52 (m, 1H), 4.88 (dd, 1H, *J* = 9.5 Hz), 4.96 (s, 2H), 7.17–7.30 (m, 5H); HRMS calculated for $C_{31}H_{46}N_8O_{14}$ + Cs^+ 887.2188, found 887.2193.

Boc-Asn(βLacNAc)-Ala-Ser-Phe-Leu-NH$_2$ (29). ^{1}H-NMR (500 MHz, DMSO-d_6) δ 0.80 (d, 3H, *J* = 6.5 Hz), 0.85 (d, 3H, *J* = 6.5 Hz), 1.14 (d, 3H, *J* = 7.0 Hz), 1.34 (s, 9H), 1.40–1.48 (m, 2H), 1.48–1.57 (m, 1H), 2.39 (dd, 1H, *J* = 8.5, 16.5 Hz), 2.54 (dd, 1H, *J* = 4.8, 16.5 Hz), 2.83 (dd, 1H, *J* = 9.0, 14.0 Hz), 3.05 (dd, 1H, *J* = 4.3, 14.0 Hz), 3.40–3.62 (m, 12H), 3.72 (dd, 1H, *J* = 8.0, 12.5 Hz), 4.13–4.26 (m, 5H), 4.39–4.46 (m, 1H), 4.53 (d, *J* = 5.0 Hz), 4.63 (t, 1H, *J* = 5.2 Hz), 4.68 (t, 1H, *J* = 4.8 Hz), 4.71 (s, 1H), 4.80–4.88 (m, 2H), 5.10–5.17 (m, 2H), 6.74 (d, 1H, *J* = 8.0 Hz), 7.03 (s, 2H), 7.12–7.24 (m, 5H), 7.82 (d, 1H, *J* = 8.5 Hz), 7.89 (d, 1H, *J* = 8.5 Hz), 7.91 (d, 1H, *J* = 7.0 Hz), 7.94 (d, 1H, *J* = 7.4 Hz), 8.07 (d, 1H, *J* = 7.5 Hz), 8.28 (d, 1H, *J* = 9.0 Hz); HRMS calculated for $C_{44}H_{70}N_8O_{19}$ + Cs^+ 1147.3812, found 1147.3750.

Cbz-Ala-Ser(βGal1,4βXyl)-Gly-Ala-NH$_2$ (30). To 1 mL of an aqueous solution (containing 15% acetone, 100 mM Hepes, 4 mM KCl, 4 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM DTT) were added 14 mg PEP, 20 mg of Gal-1-P, 20 mg of Cbz-Ala-Ser(βXyl)-OMe, 2.7 mg of UDP, and 1.5 mg of Glc-1-P. The solution was adjusted to pH 7.5. After addition of 0.4 mg of α-lactabumin, 200 units of pyruvate kinase, 10 units of UDP-glucose pyrophosphorylase, 10 units of Gal-1-P uridyltransferase, 1 units of inorganic pyrophosphatase, and 3 units of bovine galactosyltransferase, the reaction mixture was incubated at 25 °C for 72 h. After lyophilization of the reaction mixture, the product (5 mg, 21% yield) was separated by HPLC: ^{1}H-NMR (500 MHz, D_2O) δ 1.16 (d, 3H, *J* = 3.0 Hz), 1.20 (d, 3H, *J* = 3.5 Hz), 3.09 (q, 3H, *J* = 9.0 Hz), 3.28 (t, 1H, *J* = 7.0 Hz), 3.36–3.52 (m, 6H), 3.35–3.59 (m, 5H), 3.68 (s, 3H), 3.85–3.93 (m, 3H), 4.18–4.22 (m, 3H), 4.18–4.22 (m, 3H), 4.37 (s, 1H), 4.93 (s, 2H), 7.10–7.20 (m, 5H); HRMS calculated for $C_{30}H_{45}N_5O_{16}Cs^+$ (M + Cs^+) 864.1916, found 864.1916.

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