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Combined Chemical and Enzymatic Synthesis of a *C*-Glycopeptide and Its Inhibitory Activity toward Glycoamidases

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Abstract: A novel chemoenzymatic approach to synthesizing high-mannose-type *N*-glycopeptide and its *C*-linked glycopeptide analog is described. The synthesis consists of two steps: a chemical synthesis of GlcNAc-containing peptides and an enzymatic glycosyl transfer of $Man_9GlcNAc$ to the terminal GlcNAc in the peptides in an aqueous medium containing organic solvents. The essential enzyme used is an *endo-β-N*-acetyl-glucosaminidase from *Arthrobacter protophormiae* (Endo-A). This approach should be generally applicable to the synthesis of both natural and designed high-mannose-type glycopeptides. It has been found that, while the natural high-mannose-type *N*-glycopeptide **2** can be rapidly hydrolyzed by glycoamidases [commonly called *N*-glycanase or, systematically, peptide-N^4-(*N*-acetyl-β-D-glucosaminylasparagine amidase], the synthetic *C*-glycopeptide **1** with an insertion of a methylene group at the crucial asparagine−GlcNAc linkage is resistant to the enzyme-catalyzed hydrolysis and shows apparent inhibitory activity toward glycoamidases of plant, bacterial, and animal origin, with the K_i values ranging from 1 to 160 μM for different enzymes. The *C*-glycopeptide **1** is the first, broad spectrum inhibitor for glycoamidases, which is expected to be a useful tool in the study of the mechanism and biological functions of the enzymes.

Introduction

Protein glycosylation is one of the most common co- and posttranslational modifications of proteins, in which sugar chains are added to specific Asn or Ser/Thr residues to form the *N*- and *O*-linked glycoproteins, respectively.[1] Protein glycosylation plays a crucial role in the expression of most cell-surface and secreted proteins and is frequently required for the correct folding, stability, antigenicity, and biological functions of proteins, and as recognition markers for cell−cell and cell−matrix interactions.[2−4] Although it is well-known that removal of the carbohydrate moieties from certain glycoproteins could cause structural and physiological changes in their core proteins, thus affecting the properties of the proteins, little is known about the functional importance of physiological protein "de-glycosylation". However, recent findings have indicated that protein de-glycosylation mediated by glycoamidase [peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase] may be an important posttranslational modification of proteins for regulating their biological functions in living organisms.[5,6]

Glycoamidase (also called *N*-glycanase, peptide *N*-glycanase,

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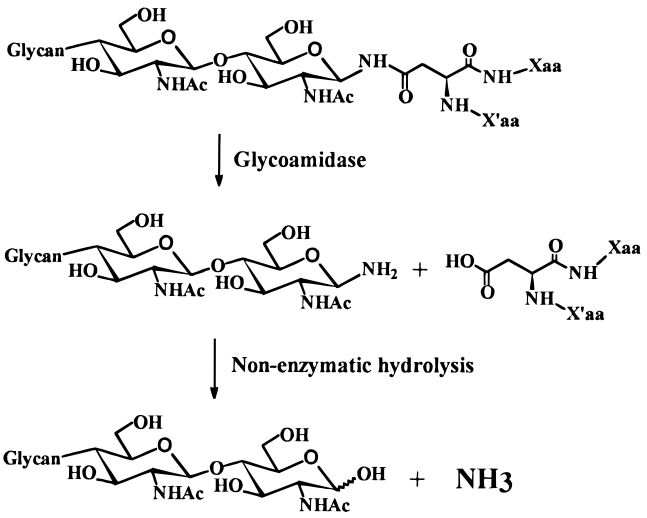

Figure 1. Schematic depiction of glycoamidase-catalyzed hydrolysis of *N*-glycopeptides.

and glycopeptidase) is an enzyme that releases the intact oligosaccharides from *N*-glycopeptides or *N*-glycoproteins by cleaving the β-aspartylglucosylamine amide linkage. Upon hydrolysis, the oligosaccharide is released in the form of glycosylamine which will be spontaneously hydrolyzed to reducing oligosaccharide, and the asparagine residue is converted to aspartic acid (Figure 1).[7,8] Two glycoamidases which have been well characterized and widely used for glycoprotein studies are the plant glycoamidase A from almond emulsin[7] and the bacterial glycoamidase F from *Flavobacterium meningosepticum*.[8] Both glycoamidases A and F can liberate *N*-glycans of high-mannose-, complex-, and hybrid-type in *N*-glycopeptides from vertebrates, given that both the amino and carboxyl groups of the glycosylated asparagine (Asn) residue are present in peptide linkage.[7,8] However, a striking difference in substrate specificity exists between the two enzymes. For example, the bacterial enzyme is unable to release *N*-glycans bearing a (1,3)-fucose residue on the Asn-linked *N*-acetylglucosamine (GlcNAc), often found in glycoproteins from insects and plants.[9,10] In addition to glycoamidase A, more glycoamidases have been subsequently isolated from different plant sources.[11] Furthermore, other glycoamidases have been found recently in animal organs and cultured cells, e.g., in early embryos of Medaka fish (Oryzias latipes),[12] in organs and tissures of mice,[13] and in cultured animal cells.[14,15] More recently, it has been shown that glycoamidase activity are present in human cytomegalovirus infected cells[16] and in human tumor cell lines,[17] in which the enzymes are assumed to be involved in the destruction of newly synthesized major histocompatibility (MHC) class I molecules and in the processing of the tumor antigen presented by MHC class I molecules.[16,17] The wide occurrence of glycoamidase strongly suggests that protein *N*-de-glycosylation mediated by the enzyme may be a universal feature in living organisms as a functionally important mechanism of regulation.[5,6,16,17]

To study the mechanism and biological functions of glycoamidases, we are interested in various glycopeptide substrates and their analogs which can serve as useful probes. Among others, substrate analogs with a modification at the crucial carbohydrate−peptide linkage in the natural glycopeptides is of great interest. For this purpose, we have chosen a high-mannose-type *N*-glycopeptide analog, a *C*-glycopentapeptide **1** as our first target molecule (Figure 2). The *C*-glycopeptide **1** contains all the structural features of the natural *N*-glycopeptide **2** except the inserted methylene group at the crucial asparagine−GlcNAc linkage. However, in comparison with peptide synthesis, glycopeptide synthesis involves many more functional groups and is a more challenging task when a complex oligosaccharide chain is attached.[18] Several approaches are available for *N*-glycopeptide synthesis, including solid phase synthesis using glycosylated amino acid building blocks,[19] the convergent coupling of Asp-containing peptide with protected or unprotected glycosylamine,[20] and combined chemical and enzymatic synthesis using peptidase and/or glycosyltransferases.[21] However, these approaches are not quite suitable for the synthesis of the target molecule **1** because they are either

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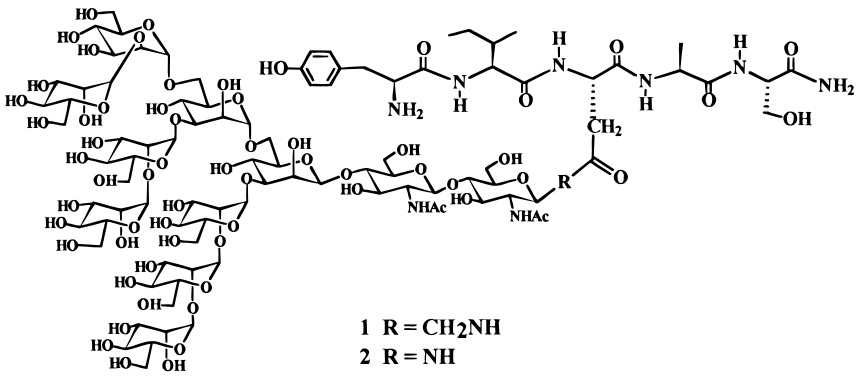

Figure 2. Structure of a high-mannose-type natural *N*-glycopeptide and the designed *C*-glycopeptide.

limited to the synthesis of glycopeptides bearing simple sugar moiety or restricted to the synthesis of glycopeptides with natural linkages.

We describe in this paper a novel strategy for the synthesis of the *C*-glycopeptide **1**[22] and the natural high-mannose *N*-glycopeptide **2**, as well as their biological activity toward various glycoamidases. The synthetic strategy consists of two key steps: a chemical synthesis of a GlcNAc containing glycopeptide or neoglycopeptide and an enzymatic transfer of a Man_n-GlcNAc moiety ($n = 5-9$) to the terminal GlcNAc residue in the peptide. The essential enzyme used is endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A),[23] which hydrolyzes the glycosidic bond in the *N*,*N*′-diacetylchitobiose moiety of high-mannose-type *N*-glycans. Endo-A was also reported to have a transglycosylation activity, being able to transfer a Man_{5-9}GlcNAc residue to the 4-OH of a terminal GlcNAc residue, although the transglycosylation activity is very low compared with its hydrolytic activity.[24] Our strategy was based on our finding that the transglycosylation yield of Endo-A could be substantially enhanced by performing the enzymatic reaction in aqueous organic solvents.[25] The synthetic *C*-glycopeptide **1** has been found to be a competitive inhibitor for all glycoamidases of plant, bacterial, and animal origins we have tested.

Results and Discussion

Stepwise Synthesis of a GlcNAc-CH_2-Pentapeptide (14). There has been an increasing interest in *C*-glycosides and related compounds in recent years, because of their mimetic effects and resistance to glycosidase-catalyzed hydrolysis.[26] However, the synthesis of *C*-glycosides of 2-acetamido-2-deoxy sugars has proven to be a difficult task because the common Lewis acid-catalyzed *C*-glycosylation and nucleophilic substitution reaction usually lead to the formation of oxazolines when applied to the 2-acetamido-2-deoxy sugars.[27] We chose a protecting group strategy for preparing the key *C*-(2-acetamido-2-deoxyglucosyl)methylamine as the building block, and the synthesis of the GlcNAc-CH_2-pentapeptide **14**, is summarized in Scheme 1. Reduction of the β-glycosyl cyanide **3**[28] via catalytic hydrogenation followed by *N*-protection of the resulting glycosylmethylamine gave **4**. A large $J_{1,2}$-value (9.8 Hz) indicated that **4** was in the desired 1,2-trans-configuration. The *N*-phthaloyl derivative **4** was successfully converted into the *N*-acetyl derivative **5** by treatment of **4** with hydrazine hydrate followed by acetylation. De-*N*-Boc-protection of **5**, followed by its condensation with Boc-Asp-OBn, provided the fully protected N^4-(2-acetamido-2-deoxy-β-D-glucopyranosylmethyl)-asparagine **7**.[29] This compound can serve as a building block for synthesizing various neo-glycopeptides containing the GlcNAc-CH_2-Asn structural unit. For the preparation of GlcNAc-CH_2-pentapeptide **14**, a stepwise solution-phase synthetic approach was used. De-*O*-benzylation of **7** followed by coupling with dipeptide H-Ala-Ser-OMe (**9**) gave the tripeptide derivative **10**, which was then elongated at the *N*-terminus to pentapeptide derivative **13** by coupling with the dipeptide Boc-Tyr-Ile-OH **12**. Deprotection of **13** afforded the GlcNAc-CH_2-pentapeptide **14**, which was purified by reversed phase high-performance liquid chromatography (RP-HPLC). The overall yield from the glycosyl cyanide **3** is 23% (Scheme 1).

A Convergent Approach to the GlcNAc-CH_2-Pentapeptide 14. Compound **14** was also synthesized by a convergent approach, as shown in Scheme 2. First, a pentapeptide **18** with a free β-carboxyl group in the Asp residue was prepared by a stepwise solution peptide synthesis. Then **18** was condensed with the glycosylmethylamine derivative **6** using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[20,30] as the coupling reagent, giving **13** in 65% yield. Deprotection of **13** followed by RP-HPLC purification provided the *C*-glycopentapeptide **14** (Scheme 2). In comparison, the convergent approach is superior to the stepwise approach in both the length of the synthetic scheme and the overall yield for the preparation of **14**.

Enzymatic Transfer of Man_9GlcNAc to the GlcNAc-CH_2-Pentapeptide. To prepare the high-mannose-type *C*-glycopeptide **1** by an Endo-A-catalyzed glycosyl transfer reaction, a

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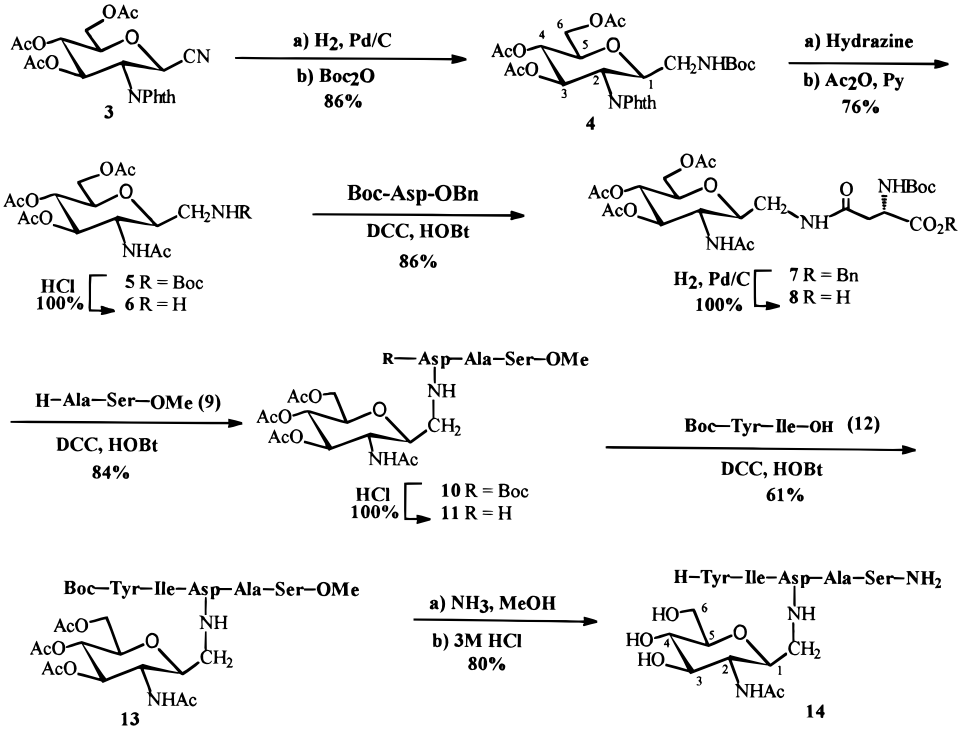
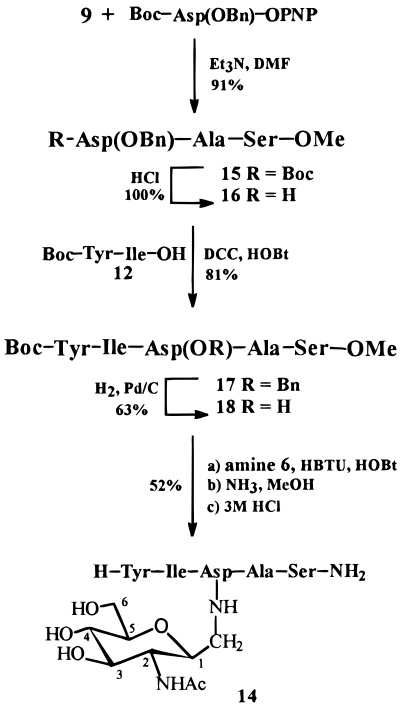
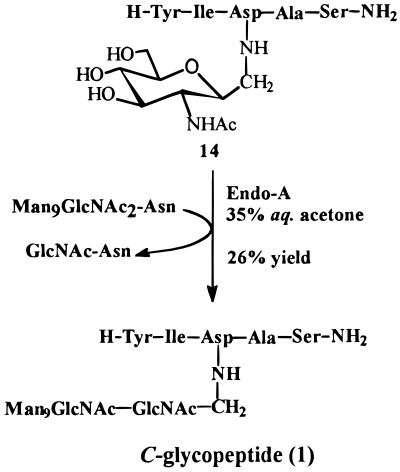
Scheme 1



Scheme 2



Scheme 3



suitable high-mannose *N*-glycan donor is required. We chose $Man_9GlcNAc_2Asn$ as the donor substrate because $Man_9GlcNAc_2$-Asn can be conveniently prepared from soybean agglutinin (isolated from soybean flour) by exhaustive protease digestion and subsequent gel filtration separation.[31] The transglycosylation was performed in acetate buffer (pH 6.0) containing 35% acetone with 4-fold excess of the acceptor **14** (50 mM) (Scheme 3). Unfortunately, the low solubility of **14** in the reaction medium prevented the use of a higher concentration of the acceptor substrate. Under an optimal condition (see Experimental Section), the transglycosylation product, H-Tyr-Ile-Asn-($Man_9GlcNAc_2CH_2$)-Ala-Ser-NH_2 (**1**), was obtained in 26% yield after RP-HPLC purification, and the excess acceptor could be effectively recovered and recycled. It is worthy to note that, in contrast to using a host of glycosyltransferases,[21] the present approach allows the installation of a complex oligosaccharide chain into glycoconjugates in *one step* using only *one enzyme*. Although Endo-A is specific for hydrolyzing and transfering high-mannose-type *N*-glycans, the approach described here should be readily extendable to complex- and hybrid-type *N*-glycopeptides and their analogs, when a suitable endoenzyme becomes available.[32]

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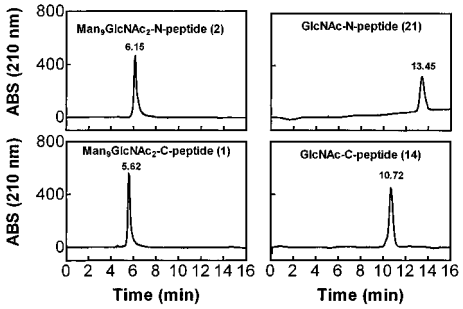


Figure 3. RP-HPLC patterns of the *N*-glycopeptides **2** and **21** and of the corresponding *C*-glycopeptides **1** and **14**. The RP-HPLC was performed on a Microsorb MW ODS column (4.6 × 250 mm, Rainin, Ridgefield, NJ) (mobile phase, 9% aqueous MeCN containing 0.05% trifluoroacetic acid; flow rate, 1.0 mL/min).

^{1}H-NMR of **1** showed eight H-1 α-Man signals at δ 5.400−4.894 and one H-1 β-Man signal at δ 4.783. The H-1 GlcNAc-2 signal appeared as a distorted doublet at δ 4.618 with a relatively large *J*-value (*cat.* 7.5 Hz), indicating a β-anomeric configuration for the newly formed glycosidic linkage. Amino acid and sugar composition analysis of **1** gave satisfactory results. Moreover, the structure of **1** was confirmed by high-resolution FAB-MS.

Synthesis of a Natural High-Mannose *N*-Glycopeptide 2. To demonstrate the general applicability of the synthetic strategy, a natural high-mannose *N*-glycopeptide **2** was also synthesized. First, 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine (**19**)[33] was condensed with the pentapeptide **18** using HBTU as the coupling reagent to give **20**, which was then converted into the GlcNAc-*N*-pentapeptide **21** *via* sequential deprotections (41% overall yield from **18**). Finally, an enzymatic transfer of a $Man_9GlcNAc$ to **21**, as in the preparation of the *C*-glycopeptide **1**, provided a 25% yield of the desired high-mannose *N*-glycopeptide **2** after RP-HPLC purification (Scheme 4).

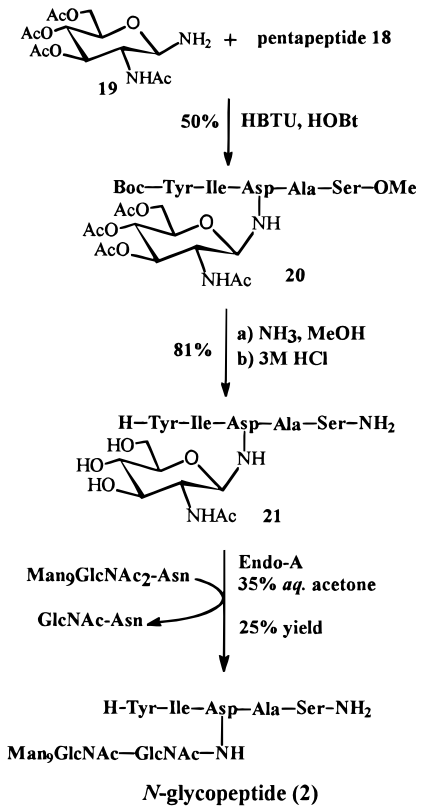
A comparison of the elution position of the *C*-glycopeptide with that of the *N*-glycopeptide in RP-HPLC reveals an interesting feature (Figure 3). Under the same HPLC conditions, the *C*-glycopeptide **1** is actually eluted earlier (t_R = 5.62 min) than the corresponding *N*-glycopeptide **2** (t_R = 6.15 min). Similarly, the GlcNAc-CH_2-pentapeptide **14** was eluted earlier (t_R = 10.72 min) than the GlcNAc-*N*-pentapeptide **21** (t_R = 13.45 min). It seems that an insertion of the hydrophobic methylene group, rather than enhancing its interaction with the hydrophobic stationary phase of the column, has a "negative" effect.

Biological Activity of the *C*-Glycopeptide toward Glycoamidases. The potential biological activity of the *C*-glycopeptide was tested with several typical glycoamidases originated from plant, bacteria, and animals. Glycoamidase A from almond emulsin and glycoamidase F from *Flavobacterium meningosepticum* are well-known and are commercially available.[7,8,34] Moreover, we isolated two new glycoamidases: a bacterial glycoamidase Sm from *Sphingobacterium multivorum* and an animal glycoamidase HO from hen oviduct. These enzymes were purified to homogeneity, completely devoid of protease and exo- and endoglycosidase activities and were used for studying the biological activity of the *C*-glycopeptide.

*(1) Hydrolysis of the synthetic C-glycopeptide **1** by various glycoamidases.* To examine whether the *C*-glycopeptide can be hydrolyzed by the glycoamidases, the *C*-glycopeptide was incubated with glycoamidases A, F, Sm, and HO, respectively, under the optimal conditions for 18 h. The reaction mixture was analyzed by RP-HPLC. In all cases, we observed no change in the *C*-glycopeptide after enzymatic incubation, nor was the formation of any detectable hydrolytic products. In contrast, under the same conditions, the synthetic natural *N*-glycopeptide **2** was rapidly hydrolyzed (data not shown). Therefore, the *C*-glycopeptide is not a substrate for all the glycoamidases so far tested, although the only structural difference between the *C*- and *N*-glycopeptides is the presence of a CH_2 group between the anomeric carbon and the amide nitrogen. The results suggest a strict structural requirement by the glycoamidases at the cleaving site of the substrate. Alternatively, the nature of the potential leaving group of the enzymatic hydrolysis (glycosylmethylamine in the *C*-glycopeptide versus glycosylamine in the *N*-glycopeptide) may also account for the lack of hydrolytic activity of the *C*-glycopeptide, considering that the pK_a of glycosylmethylamine is usually 2−3 units higher than that of the corresponding glycosylamine.[35]

(2) Inhibition of Glycoamidase-Catalyzed Hydrolysis by the C-Glycopeptide. The effects of the C-glycopeptide **1** on the glycoamidase-catalyzed hydrolysis were studied. The ^{14}C-labeled asialofetuin glycopeptide I (asialo-fetGP I), Leu-Asn($Man_3Gal_3GlcNAc_5$)-Asp-Ser-Arg, which was prepared from fetal calf serum fetuin with a triantennary complex-type glycan chain,[14] was used as the substrate for glycoamidases Sm and HO. In contrast, the synthetic *N*-glycopeptide **2** was used as the substrate for glycoamidases A and F. The K_m of the asialo-fetGP I was determined to be 170 and 110 μM for glycoamidases Sm and HO, respectively; and the K_m of **2** for glycoamidases A and F was determined to be 8 and 80 μM, respectively. As can be seen in Figure 4, the *C*-glycopeptide **1** shows apparent

Scheme 4



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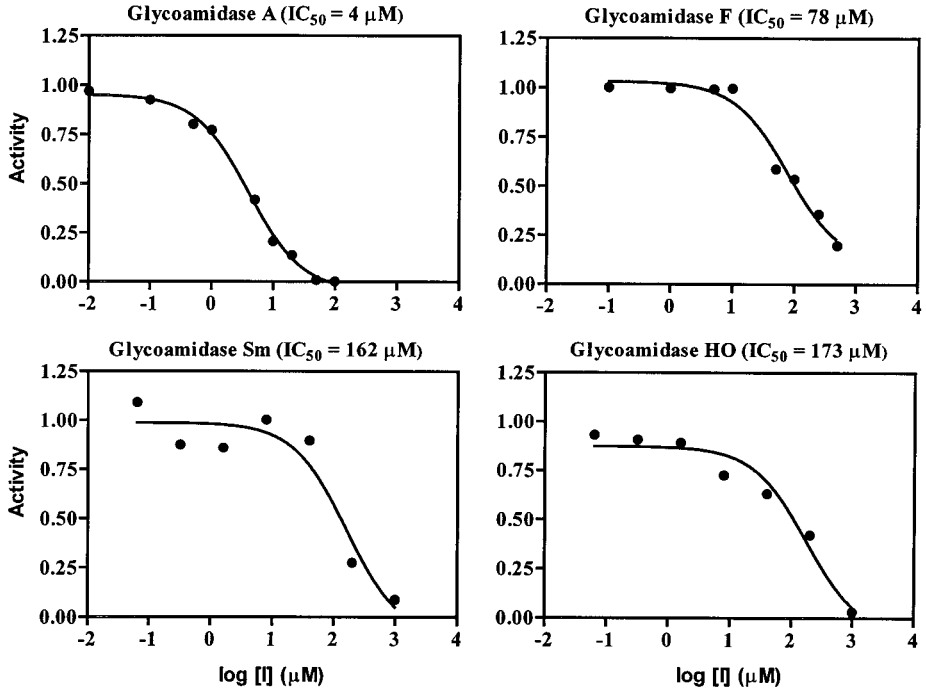

Figure 4. Inhibition of various glycoamidases by the *C*-glycopeptide **1**. The *N*-glycopeptide **2** was used as the substrate for glycoamidases A and F; in contrast, a radiolabeled glycopeptide asialo-[^{14}C]fetGP I was used as the substrate for glycoamidases Sm and HO. The substrate concentration was fixed at 11 μM. The IC_{50} were obtained by using nonlinear regression (logistics equation) with the Graphpad Prism program (GraphPad Software, Inc., Grand Junction, CO).

inhibitory activity toward all the glycoamidases tested. Anaylsis by the Lineweaver−Burk plot (1/*V* vs 1/[S]) in the presence of **1** at various concentrations reveals that the inhibition of the *C*-glycopeptide **1** against the glycoamidases is competitive in nature (data not shown), and the enzyme-inhibitor dessociation constant K_i of **1** for glycoamidases A, F, Sm, and HO was determined to be 1, 43, 152, and 157 μM, respectively. This is the first, broad-spectrum inhibitor for glycoamidases.

Conclusion

We have described a novel strategy for the synthesis of high-mannose-type *N*-glycopeptide and its *C*-linked glycopeptide analog, which allows the installation of a complex oligosaccharide moiety into glycoconjugate in one single step using one enzyme. This strategy should be generally applicable to the preparation of many desirable high-mannose-type glycopeptides and their analogs and can be extended to the hybrid- and complex-types, when a suitable endoenzyme becomes available. The synthetic *C*-glycopeptide **1** with a CH_2 between the β-amide and the anomeric carbon demonstrates an apparent inhibitory activity toward various glycoamidases of plant, bacterial, and animal origin, which comprises the first and broad-spectrum inhibitor for the enzymes and should have considerable potential as a tool in the study of the mechanism and biological functions of diverse glycoamidases.

Experimental Section

Materials and Methods. $Man_9GlcNAc_2Asn$ was prepared from soybean agglutinin by exhaustive pronase digestion, followed by gel filtration on Sephadex G-50.[31] *Endo-N*-acetyl-β-glucosaminidase from *Arthrobacter protophormiae* (Endo-A) is a gift from Takara Shuzo Co. (Ohtsu, Japan). Glycoamidase A (from almond) and glycoamidase F (from *Flavobacterium*) were purchased from Seikagaku America, Inc. (Rockville, MD) and Glyko, Inc. (Novato, CA), respectively.

Asialofetuin glycopeptide I (asialo-fetGP I) having a triantennary complex-type glycan chain, Leu-Asn($Man_3Gal_3GlcNAc_5$)-Asp-Ser-Arg, was prepared from fetal calf serum fetuin (Nacalai Tesque, Co., Japan) as previously described.[14] The glycopeptide obtained was ^{14}C-labeled at the amino terminal residue by reductive methylation[13] at the Radioisotope Centre, University of Tokyo. Specific radioactivity for asialo-[^{14}C]fetGP I was determined to be 1.1×10^5 dpm/nmol.

Glycoamidase Sm was purified from *Sphingobacterium multivorum* to homogeneity, completely devoid of protease and exo- and endoglycosidase activities (details will be reported elsewhere). The molecular weight of the enzyme was estimated to be 86 000 by Sephacryl S-200 gel filtration.

Glycoamidase HO was purified from hen oviduct as follows: 270 g of hen oviduct was homogenized in a Waring blender in 2 volumes (w/v) of 50 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.5) containing 2 mM DTT (dithiothreitol) and various protease inhibitors, i.e., leupeptin, 0.5 μg/ml; aprotinin, 1.0 μg/mL; pepstatin, 0.7 μg/mL; soybean trypsin inhibitor, 0.5 μg/mL; phenylmethanesulfonyl fluoride, 100 μM (all the purification procedures were carried out at 4 °C or on ice). The homogenate was centrifuged at 10 000*g* for 10 min, and the precipitate was washed by suspension with an appropriate volume of the homogenization buffer. NaCl was then added to the combined supernatant to a final concentration of 2.4 M, and the solution was applied on a TSK butyl-Toyopearl 650M column (Tosoh; 5.5 × 10 cm) equilibrated with 50 mM HEPES buffer (pH 7.5) containing 2.4 M NaCl and 2 mM DTT. The column was washed first with 500 mL of equilibration buffer, followed by elution with 700 mL of 50 mM HEPES buffer (pH 7.5) containing 0.4 M ammonium sulfate and 2 mM DTT. Glycoamidase-positive fraction was then eluted with 500 mL of 50 mM HEPES buffer (pH 7.5) containing 2 mM DTT, and the fraction obtained was concentrated by ultrafiltration with YM-30 membrane (Amicon) to 100 mL. Ammonium sulfate was then added to the fraction up to 0.4 M, and the resulting solution was applied to a butyl-Toyopearl 650 M column (1.7 × 9 cm) equilibrated with 50 mM HEPES buffer (pH 7.5) containing 0.4 M ammonium sulfate and 2 mM DTT and was washed with 100 mL of equilibration buffer. Glycoamidase-positive fraction was then eluted with 50 mM HEPES buffer (pH 7.5) containing 2 mM DTT, concentrated by YM-30 membrane, and applied to a Sephacryl S-300 column (2.5 × 155 cm) equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 4 mM DTT, collecting 5.6 mL fractions. The glycoamidase-active fractions (41−45) were pooled and concentrated by ultrafiltration with a YM-30 membrane to

15 mL and the mixture was applied to a DEAE-Memsep 1010 column (Millipore). The flow-through fraction containing glycoamidase activity was concentrated by absorption on a Gigapite (Hydroxyapatite) column (Seikagaku Kogyo Co., Tokyo; 1.2 × 3 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM DTT, and eluted with 0.1 M sodium phosphate buffer (pH 7.0) containing 50 mM HEPES buffer (pH 7.5) and 2 mM DTT. The enzyme fraction was purified 87-fold to 5.2 milliunit/mg protein in a 2.0% yield from the supernatant of 10 000*g* centrifugation. This fraction is devoid of *endo*-β-*N*-acetylglucosaminidase, β-*N*-acetylglucosaminidase, β-galactosidase, and α-mannosidase activities under the glycoamidase assay conditions.

HPLC was performed on a Gilson HPLC system equipped with a UV detector (ISCO, Lincoln, NE), a Rheodyne 7125 injector, and a Fiatron CH-30 column heater. High-performance anion exchange chromatography (HPAEC) was performed on a Bio-LC [(Dionex Corp., Sunnyvale, CA) HPAEC system], equipped with a pulsed amperometric detector (PAD-II), and was managed with AI-450 chromatography software (Dionex).

Melting points were determined with a Fisher-Johns apparatus and uncorrected. Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN. ^{1}H-NMR spectra were recorded with a Bruker AMX-300 or a Varian Unity Plus 500 NMR spectrometer at 25 °C unless otherwise specified. Multiplicities are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). HR-FABMS was recorded with a VG ProSpec mass spectrometer, and the ES-MS was obtained with a Hewlett-Packard Model 5989 B electrospray ionization mass spectrometer. Thin layer chromatography (TLC) was carried out on precoated plates of silica gel (E. Merck, $60F_{254}$, 0.25 mm layer thickness). Column chromatography was performed on silica gel (E. Merck). Ratios of solvents for TLC and column chromatography were expressed in volume.

General Procedure for Peptide Synthesis. An *O*-protected amino acid hydrochloride (1.0 equiv) and an *N*-protected amino acid (1.0 equiv) were dissolved in CH_2Cl_2 or DMF at 0 °C. To the solution were sequentially added triethylamine (1.1 equiv), HOBt (1.0 equiv), and DCC (1.1 equiv). The mixture was stirred at 0 °C for 30 min, then at room temperature for 10−20 h (monitored by TLC). White precipitate was removed by filtration, and the filtrate was evaporated *in vacuo* to dryness. The desired peptide was finally purified by column chromatography on silical gel.

Deblocking of *N*-Boc Group in Peptide Synthesis. The *N*-Boc-protected compound was dissolved in 4 M HCl in anhydrous dioxane at 0 °C and the mixture was stirred at the same temperature. After completion of the reaction (monitored by TLC), the reaction mixture was evaporated and the residue was coevaporated with toluene *in vacuo*. Trituration of the residue with ether gave the peptide as its hydrochloride.

***N*-*tert*-Butoxycarbonyl-*C*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)methylamine (4).** 3,4,6-Tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl cyanide (**3**)[28] (955 mg, 2.15 mmol) was dissolved in THF−EtOH (9:1, v/v, 20 mL), to which 10% palladium on charcoal (110 mg) was added, and the mixture was stirred under hydrogen atmosphere for 16 h. Then the reaction mixture was filtered. The filtrate was evaporated to give a white foam, which was treated with di-*tert*-butyl dicarbonate (1.41 g, 6.45 mmol) and triethylamine (2 mL) in THF (15 mL) at 30 °C for 10 h. After concentration, the reaction mixture was diluted with CH_2Cl_2 (60 mL), washed with saturated $NaHCO_3$, brine, and water, dried (Na_2SO_4), and filtered. The filtrate was evaporated and the residue was chromatographed on silica gel, eluting with 35:1 $CHCl_3$−EtOH to provide **4** (1.02 g, 86%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.787−7.666 (m, 4 H, phthaloyl), 5.719 (t, 1 H, J = 9.8 Hz, H-3), 4.104 (t, 1 H, J = 10.0 Hz, H-4), 4.847 (t, 1 H, J = 5.9 Hz, NH), 4.490 (dt, 1 H, J = 1.2 and 10.3 Hz, H-1), 4.300 (t, 1 H, J = 9.8 Hz, H-2), 4.298 (dd, 1 H, J = 4.6, 12.3 Hz, H-6a), 4.085 (dd, 1 H, J = 2.1, 12.3 Hz, H-6b), 3.799 (dd, 1 H, J = 2.1, 4.6, 9.8 Hz, H-5), 3.272 (m, 2 H, CH_2N), 2.074, 1.977, and 1.780 (s, each 3 H, 3 Ac), 1.335 (s, 9 H, Boc). Anal. Calcd for $C_{26}H_{32}N_2O_{11}$ H_2O: C, 55.12; H, 6.05; N, 4.94. Found: C, 54.82; H, 5.91; N, 4.68.

***N*-*tert*-Butoxycarbonyl-*C*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)methylamine (5)**. A mixture of **4** (540 mg, 0.984 mmol) in ethanol (10 mL) containing hydrazine monohydrate (1.5 mL) was heated at 80 °C for 2 h. The mixture was evaporated and trace hydrazine was removed by coevaporation with toluene (3 × 10 mL). The residue was then treated with acetic anhydride (2 mL) and pyridine (4 mL) at 20 °C for 6 h. The solution was poured into ice water (15 mL), stirred at 20 °C for 3 h, and evaporated. The residue was dissolved in $CHCl_3$ (50 mL), washed with 0.1 N HCl, saturated $NaHCO_3$, and water; dried (Na_2SO_4); and filtered. The filtrate was evaporated and the residue was chromatographed on silica gel with 35:1 $CHCl_3$−EtOH as the eluent to afford **5** (345 mg, 76%): mp 171−172 °C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.740 (d, 1 H, J = 9.3 Hz, NH), 5.124−5.044 (m, 3 H, H-3,4 and NH), 4.248 (dd, 1 H, J = 4.5, 12.1 Hz, H-6a), 4.108 (dd, 1 H, J = 1.9, 12.1 Hz, H-6b), 3.952 (q, 1 H, J = 9.4 Hz, H-2), 3.650−3.525 (m, 2 H, H-1,5), 3.462 and 3.051 (m, each 1 H, CH_2N), 2.095, 2.038, 2.031, and 1.965 (s, each 3 H, 4 Ac), 1.445 (s, 9 H, Boc). Anal. Calcd for $C_{20}H_{32}N_2O_{10}$: C, 52.16; H, 7.00; N, 6.08. Found: C, 52.15; H, 7.17; N, 5.98.

***N*^{4}-[*C*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)methyl]-*N*^{2}-(*tert*-butoxycarbonyl)-L-asparagine Benzyl Ester [Boc-Asn(tri-*O*-acetyl-β-GlcNAc-CH_2)-OBn] (7)**. To a solution of **5** (168 mg, 0.365 mmol) in CH_2Cl_2 (6 mL) was added a solution of HCl in anhydrous dioxane (4 M, 2.0 mL), and the mixture was stirred at 20 °C for 30 min when TLC (9:1 $CHCl_3$−EtOH) showed the disappearance of starting material. The reaction mixture was evaporated and the residue was coevaporated with toluene (3 × 6 mL). Trituration of the residue with ether gave **6** as its hydrochloride salt (146 mg, quantitative), which was used in the next step without further purification. The solid was suspended in CH_2Cl_2 (6 mL) and THF (2 mL), to which were added triethylamine (55.8 mL, 0.402 mmol), HOBt (59.2 mg, 0.438 mmol), and Boc-Asp-OBn (142 mg, 0.438 mmol). The solution was cooled to 0 °C, and DCC (107 mg, 0.438 mmol) was added. After stirring at 0 °C for 30 min and at 20 °C for 10 h, the white suspension was filtered and the precipitate was washed with CH_2Cl_2 (2 × 3 mL). The filtrate and washings were combined and evaporated. The residue was chromatographed on silica gel with 30:1 $CHCl_3$−EtOH as the eluent to yield **7** (209 mg, 86%): mp 190−192 °C; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.962 (d, 1 H, J = 9.3 Hz, NH), 7.900 (t, 1 H, J = 7.2 Hz, CH_2NH), 7.344 (s, 5 H, phenyl), 7.154 (d, 1 H, J = 7.1 Hz, NH), 5.093 (s, 2 H, $PhCH_2$), 5.030 (t, 1 H, J = 9.7 Hz, H-3), 4.839 (t, 1 H, J =9.7 Hz, H-4), 4.351 (m, 1 H, α-CH Asp), 4.187 (dd, 1 H, J =4.6, 12.1 Hz, H-6a), 3.963 (dd, 1 H, J = 1.8, 12.2 Hz, H-6b), 3.795 (q, 1 H, J = 9.8 Hz, H-2), 3.685 (ddd, 1 H, J = 1.8, 4.6, 9.7, H-5), 3.529 (m, 1 H, H-1), 3.352 and 2.948 (m, each 1 H, CH_2N), 2.595 and 2.465 (each dd, each 1 H, J = 5.9, 13 Hz, β-CH_2 Asp), 1.997, 1.960, 1.908, and 1.776 (s, each 3 H, 4 Ac), 1.358 (s, 9 H, Boc). Anal. Calcd for $C_{31}H_{43}N_3O_{13}$: C, 55.93; H, 6.51; N, 6.31. Found: C, 56.04; H, 6.41; N, 5.96.

***N*^{4}-[*C*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)methyl]-*N*^{2}-(*tert*-butoxycarbonyl)-L-asparagine [Boc-Asn(tri-*O*-acetyl-β-GlcNAc-CH_2)-OH] (8)**. Compound **7** (195 mg, 0.293 mmol) was hydrogenated in THF-EtOH (1:2, v/v, 9 mL) in the presence of 10% palladium on charcoal (20 mg) under hydrogen atmosphere. After 10 h, the mixture was filtered through a Celite pad and the filtrate was evaporated *in vacuo* to provide **8** (169 mg, quantitative): mp 192−194 °C; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 12.445 (s, 1 H, CO_2H), 7.981 (d, 1 H, J = 9.4 Hz, NH), 7.858 (t, 1 H, J = 6.5 Hz, CH_2NH), 6.898 (d, 1 H, J = 8.1 Hz, NH), 5.030 (t, 1 H, J = 9.5 Hz, H-3), 4.840 (t, 1 H, J = 9.5 Hz, H-4), 4.312−4.206 (m, 2 H, H-6a and α-CH Asp), 3.973 (dd, 1 H, J = 1.8, 12.1 Hz, H-6b), 3.782 (q, 1 H, J = 9.5 Hz, H-2), 3.703 (m, 1 H, H-5), 3.450 (m, 1 H, H-1), 3.418 and 2.904 (m, each 1 H, CH_2N), 2.563−3.390 (m, 2 H, β-CH_2 Asp), 2.005, 1.954, 1.904, and 1.778 (s, each 3 H, 4 Ac), 1.362 (s, 9 H, Boc). Anal. Calcd for $C_{24}H_{37}N_3O_{13}$: C, 50.08; H, 6.48; N, 7.30. Found: C, 49.83; H, 6.79; N, 6.90.

L-Alanyl-L-serine Methyl Ester (H-Ala-Ser-OMe) (9). H-Ser-OMe hydrochloride (1.56 g, 10 mmol) and Boc-Ala-OH (1.89 g, 10 mmol) were coupled in CH_2Cl_2 (30 mL) according to the standard procedure. The product was purified by chromatography on silica gel using 25:1 $CHCl_3$−EtOH as the eluent, giving *tert*-butoxycarbonyl-L-alanyl-L-serine methyl ester (Boc-Ala-Ser-OMe) (2.76 g, 95%) as a colorless syrup: ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.006 and 6.943 (each d, each 1 H, J = 7.8 Hz, 2 NH), 5.061 (t, 1 H, J = 5.6 Hz, OH Ser), 4.325 and 4.056 (m, each 1 H, α-CH Ser and Ala), 3.760−3.558 (m, 2 H, β-CH_2 Ser), 3.617 (s, 3 H, OMe), 1.370 (s, 9 H, Boc), 1.168

(d, 3 H, $J = 7.1$ Hz, Me Ala). Anal. Calcd for $C_{12}H_{22}N_2O_6$: C, 49.64; H, 7.64; N, 9.65. Found: C, 49.50; H, 7.71; N, 9.58. Cleavage of the *N*-Boc protecting group in Boc-Ala-Ser-OMe using the standard procedure afforded **9** in quantitative yield as its hydrochloride, which was used for peptide synthesis without purification.

***N*⁴-[*C*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)methyl]-*N*²-(*tert*-butoxycarbonyl)-L-asparaginyl-L-alanyl-L-serine Methyl Ester [Boc-Asn(tri-*O*-acetyl-β-GlcNAc-CH₂)-Ala-Ser-OMe] (10)**. Compound **8** (158 mg, 0.274 mmol) and the hydrochloride salt of H-Ala-Ser-OMe **9** (98 mg, 0.431 mmol) were coupled in DMF (5 mL) according to the standard procedure. The product was purified by chromatography on silica gel using 30:1 to 15:1 $CHCl_3$−EtOH as the eluent, giving **10** (172 mg, 84%): mp 177−179 °C; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.256 (d, 1 H, $J = 7.6$ Hz, NH), 7.960 (d, 1 H, $J = 9.5$ Hz, NH), 7.851 (m, 2 H, 2 NH), 6.962 (d, 1 H, $J = 7.5$ Hz, NH), 5.056 (m, 2 H, H-3 and OH Ser), 4.850 (t, 1 H, $J = 9.6$ Hz, H-4), 4.401−4.285 (m, 2 H, α-CH Ser and Asn), 4.250−4.105 (m, 2 H, H-6a and α-CH Ala), 3.983 (dd, 1 H, $J = 1.9$, 10.8 Hz, H-6b), 3.816 (q, 1 H, $J = 8.8$ Hz, H-2), 3.792−3.650 (m, 3 H, H-5 and β-CH_2 Ser), 3.618 (s, 3 H, OMe), 3.507 (m, 1 H, H-1), 3.350 and 2.985 (m, each 1 H, CH_2N), 2.585−2.360 (m, 2 H, β-CH_2 Asn), 2.013, 1.962, 1.911, and 1.779 (s, each 3 H, 4 Ac), 1.366 (s, 9 H, Boc), 1.202 (d, 3 H, $J = 7.0$ Hz, Me Ala). Anal. Calcd for $C_{31}H_{49}N_5O_{16}\,H_2O$: C, 48.62; H, 6.71; N, 9.14. Found: C, 48.45; H, 6.50; N, 9.22.

***N*-*tert*-Butoxycarbonyl-L-tyrosyl-L-isoleucine (Boc-Tyr-Ile-OH) (12)**. Boc-Tyr-OH (563 mg, 2 mmol) and H-Ile-OBn *p*-toluenesulfonate (787 mg, 2 mmol) were coupled by the standard procedure to give, after chromatographic purification, *N*-*tert*-butoxycarbonyl-L-tyrosyl-L-isoleucine benzyl ester (Boc-Tyr-Ile-OBn) (737 mg, 76%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.998 (s 1 H, OH Tyr), 7.327 (s, 5 H, Ph), 6.997 and 6.735 (d, each 2 H, 4 aromatic H Tyr), 6.468 (d, 1 H, $J = 8.5$ Hz, NH), 5.114 (s, 2 H, PhCH_2), 5.080 (d, 1 H, $J = 8.1$ Hz, NH), 4.522 (dd, 1 H, J =5.1, 8.4 Hz, α-CH Ile), 4.265 (m, 1 H, α-CH Tyr), 2.945−2.870 (m, 2 H, β-CH_2 Tyr), 1.820−-1.050 (m, 4 H, β-CH_2 and γ-CH_2 Ile), 1.398 (s, 9 H, Boc), 0.844−0.777 (m, 6 H, 2 Me Ile). Anal. Calcd for $C_{27}H_{36}N_2O_6$: C, 66.92; H, 7.49; N, 5.78. Found: C, 66.78; H, 7.82; N, 5.65.

Hydrogenation of Boc-Tyr-Ile-OBn (680 mg, 1.40 mmol) in THF−EtOH (1:1, 15 mL) was performed in the presence of 10% Pd/C (100 mg) under hydrogen atmosphere at 20 °C. After 12 h, the catalyst was removed by filtration and the filtrate was evaporated. The product was purified by chromatography on silica gel using 7:1 $CHCl_3$−MeOH as the eluent to provide **12** (492 mg, 89%) as a colorless syrup. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.013 (s, 1 H, OH Tyr), 6.978 and 6.708 (d, each 2 H, 4 aromatic H Tyr), 6.519 (d, 1 H, $J = 8.1$ Hz, NH), 5.378 (d, 1 H, $J = 7.9$ Hz, NH), 4.456 and 4.310 (m, each 1 H, α-CH Tyr and Ile), 2.980−2.820 (m, 2 H, β-CH_2 Tyr), 1.825−1.170 (m, 3 H, β-CH and g-CH_2 Ile), 1.427 (s, 9 H, Boc), 0.899−0.862 (m, 6 H, 2 Me Ile). Anal. Calcd for $C_{20}H_{30}N_2O_6$: C, 60.89; H, 7.67; N, 7.10. Found: C, 60.80; H, 7.92; N, 6.90.

***N*⁴-[*C*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)methyl]-*N*²-(*N*-*tert*-butoxycarbonyl-L-tyrosyl-L-isoleucyl)-L-asparaginyl-L-alanyl-L-serine Methyl Ester [Boc-Tyr-Ile-Asn(tri-*O*-acetyl-β-GlcNAc-CH₂)-Ala-Ser-OMe] (13)**. A solution of **10** (150 mg, 0.201 mmol) in CH_2Cl_2 (6 mL) was treated with 4M HCl in anhydrous dioxane (2 mL) at 20 °C for 2 h, the reaction mixture was evaporated *in vacuo*, and coevaporated with toluene (3 × 6 mL). Trituration of the residue with diethyl ether gave the hydrochloride salt of **11** (139 mg, quantitative) as a white solid, which was used immediately without characterization. The white solid was suspended in DMF (5 mL), followed by the sequential addition of triethylamine (33.4 mL, 0.24 mmol), Boc-Tyr-Ile-OH (**12**) (103 mg, 0.26 mmol), HOBt (35 mg, 0.26 mmol), and DCC (62 mg, 0.30 mmol). The mixture was stirred at 0 °C for 30 min and at 20 °C for 16 h. After water was added (0.3 mL), the resulting suspension was filtered and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel using 20:1 to 9:1 $CHCl_3$−EtOH as the eluent to give **13** (126 mg, 61%): ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.141 (s, 1 H, OH Tyr), 8.214 (d, 2 H, J =7.6 Hz, 2 NH), 7.943 (m, 2 H, 2 NH), 7.839 (d, 1 H, J = 7.6 Hz, NH), 7.712 (d, 1 H, J =8.1 Hz, NH), 7.023 and 6.628 (d, each 2 H, $J = 8.1$ Hz, 4 aromatic H Tyr), 6.939 (d, 1 H, $J = 7.8$ Hz, NH), 5.060−4.979 (m, 2 H, H-3 and OH Ser), 4.885 (t, 1 H, $J = 9.6$ Hz, H-4), 4.460−4.025 (m, 6 H, H-6a and 5 α-CH), 3.985 (dd, 1 H, J = 1.8, 11.2 Hz, H-6b), 3.820 (q, 1 H, $J = 8.5$ Hz, H-2), 3.790−3.598 (m, 3 H, H-5 and β-CH_2 Ser), 3.610 (s, 3 H, OMe), 3.510 (m, 1 H, H-1), 3.348 and 2.982 (m, each 1 H, CH_2N), 2.910−2.520 (m, 4 H, β-CH_2 Asn and Tyr), 2.005, 1.954, 1.902, and 1.766 (s, each 3 H, 4 Ac), 1.715, 1.390, and 1.260 (m, 2 H, β-CH and g-CH_2 Ile), 1.297 (s, 9 H, Boc), 1.181 (d, 1 H, $J = 7.0$ Hz, Me Ala), 0.803 (m, 6 H, 2 Me Ile). Anal. Calcd for $C_{46}H_{69}N_7O_{19}\,H_2O$: C, 53.01; H, 6.87; N, 9.41. Found: C, 52.83; H, 7.06; N, 9.20.

***N*⁴-[*C*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)methyl]-*N*²-(L-tyrosyl-L-isoleucyl)-L-asparaginyl-L-alanyl-L-serine amide [H-Tyr-Ile-Asn(GlcNAc-CH₂)-Ala-Ser-NH₂] (14)**. A solution of **13** (42 mg, 41 μmol) in methanol (30 mL) was saturated with ammonia at −5 °C for 30 min. The solution was kept at 20 °C for 3 days, then evaporated to dryness. Trituration of the residue with 2-propanol−water gave a white solid (35 mg), which was then treated with aqueous 3 M HCl at 20 °C for 1 h. The clear solution was evaporated to dryness and the product was purified by RP-HPLC on a Microsorb MW ODS column (4.6 × 250 mm, Rainin, Ridgefield, NJ) with a UV detector (double detection at 215 and 280 nm) (mobile phase, 9% aqueous MeCN containing 0.05% trifluoroacetic acid; flow rate, 1.0 mL/min). The product ($t_R = 10.72$ min) was collected and lyophilized to provide the GlcNAc-CH_2-pentapeptide **14** (25.7 mg, 80%): mp 226−-229 °C; ^{1}H-NMR (300 MHz, D_2O) δ 7.046 and 6.807 (each d, each 2 H, $J = 8.3$ Hz, 4 aromatic H Tyr), 4.590 (t, 1 H, $J = 6.8$ Hz, α-CH Tyr), 4.350 (t, 1 H, $J = 4.9$ Hz, α-CH Ser), 4.265 (q, 1 H, $J = 7.3$ Hz, α-CH Ala), 4.115−4.020 (m, 2 H, H-6a and α-CH Asn), 3.905−3.834 (m, 3 H, α-CH Ile and β-CH_2 Ser), 3.720−3.620 (m, 2 H, H-2,6b), 3.503−3.335 (m, 5 H, H-1,3,4,5 and $^1/_2$ CH_2N), 3.192 (dd, 1 H, $J = 7.5$, 12.5 Hz, 1/2 CH_2N), 2.994 (m, 2 H, β-CH_2 Asn), 2.845 and 2.682 (dd, each 1 H, $J = 6.8$, 13.0 Hz, β-CH_2 Tyr), 1.959 (s, 3 H, *N*-Ac), 1.710 (m, 1 H, β-CH Ile), 1.412 and 1.065 (m, each 1 H, g-CH_2 Ile), 1.358 (d, 1 H, J = 7.2 Hz, Me Ala), 0.805−0.764 (m, 6 H, 2 Me Ile); HR-FABMS calcd for $C_{34}H_{54}N_8O_{13} + H^+$ 783.3928, found: 783.3902.

***N*-*tert*-Butoxycarbonyl-β-benzyl-L-aspartyl-L-alanyl-L-serine Methyl Ester [Boc-Asp(OBn)-Ala-Ser-OMe] (15).** The hydrochloride of H-Ala-Ser-OMe (**9**) (795 mg, 3.5 mmol) was dissolved in DMF (10 mL). Triethylamine (0.73 mL, 5.25 mmol) and *p*-nitrophenyl *N*-*tert*-butoxycarbonyl-β-benzyl-L-aspartate [Boc-Asp(OBn)-OPNP] (3.11 g, 7.0 mmol) were added, and the mixture was stirred at 20 °C for 16 h. After evaporation of the solvent, the residue was dissolved in $CHCl_3$ (60 mL), washed with saturated $NaHCO_3$ and water, dried (Na_2SO_4), and evaporated. The product was purified by chromatography on silica gel using 30:1 $CHCl_3$−EtOH as the eluent to give **15** (1.58 g, 91%): ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.260, 7.862, and 7.232 (each d, each 1 H, $J = 7.6$ Hz, 3 NH), 7.356 (s, 5 H, Ph), 5.096−5.038 (m, 3 H, PhCH_2 and OH Ser), 4.362−4.302 (m, 3 H, 3 α-CH), 3.741−3.564 (m, 2 H, β-CH_2 Ser), 3.634 (s, 3 H, OMe), 2.778 (dd, 1 H, $J = 5.1$, 16 Hz, $^1/_2$ β-CH_2 Asp), 2.574 (dd, 1 H, $J = 9.1$, 16 Hz, $^1/_2$ β-CH_2 Asp), 1.369 (s, 9 H, Boc), 1.195 (d, 2 H, $J = 7.0$ Hz, Me Ala).

***N*²-(*N*-*tert*-butoxycarbonyl-L-tyrosyl-L-isoleucyl)-β-benzyl-L-aspartyl-L-alanyl-L-serine Methyl Ester [Boc-Tyr-Ile-Asp(OBn)-Ala-Ser-OMe] (17)**. De-*N*-Boc-protection of Boc-Asp(OBn)-Ala-Ser-OMe (**15**) (495 mg, 1.0 mmol) with 4 M HCl in anhydrous dioxane (4 mL) was performed by the standard procedure to obtain H-Asp(OBn)-Ala-Ser-OMe (**16**) (432 mg, quantitative) as its hydrochloride salt. Then **16** and Boc-Tyr-Ile-OH (**12**) (395 mg, 1.0 mmol) were coupled in DMF (8 mL) by the standard procedure, and the product was purified by chromatography on silica gel using 9:1 $CHCl_3$−EtOH as the eluent to give the pentapeptide **17** (638 mg, 81%): mp 196−198 °C; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.149 (s, 1H, OH Tyr), 8.414, 8.220, 7.808, 7.730, and 6.930 (each d, each 1 H, $J = 7.5$−7.8 Hz, 5 NH), 7.345 (s, 5 H, Ph), 7.014 and 6.630 (each d, each 2 H, $J = 8.3$ Hz, 4 aromatic H Tyr), 5.067 (m, 3 H, PhCH_2 and OH Ser), 4.715−3.400 (m, 5 H, 5 α-CH), 3.692 (m, 2 H, α-CH_2 Ser), 3.618 (s, 3 H, OMe), 2.910−2.520 (m, 4 H, α-CH_2 Tyr and Asp), 1.705 (m, 1 H, β-CH Ile), 1.460 and 1.085 (m, each 1 H, r-CH_2 Ile), 1.297 (s, 9 H, Boc), 1.181 (d, 3 H, J = 7.0 Hz, Me Ala), 0.828−0.795 (m, 6 H, 2 Me Ile).

***N*²-(*N*-*tert*-butoxycarbonyl-L-tyrosyl-L-isoleucyl)-L-aspartyl-L-alanyl-L-serine Methyl Ester [Boc-Tyr-Ile-Asp(OH)-Ala-Ser-OMe] (18)**. Compound **17** (394 mg, 0.5 mmol) was dissolved in THF−EtOH−MeOH (1:1:1, 18 mL) and subjected to hydrogenation in the

presence of 10% Pd/C (80 mg) under hydrogen atmosphere. After 16 h, the catalyst was removed by filtration through a Celite pad, and the filtrate was evaporated and chromatographed on silica gel using 5:1 to 2:1 $CHCl_3$−MeOH as the eluent to give **18** (220 mg, 63%): mp 170 °C (dec); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.210 (s, 1H, OH Tyr), 8.670, 8.192, 8.038, 7.703, and 6.950 (each d, each 1 H, J = 5.8−8.0 Hz, 5 NH), 7.345 (s, 5 H, Ph), 7.016 and 6.632 (each d, each 2 H, J = 8.1 Hz, 4 aromatic H Tyr), 4.525−4.080 (m, 6 H, 5 α-CH and OH Ser), 3.710 (m, 2 H, β-CH_2 Ser), 3.612 (s, 3 H, OMe), 2.880−2.215 (m, 4 H, β-CH_2 Tyr and Asp), 1.710 (m, 1 H, β-CH Ile), 1.415 and 1.065 (m, each 1 H, r-CH_2 Ile), 1.308 (s, 9 H, Boc), 1.190 (d, 3 H, J = 7.1 Hz, Me Ala), 0.850−0.710 (m, 6 H, 2 Me Ile).

Condensation of Pentapeptide (18) with Glycosylamine (6). A Convergent Approach to the Synthesis of 14. Compound **18** (50 mg, 71.6 μmol) and the hydrochloride salt of **6** (43 mg, 107 μmol) were dissolved in DMF (2 mL), then *N*,*N*′-diisopropylethylamine (38 μL, 215 μmol) , HOBt (14.5 mg, 107.5 μmol), and HBTU (82 mg, 215 μmol) were added sequentially. The mixture was stirred at 0 °C for 2 h and at 20 °C for 40 h. DMF was evaporated and the residue was applied to a column (2 × 45 cm) of Sephadex LH-20. The column was preequilibrated and eluted with 1:1 $CHCl_3$−95% EtOH to give a crude product (65 mg), which was further purified by column chromatography on silica gel eluted with 9:1 $CHCl_3$−EtOH to afford **13** (48 mg, 65%), which was identical to the product obtained by the stepwise synthesis. Removal of the protecting groups in **13** by sequential treatment with ammonia-saturated methanol and 3 M HCl as described gave **14** after RP-HPLC purification.

Transfer of $Man_9GlcNAc$ to the GlcNAc-CH_2-Pentapeptide Using Endo-A. Synthesis of the *C*-Glycopeptide 1. A mixture consisting of $Man_9GlcNAc_2Asn$ (2.4 μmol), the GlcNAc-CH_2-pentapeptide **14** (12 μmol), and enzyme (100 mU) in 25 mM NH_4OAc buffer (240 mL, pH 6.0) containing 35% acetone was incubated at 37 °C for 20 min. The reaction was stopped by boiling in a 100 °C water bath (3 min). The product was purified by RP-HPLC on a Microsorb MW ODS column (4.6 × 250 mm, Rainin, Ridgefield, NJ) with a UV detector (double detection at 215 and 280 nm) (mobile phase, 9% aqueous MeCN containing 0.05% trifluoroacetic acid; flow rate, 1.0 mL/min). The transglycosylation product eluted at 5.62 min was collected and lyophilized to obtain **1** (1.56 mg, 0.634 μmol, 26%). The excess GlcNAc-CH_2-pentapeptide (acceptor) was eluted after 10 min under the conditions and was efficiently recovered: ^{1}H-NMR (500 MHz, 60 °C in D_2O, set the DHO signal at δ 4.441) of **1** δ 7.112 and 6.855 (each d, 4 H, J = 8.3Hz, 4 aromatic H Tyr), 5.400, 5.344, 5.312, 5.143, 5.094, 5.083, 5.078, and 4.894 (each br. s, each 1 H, 8 H-1 α-Man), 4.783 (s, 1 H, H-1 β-Man), 4.687 (t, 1 H, J = 6.8 Hz, α-CH Tyr), 4.618 (distorted d, J = 7.5 Hz, H-1 GlcNAc-2), 4.373−3.448 (m, multiple protons), 3.174 (dd, 1 H, J = 7.5, 14 Hz, 1/2 CH_2N), 2.889 (d, 2 H, J = 6.4 Hz, β-CH_2 Asn), 2.865 and 2.714 (each dd, 2 H, J = 6.9, 14.5 Hz, β-CH_2 Tyr), 2.086 and 2.044 (s, each 3 H, 2 NAc), 1.765 (m, 1 H, β-CH Ile), 1.426 (d, 3 H, J = 7.3 Hz, Me Ala), 1.314 and 1.100 (m, 2 H, g-CH_2 Ile), 0.868−0.838 (m, 6 H, 2 Me Ile); HR-FABMS calculated for $C_{96}H_{157}N_9O_{63}$ + H^+ 2444.9436; Found 2444.9462.

Compositional Analysis of 1. The monosaccharide analysis was carried out by the established HPAEC-PAD method.[36] In brief, a sample was hydrolyzed with 4N HCl at 100 °C for 6 h (for GlcN) or with 2M trifluoroacetic acid at 100 °C for 4 h (for Man), then evaporated by speed-vac and diluted to a suitable concentration for HPAEC-PAD analysis. The ratio between Man and GlcNAc was found to be 9:1. The released GlcNAc-CH_2NH_2 was detected without quantitative analysis. For amino acid analysis, the sample was hydrolyzed using 4 N HCl at 110 °C for 20 h. TLC (5:5:1:3 EtOAc−pyridine−AcOH−H_2O) of the hydrolysate clearly showed the presence of Tyr, Ile, Asp, Ala, Ser, and GlcN-CH_2NH_2.

***N*^{4}-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-*N*^{2}-(*N*-*tert*-butoxycarbonyl-L-tyrosyl-L-isoleucyl)-L-asparaginyl-L-alanyl-L-serine methyl ester [Boc-Tyr-Ile-Asn(tri-*O*-acetyl-β-GlcNAc)-Ala-Ser-OMe] (20)**. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine (**19**)[33] (88 mg, 254 μmol), compound **18** (59 mg, 84.7 μmol), diisopropylethylamine (12 mL, 85 μmol), HOBt (13.5 mg, 100 μmol), and HBTU (160 mg, 84.7 μmol) were mixed in DMF (2.5 mL). After 48 h, DMF was evaporated *in vacuo* and the residue was chromatographed on a column (2 × 45 cm) of Sephadex LH-20 in 1:1 $CHCl_3$−95% EtOH to give a crude product, which was crystallized from EtOH to afford **20** (43.5 mg, 50%): ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.165 (s, 1 H, OH Tyr), 8.697, 8.370, 8.212, 7.925, 7.750, 7.665, and 6.920 (each d, each 1 H, J = 7.0−8.0 Hz, 7 NH), 7.024 and 6.636 (each d, each 2 H, J = 8.4 Hz, 4 aromatic H Tyr), 5.170−4.950 (m, 3 H, H-1,3, and OH Ser), 4.830 (t, 1 H, J = 9.5 Hz, H-4), 4.550−3.665 (m, 11 H, H-2,5,6a,6b,5 α-CH and β-CH_2 Ser), 3.619 (s, 3 H, OMe), 2.890−2.285 (m, 4 H, β-CH_2 Asn and Tyr), 1.993, 1.963, 1.909, and 1.745 (s, each 3 H, 4 Ac), 1.690, 1.405, and 1.060 (m, 3 H, β-CH and r-CH_2 Ile), 1.307 (s, 9 H, Boc), 1.178 (d, 3 H, J = 7.0 Hz, Me Ala), 0.799 (m, 6 H, 2 Me Ile).

***N*^{4}-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-*N*^{2}-(L-tyrosyl-L-isoleucyl)-L-asparaginyl-L-alanyl-L-serine Amide [H-Tyr-Ile-Asn-(GlcNAc)-Ala-Ser-NH_2] (21).** Compound **20** (35 mg, 34 μmol) was treated with ammonia-saturated MeOH (30 mL) for 2 days, then the solution was evaporated to dryness. Trituration of the residue with EtOH gave a white solid. The solid was treated with aqueous 3 M HCl at 20 °C for 1 h and evaporated to dryness. The product was purified by RP-HPLC on a Microsorb MW ODS column (4.6 × 250 mm, Rainin, Ridgefield, NJ) with a UV detector (double detection at 215 and 280 nm) (mobile phase: 9% aqueous MeCN containing 0.05% trifluoroacetic acid; flow rate: 1.0 mL/min). The product, eluted at 13.45 min, was collected and lyophilized to obtain the GlcNAc-pentapeptide (**21**) (22 mg, 81%): mp 223−225 °C; ^{1}H-NMR (300 MHz, D_2O) δ 7.077 and 6.809 (each d, each 2 H, J = 8.3 Hz, 4 aromatic H Tyr), 5.019 (d, 1 H, J = 9.7 Hz, H-1), 4.643 (t, 1 H, J = 6.8 Hz, α-CH Tyr), 4.359 (t, 1 H, J = 5.3 Hz, α-CH Ser), 4.283 (q, 1 H, J = 7.3 Hz, α-CH Ala), 4.203 (t, 1 H, J = 7.1 Hz, α-CH Asn), 4.074 (d, 1 H, J = 8.3 Hz, α-CH Ile), 3.859−3.421 (m, 8 H, H-2,3,4,5,6a,6b, and β-CH_2 Ser), 3.150−2.980 (m, 2 H, β-CH_2 Asn), 2.895 (dd, 1 H, J = 7.7, 16 Hz, 1/2 β-CH_2 Tyr), 2.719 (dd, 1 H, J = 5.8, 16 Hz, 1/2 β-CH_2 Tyr), 1.947 (s, 3 H, Ac), 1.706 (m, 1 H, β-CH Ile), 1.378 and 1.086 (m, each 1 H, r-CH_2 Ile); 1.367 (d, 3 H, J = 7.2 Hz, Me Ala), 0.827−0.789 (m, 6 H, 2 Me Ile). HR-FABMS calcd for $C_{33}H_{52}N_8O_{13}$ + H^+ 769.3732, Found 769.3734.

Transfer of $Man_9GlcNAc$ to the GlcNAc-Pentapeptide by Endo-A. Synthesis of the Natural High-Mannose *N*-Glycopeptide (2). A mixture consisting of $Man_9GlcNAc_2Asn$ (2 μmol), the GlcNAc-pentapeptide **21** (10 μmol), and enzyme (70 mU) in 25 mM NH_4OAc buffer (100 μL, pH 6.0) containing 35% acetone was incubated at 37 °C for 20 min. The reaction was stoped by boiling at 100 °C for 3 min, and the transglycosylation product was purified by RP-HPLC on a Microsorb MW ODS column (4.6 × 250 mm, Rainin, Ridgefield, NJ) detected at 215 and 280 nm (mobile phase, 9% aqueous MeCN containing 0.05% TFA; flow rate, 1 mL/min). The transglycosylation product, eluted at 6.15 min, was collected and lyophilized to give **2** (1.25 mg, 0.51 μmol, 25%). The excess GlcNAc-pentapeptide acceptor **21**, which was eluted much later, was efficiently recovered: ^{1}H-NMR (500 MHz, 60 °C in D_2O, set the DHO signal at δ 4.441) of **2** δ 7.136 and 6.874 (each d, each 2 H, J = 8.4Hz, 4 aromatic H Tyr), 5.402, 5.349, 5.315, 5.146, 5.099, 5.084, 5.078, and 4.901 (each br. s, each 1 H, 8 H-1 α-Man), 5.048 (d, 1 H, J = 9.5 Hz, H-1 GlcNAc-1), 4.790 (s, 1 H, H-1 β-Man), 4.711 (t, 1 H, J = 6.6 Hz, α-CH Tyr), 4.639 (distorted d, 1 H, J = 6.6 Hz, H-1 GlcNAc-2), 4.484−3.539 (m, multiple protons), 2.959 (d, 2 H, J = 6.6 Hz, β-CH_2 Asn), 2.927 and 2.750 (each dd, each 1 H, J = 6.6, 16 Hz, β-CH_2 Tyr), 2.092 and 2.028 (s, each 3 H, 2 NAc), 1.790 (m, 1 H, β-CH Ile), 1.423 (d, 3 H, J = 7.0 Hz, Me Ala), 1.395 and 1.150 (m, 2 H, g-CH_2 Ile), 0.868−0.835 (m, 6 H, 2 Me Ile); ESI-MS calcd for $C_{95}H_{155}N_9O_{63}$ (M) 2431 (average mass), and 2429.9 (exact mass); Found 1217 [$(M + 2H)^{2+}$], 1228 [$(M + H + Na)^{2+}$], and 1239 [$(M + 2Na)^{2+}$].

Assay of the Activity of Glycoamidases. The activity of glycoamidases HO and Sm was assayed using asialo-[^{14}C]fetGP I as the substrate as previously described.[14] Briefly, the reaction mixture containing the glycoamidase in a total volume of 5 μL of 50 mM HEPES buffer (pH 7.5) and 2 mM DTT together with 11 μM of asialo-[^{14}C]fetGP I (6,100 dpm) was incubated in a polypropylene microtube at 37 °C for 3 h. For bacterial glycoamidase Sm, the substrate was

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incubated at 25 °C for 20 min in 5 μL of 20 mM Tris-HCl buffer (pH 7.1). Reaction products were separated by paper chromatography followed by measurement of the radioactivities by the Bio-Imaging analyzer (Fujix BAS 2000).

The activity of glycoamidases A and F was assayed using the synthetic *N*-glycopeptide **2** [H-Tyr-Ile-Asn($Man_9GlcNAc_2$)-Ala-Ser-NH_2] as substrate. Briefly, the reaction mixture containing the glycoamidase and the substrate was incubated in a total volume of 50 μL of 40 mM ammonium acetate buffer (pH 5 for glycoamidase A and pH 8 for glycoamidase F) for a predetermined time. The enzymatic reactions were stopped by boiling in a water bath for 3 min and the hydrolytic product, the pentapeptide H-Tyr-Ile-Asp-Ala-Ser-NH_2, was analyzed by RP-HPLC, which was quantitated by intergrating the peak observed at 210 and/or 280 nm.

Inhibition of Glycoamidases by the C-Glycopeptide (1). For practical reasons, the synthetic *N*-glycopeptide **2** was used as the substrate for glycoamidases A and F, but a radiolabeled glycopeptide asialo-[^{14}C]fetGP I was used as the substrate for glycoamidases Sm and HO in the inhibitory experiments. In all the cases, enzymes were used at the amount that would give less than 10% hydrolysis of the respective substrate. The initial rates of the enzymatic reactions were determined by quantitating the peptide released. The K_m values of the two substrates for the respective enzymes were obtained by Lineweaver−Burk double-reciprocal plots (1/*V* vs. 1/[S]). The inhibitory activity of **1** against glycoamidases was examined by performing the enzymatic reaction at a fixed substrate concentration ([S] = 11 μM) for a pre-determined time when the inhibitor was present at various concentrations (0.1−1000 μM). The IC_{50} values were obtained by using nonlinear regression (logistics equation) with the Graphpad Prism program (GraphPad Software, Inc., Grand Junction, CO) and the results were shown in Figure 4. To determine the Ki values for glycoamidases A and F, the enzymatic reactions were performed at various substrate concentrations (0.1−1000 μM) when the inhibitor was present at various concentrations (10, 50, and 100 μM). The Lineweaver−Burk double-reciprocal plots (1/*V* vs 1/[S]) were created, and the slops of each of these lines were then plotted against [I], which was fitted to a straight line. The intercept at the [I] axes is equal to $-K_i$. By this method, the K_i of the *C*-glycopeptide **1** for glycoamidases A and F was determined to be 1 and 43 μM, respectively. The Lineweaver−Burk plots at different [I] also revealed that the inhibition by the *C*-glycopeptide **1** is competitive in nature. For glycoamidases Sm andHO, the Ki values were calculated by the equation of Cheng and Prusoff,[37] $K_i = IC_{50} / (1 + [S]/K_m)$, taking the above determined data of IC_{50} ([S] = 11 μM) and K_m.

Hydrolysis of the *C*-Glycopeptide by Glycoamidases. To examine whether *C*-glycopeptide **1** would be hydrolyzed by the glycoamidases, 10 nmol of the *C*-glycopeptide was incubated with the respective glycoamidase under the conditions described for assaying enzymatic activity for 18 h. The reaction mixture was then evaporated by a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, NY). RP-HPLC analysis showed neither the change of the C-glycopeptide nor the presence of any hydrolytic products.

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