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Convergent synthesis of a fluorescence-quenched glycopeptide as a potential substrate for peptide: *N*-glycosidases

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Abstract

The fluorescence-quenched glycopeptide N^{γ}-[2-acetamido-6-(2′-amino)benzamido-2,6-dideoxy-β-D-glucopyranosyl]-N^{α}-benzoyl-Asn-Tyr(NO_2)-OMe, having anthranilamide/3-nitrotyrosine as the donor/acceptor pair, was synthesized via a convergent route involving the EEDQ-mediated coupling of 2-acetamido-2,3-di-O-acetyl-6-(2′-*tert*-butoxycarbonylamino)benzamido-2,6-dideoxy-β-D-glucopyranosylamine with *N*-benzoyl-Asp-Tyr(NO_2)-OMe, followed by deprotection.

Keywords: PNGase assay; fluorimeter; peptide; nitrotyrosine

1. Introduction

Peptide-N^4-(N-acetyl-β-D-glucosaminyl)asparagine amidase F [1] (peptide: *N*-glycosidase F, glycopeptidase F or PNGase F) from *Flavobacterium meningosepticum* is an amidase/amidohydrolase that cleaves the β-aspartylglucosylamine bond of *N*-linked glycoproteins, converting the asparagine residue to an aspartic acid [2]. The released 1-aminooligosaccharide spontaneously degrades to ammonia and the free oligosaccharide [3,4]. PNGases are also found in plants, and recently they have been found to be widely distributed in animals [5]. It is speculated that they may play an important role in the non-lysosomal regulation of various physiological and/or physicochemical properties of certain target proteins involved in embryonal development [5]. The enzyme hydrolyzes all types of asparagine-linked oligosaccharides, provided that both the amino and carboxyl groups of the asparagine are involved in amide bonds. The enzyme has a broad substrate specificity [2], liberating all *N*-linked oligosaccharides, regardless of their structure, size or charge, from glycoproteins of vertebrate origin. However, the binding of the enzyme to the inner di-*N*-acetylchitobiose core of the carbohydrate chain is more specific [6]. For example, *N*-glycans with a fucose linked α-(1→6) to the asparagine-proximal *N*-acetylglucosamine are good substrates, but if the fucose is in an α-(1→3) linkage as found in glycoproteins from plants and insects, the enzyme is inactive [7].

PNGase F is probably the most widely used agent for the deglycosylation of glycoproteins [8]

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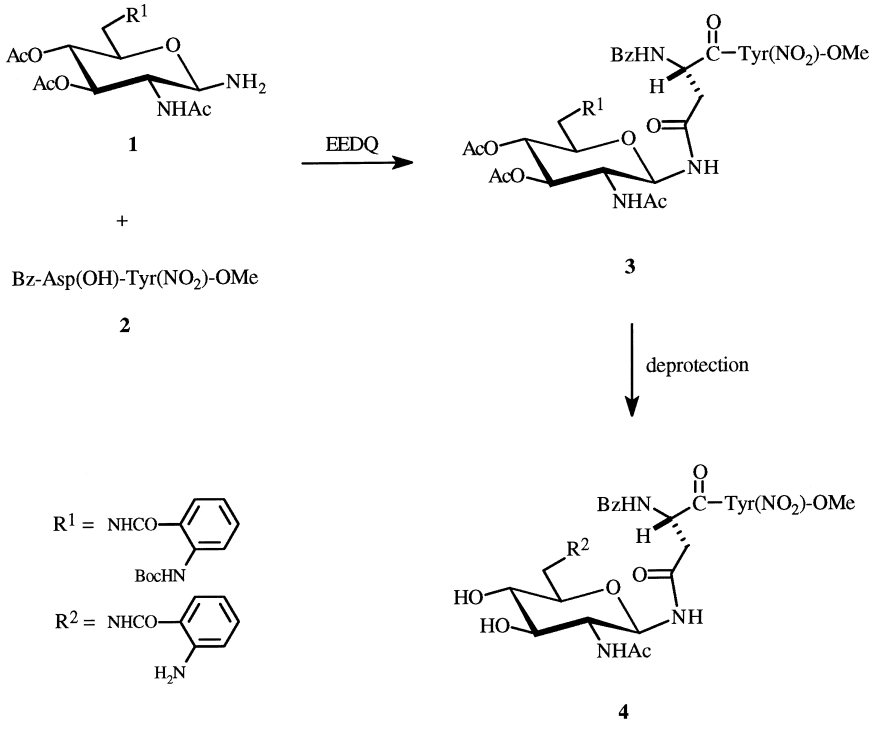
and is thus a valuable biochemical tool for investigating oligosaccharide structure and the biosynthesis of *N*-linked glycoproteins [9]. Despite the importance of these enzymes, and the fact that the three-dimensional X-ray crystallographic structure of PNGase F was recently determined [10,11], very few kinetic studies have been performed beyond recent kinetic comparisons of PNGase F and PNGase A (from almond) which revealed several differences in substrate specificity [12].

The currently available assays for PNGases are quite tedious and time consuming, involving the extensive use of HPLC [8]. In addition, the expensive glycopeptide substrates required for the assay must first be derivatized with a fluorescent label. A more sensitive and convenient assay would therefore be valuable. This paper describes the design and synthesis of a fluorescence-quenched glycopeptide substrate as a first step towards the development of a more convenient assay for PNGase activity.

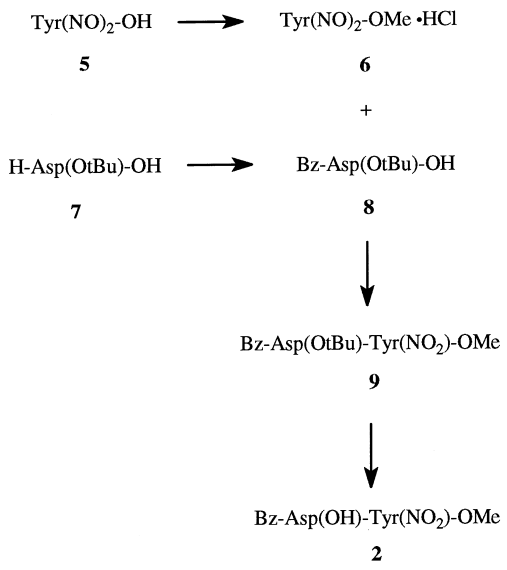
2. Results and discussion

A substrate was desired having both the minimum requirements for recognition by the enzyme PNGase. The glycopeptide **4** was thus designed as the minimal structure to meet these requirements. Glycopeptide **4** possesses an *N*-acetylglucosamine derivative *N*-linked via asparagine to a dipeptide. The donor/acceptor pair chosen to provide the fluorescence quenching via resonance energy transfer was anthranilamide/nitrotyrosine [13], a highly efficient pair that has found utility in fluorescence-quenched assays of proteolytic enzymes [14,15]. This donor/acceptor pair is also the only one currently available that is amenable to solid-phase synthesis techniques [13], an important consideration for ultimately producing substrates of varying peptide length. The anthranilamide donor was placed at C-6 of the *N*-acetylglucosamine residue for ease of synthesis and because PNGases are known to accept substrates with substitution at C-6 [7]. Glycopeptide **4** was prepared by a convergent approach [16–19] involving syntheses of the glycosylamine **1** and the dipeptide **2** and then coupling as outlined in Scheme 1.

The dipeptide **2** was prepared by conventional solution synthesis techniques as shown in Scheme 2. Commercially available *β-tert*-butyl-L-aspartate (**7**) was *N*-benzoylated in 94% yield with benzoyl chloride and sodium carbonate in aqueous dioxane and then coupled with 3-nitro-L-tyrosine methyl ester hydrochloride **6** [20] under standard peptide coupling conditions (DCC/DhbtOH) to give the protected dipeptide **9** in 91% yield. The terminal



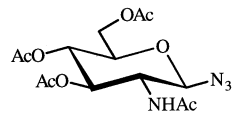
Scheme 1.



Scheme 2.

methyl ester of the dipeptide proved to be very stable, and all attempts to convert it into an amide by treatment with ammonia in methanol led to recovery of starting material. The *tert*-butyl group of **9** was then quantitatively removed by brief exposure to neat trifluoroacetic acid (TFA) at room temperature to give **2**.

The synthesis of the glycosylamine **1** began from the readily available glycosyl azide **10** [21]. Compound **10** was hydrogenated in ethanol with platinum oxide catalyst for 5 h. The resulting glycosylamine is known to be unstable, giving rise to dimerization products even when stored at room temperature after recrystallization [21]. The crude amine was thus treated immediately with benzyl chloroformate and pyridine in dichloromethane at 0 °C to give the carbamate **11** in 61% overall yield, after recrystallization. Compound **11** was then deacetylated with ammonia in methanol to give the triol **12**. Crude **12** was selectively tosylated in 82% overall yield by treatment with 1.2 equivalents of *p*-toluenesulfonyl chloride in pyridine at 0 °C to give the tosylate **13**. The tosylate **13** was smoothly converted into the azide **14** in good yield by heating with sodium azide in DMF. The fluorescent anthranilamide group was then readily introduced in an efficient, one-pot procedure. The azido group was first reduced by heating with triphenylphosphine in 7:1 THF–water [22] and was then coupled with Boc-ABz-ODhbt [23]. The crude reaction mixture was acetylated (excess acetic anhydride–pyridine), and the desired product **15** was obtained as a crystalline solid in 91% overall yield following purification by flash chromatography. The benzyloxycarbonyl group of compound **15** was quantitatively removed by hydrogenolysis with palladium-on-charcoal as catalyst to give the glycosylamine **1**, in readiness for coupling with the dipeptide (**2**). Compound **1**, isolated as a foam, was pure by TLC and ^{1}H NMR spectroscopy and was used immediately in the coupling step without further purification.



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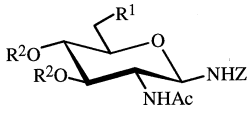
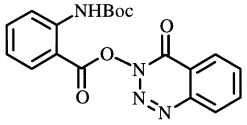
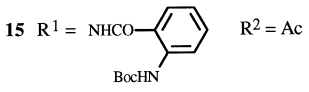


11 R^1 = OAc R^2 = Ac
12 R^1 = OH R^2 = H
13 R^1 = OTs R^2 = H
14 R^1 = N_3 R^2 = H



Boc-ABz-ODhbt



15 R^1 = NHCO–(BocHN) R^2 = Ac

The coupling of glucosylamine **1** with dipeptide **2** in DMF at room temperature via the agency of 2-ethoxy-l-N-ethoxycarbonyl-1,2-dihydroquinoline EEDQ [24] afforded the fully protected glycopeptide **3** in 43% yield after flash chromatography. The coupling could also be performed in similar yield using DCC in the presence of DhbtOH. As a consequence of its poor solubility properties (soluble only in Me_2NCHO, Me_2SO or MeOH–CH_2Cl_2 mixtures), compound **3** proved to be difficult to purify, and repeated chromatography was required, which partly accounted for the moderate yield. Compound **3** was fully characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. Deprotection to give the target compound **4** was achieved by deacetylation with ammonia in methanol followed by brief treatment with aqueous TFA. The crude product, obtained as the TFA salt in essentially quantitative yield, was approximately 92% pure as estimated by ^{1}H NMR spectroscopy, analytical reversed-phase HPLC and ESIMS (M, 780.5). Final purification was performed by semi-preparative reversed-phase HPLC to give pure glycopeptide **4** as a yellow, amorphous solid.

3. Experimental

General.—Melting points (mp) were determined on a Laboratory Devices Mel-Temp II melting

point apparatus and are uncorrected. Optical rotations were determined at 25 °C on a Perkin–Elmer 241 MC polarimeter. NMR spectra were recorded on Bruker AC 200, Bruker WH 400 or Varian XL 300 instruments and are referenced to the solvent peak unless otherwise stated. Where required, the interpretations were supported by COSY or APT NMR experiments. Mass spectra were obtained by desorption chemical-ionization (DCI) on a Delsi Nermag R10-10C mass spectrometer with ammonia as the reactive gas, by the liquid secondary-ion (LSIMS) technique on a Kratos Concept mass spectrometer using thioglycerol–water as the matrix, or by electrospray-ionization (ESIMS) on a PE/Sciex API 300 mass spectrometer. Semi-preparative and analytical reversed-phase HPLC were performed on a Waters 600E HPLC system using a Waters 10 μm RCM C_{18} (8 mm×10 cm) column with a flow rate of 2 mL min^{-1} and detection at 254 nm using a Waters 486 tunable absorbance detector. Solvent system A: 0.05% TFA in 49:1 water–acetonitrile; B: 0.05% TFA in 1:4 water–acetonitrile. Microanalyses were performed by Mr. Peter Borda in the Microanalytical Laboratory of the University of British Columbia. Solvents and reagents used were either reagent grade, certified, or spectral grade and where necessary were dried by standard procedures. Petroleum ether had a boiling range of 35–60 °C. Reactions were monitored by thin-layer chromatography (TLC) using E. Merck Kieselgel 60F_{254} aluminium-backed sheets. Compounds were detected (where possible) under UV light or by charring with 10% sulfuric acid in MeOH or with 10% ammonium molybdate in 2 M sulfuric acid. Flash chromatography was performed on short columns (10–15 cm) of E. Merck Kieselgel 60 (230–400 mesh) under a positive pressure with specified eluants. 3-Nitro-L-tyrosine and H-Asp(OtBu)-OH were from Aldrich Chemical Company and Bachem, respectively.

3-Nitro-L-tyrosine methyl ester hydrochloride (**6**).—The title compound was prepared according to the method of Hanson and Law [20] in 80% yield as fine yellow needles: mp 195–197 °C (EtOH/EtOAc; lit [20]. 195–197 °C); $[\alpha]_D$ + 8.6° (c 0.8, MeOH); lit [20]. + 8.9 °; ^{1}H NMR (200 MHz, D_2O, ext. DSS): δ 3.12–3.34 (m, 2 H, AB part of an ABX system, J_{AB} 14.7, $J_{AX} \sim J_{BX}$ = 6.7 Hz, β-H), 3.79 (s, Me), 4.40 (t, J 6.7 Hz, α-H), 7.09 (d, $J_{5,6}$ 8.5 Hz, H-5), 7.47 (dd, $J_{2,6}$ 2.2 Hz, H-6), 7.92 (d, H-2); ^{13}C NMR (50 MHz, D_2O): δ 35.0 (C-β), 54.4 (2 C, C-α, Me), 121.2–139.0 (5 C, Ar), 153.6 (ArOH), 170.4 (C=O).

N-*Benzoyl-L-aspartic acid* β-tert-*butyl ester* (**8**).—H-Asp(OtBu)-OH (**7**) (378 mg, 2.00 mmol) was dissolved in aq Na_2CO_3 (4.8 mL of 1 M, 4.8 mmol) and dioxane (4 mL). A solution of benzoyl chloride (338 mg, 278 μL, 2.40 mmol) in dioxane (2 mL) was added, followed by water (5 mL). After stirring for 20 min at room temperature, a clear, homogeneous solution resulted. The solution was stirred for a further 16 h at room temperature and then was extracted with ether. The aqueous phase was acidified to pH 2 with M HCl and extracted with EtOAc. The EtOAc extracts were washed successively with water and brine, dried ($MgSO_4$), filtered and concentrated. The residue was recrystallized from diisopropyl ether–petroleum ether to give title compound **8** as a white microcrystalline solid (550 mg, 94%): mp 162–163 °C; $[\alpha]_D$ −2° (*c* 1.5, MeOH); ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 1.36 (s, Me_3), 2.66, 2.84 (2 dd, AB part of an ABX system, J_{AB} 15.9, J_{AX} 8.6, J_{BX} 5.9 Hz, β-H), 4.78 (m, α-H), 7.40–7.60, 7.78–7.89 (2 m, 5 H, Ph), 8.74 (d, J 8.3 Hz, NH); ^{13}C NMR (50 MHz, Me_2SO-d_6): δ 27.5 (Me_3), 37.0 (C-β), 49.5 (C-α), 80.3 (CMe_3), 127.4–134.0 (Ph), 166.2, 169.5, 172.5 (C=O); DCI: *m/z* 294 ($M + H^+$, 60.3%), 311 ($M + NH_4^+$, 58.8%). Anal. Calcd for $C_{15}H_{19}NO_5$: C, 61.42; H, 6.53; N, 4.78. Found: C, 61.57; H, 6.64; N, 4.73.

N-*Benzoyl-Asp(β-OtBu)-Tyr(NO$_2$)-OMe* (**9**).—A solution of **8** (300 mg, 1.02 mmol), DhbtOH (167 mg, 1.02 mmol) and **6** (304 mg, 1.10 mmol) in DMF (10 mL) was cooled to 0 °C. DCC (210 mg, 1.02 mmol) and *N*-ethyldiisopropylamine (142 mg, 192 μL, 1.10 mmol) were then added, and the mixture was stirred at 0 °C for 1 h and then at room temperature for 16 h. The mixture was filtered (Celite), and the filter cake was washed well with EtOAc. The combined filtrate and washings were washed with successively with M HCl, water and brine, dried ($MgSO_4$), filtered and concentrated. Flash chromatography (3:7 EtOAc–petroleum ether) gave the dipeptide **9** (480 mg, 91%) as yellow needles: mp 102–105 °C (EtOAc–petroleum ether); $[\alpha]_D$ + 22° (*c* 0.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, Me_3), 2.56, 2.94 (2 dd, AB part of an ABX system, J_{AB} 16.9, J_{AX} 6.2, J_{BX} 4.0 Hz, Asp β-H), 2.97, 3.15 (2 dd, AB part of an ABX system, J_{AB} 14.2, J_{AX} 6.9, J_{BX} 5.2 Hz, Tyr β-H), 3.73 (s, OMe), 4.81 (ddd, $J_{H,NH}$ 12.8 Hz, Tyr α-H), 4.92 (ddd, $J_{H,NH}$ 10.2 Hz, Asp α-H), 6.87–7.83 (10

H, Ar*H*, NH), 10.36 (s, OH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.9 (Me_3), 35.8, 36.5 (C-β), 49.6 (C-α Asp), 52.6, 53.0 (C-α Tyr, OMe), 82.1 (*C*Me_3), 120.0–138.5 (11 C, Ar), 154.0 (ArOH), 167.2, 170.4, 170.9, 172.0 (C=O); DCI: *m/z* 516 ($M+H^+$, 58.4%), 533 ($M+NH_4^+$, 100%). Anal. Calcd for $C_{25}H_{29}N_3O_9$: C, 58.24; H, 5.67; N, 8.15. Found: C, 58.27; H, 5.72; N, 8.14.

N-*Benzoyl-Asp-Tyr(NO$_2$)-OMe* (**2**).—The dipeptide **9** (270 mg, 0.52 mmol) was dissolved in neat trifluoroacetic acid (1.5 mL) and stirred at room temperature for 1 h. The mixture was then diluted with toluene and concentrated. The residue was treated with toluene and concentrated (×2) to give title compound **2** (240 mg, 100%) as a yellow solid: mp 204–205 °C (MeOH); $[\alpha]_D$ −33° (*c* 1.1, DMF); ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 2.60, 2.71(2 dd, AB part of an ABX system, J_{AB} 16-6, J_{AX} 8.5, J_{BX} 5.4 Hz, Asp β-H) 2.92, 3.02 (2 dd, AB part of an ABX system, J_{AB} 14.2, J_{AX} 8.6, J_{BX} 5.6 Hz Tyr β-H) 3.60 (s, OMe), 4.39–4.50, 4.73–4.84 (2 m, α-H), 6.90–8.58 (10 H, Ar*H*, NH), 10.81, 12.24 (2 bs, OH, CO_2H); ^{13}C NMR (75 MHz, Me_2SO-d_6): δ 34.9, 35.8 (C-β), 49.9 (C-α Asp), 52.0, 53.5 (C-α Tyr, OMe), 119.0–136.2 (11 C, Ar), 150.9 (ArOH), 166.2 (CO_2H), 171.0, 171.6, 171.8 (C=O); DCI: *m/z* 460 ($M+H^+$, 100%). Anal. Calcd for $C_{21}H_{21}N_3O_9$: C, 54.90; H, 4.61; N, 9.15. Found: C, 54.70; H, 4.70; N, 9.02.

2-Acetamido-3,4,6-tri-O-*acetyl-2-deoxy*-N-*(benzyloxycarbonyl)-β*-D-*glucopyranosylamine* (**11**).—A solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl azide (**10**) [21] (3.00 g, 8.06 mmol) in EtOH (80 mL) was hydrogenated at room temperature and atmospheric pressure in the presence of platinum oxide (250 mg) for 5 h. The mixture was then filtered (Celite) and concentrated. The residue was dissolved in a mixture of CH_2Cl_2 (25 mL) and pyridine (5 mL) and cooled to 0 °C. Benzyl chloroformate (2.17 g of 95%, 1.82 mL, 12.1 mmol) was then added dropwise, and the mixture was stirred at 0 °C for 1 h and then at room temperature for 16 h. The mixture was then diluted with CH_2Cl_2 and poured into water. The organic phase was separated, and the aqueous phase was extracted with CH_2Cl_2. The combined organic phases were washed successively with M HCl, water and brine, dried ($MgSO_4$), filtered and concentrated to a solid. Recrystallization from MeOH–diethyl ether then gave the carbamate **11** (2.36 g, 61%) as needles: mp 221–222 °C; $[\alpha]_D$ −32° (*c* 0.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.88, 2.01, 2.03, 2.06 (4 s, Me), 3.71 (m, H-5), 4.06–4.15 (m, 2 H, H-2, 6A), 4.27 (dd, B part of an ABX system, $J_{6A,6B}$ 12.5, $J_{5,6B}$ 4.0 Hz, H-6B), 4.84 (dd, $J_{3,4\approx J_{4,5}}$ = 9.2 Hz, H-4), 4.99–5.12 (m, 4 H, H-l, 3, PhCH_2), 5.88 (d, *J* 8.2 Hz, NH), 6.20 (d, *J* 8.6 Hz, NH), 7.27–7.39 (m, Ph); ^{13}C NMR (50 MHz, $CDCl_3$): δ 20.5, 20.7, 23.0 (4×CO*Me*), 52.9 (C-2), 61.9 (C-6), 67.1 (Ph*C*H_2), 68.0 (C-4), 73.1, 73.2 (C-3, 5), 82.5 (C-1), 127.9–135.9 (Ph), 156.0 (NCO_2), 169.3–171.6 (C=O). Anal. Calcd for $C_{22}H_{28}N_2O_{10}$: C, 54.99; H, 5.87; N, 5.83. Found: C, 54.95; H, 5.84; N, 5.87.

*2-Acetamido-2-deoxy-6-*O-p-*toluenesulfonyl*-N-*(benzyloxycarbonyl)-β*-D-*glucopyranosylamine* (**13**).—The carbamate **11** (2.13 g, 4.44 mmol) was suspended in MeOH (50 mL), cooled to 0 °C, and the mixture was saturated with ammonia. The mixture was stirred at 0 °C for 6 h and then concentrated. The residue was dissolved in pyridine (20 mL) and cooled to 0 °C, *p*-toluenesulfonyl chloride (1.02 g, 5.35 mmol) was added, and the mixture was stirred at 0 °C for 1 h and then at room temperature for 16 h. Water (0.5 mL) was added, and the mixture was stirred for 20 min and then concentrated. Flash chromatography (1:9 MeOH–EtOAc) then gave the *p*-toluenesulfonate **13** (1.84 g, 82%) as needles: mp 116–118 °C (dec.; EtOH–diisopropyl ether); $[\alpha]_D$ −5.5° (*c* 0.2, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.88 (s, Me), 2.36 (s, Ar*Me*), 3.40–3.65 (m, H-3, 4, 5), 3.80 (m, H-2), 4.14, 4.39 (2 bd, AB part of an ABX system, $J_{6A,6B}$ 10.1 Hz, H-6A, 6B), 4.78 (dd, $J_{1,2}$ 9.3 $J_{1,NH}$ 8.9 Hz, H-1), 5.06 (s, PhCH_2), 5.10–5.27 (bm, 2 H, OH), 6.56 (d, N*H*Z), 6.85 (d, $J_{2,NH}$ 7.6 Hz, N*H*Ac), 7.12–7.80 (m, 9 H, Ar); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.6 (Ar*Me*), 23.0 (Me), 54.4 (C-2), 66.9, 69.1 (C-6, Ph*C*H_2), 69.8 (C-4), 74.6, 74.8 (C-3, 5), 81.9 (C-l), 127.9–145.0 (Ar), 156.2 (NCO_2), 173.3 (C=O). Anal. Calcd for $C_{23}H_{28}N_2O_9S$: C, 54.32; H, 5.55; N, 5.51. Found: C, 54.24; H, 5.52; N, 5.38.

2-Acetamido-6-azido-2,6-dideoxy-N-*(benzyloxycarbonyl)-β*-D-*glucopyranosylamine* (**14**).—A mixture of the *p*-toluenesulfonate **13** (2.08 g, 4.09 mmol) and sodium azide (1.33 g, 20.5 mmol) in DMF (40 mL) was heated at 80 °C for 16 h. The mixture was then poured into water and extracted with EtOAc. The combined extracts were washed successively with water and brine, dried ($MgSO_4$), filtered and concentrated to give a white solid. Recrystallization from MeOH–diethyl ether then gave the azide **14** (1.16 g, 75%) as needles: mp 203–205 °C (dec.); $[\alpha]_D$ −33° (*c* 1.7, Me_2NCHO); ^{1}H

NMR (200 MHz, Me_2SO-d_6): δ 1.78 (s, Me), 3.06 (bddd, $J_{1,2}$ 9.3, $J_{2,3}$ 8.3, $J_{2,NH}$ 8.0 Hz, H-2), 3.19–3.60 (m, 5 H, H-3, 4, 5, 6A, 6B), 4.70 (bdd, $J_{1,NH}$ 9.0 Hz, H-l), 5.04 (s, PhCH_2), 5.08 (d, 1 H, J 5.4 Hz, OH), 5.26 (d, 1 H, J 5.9 Hz, OH), 7.09–7.23 (m, Ph), 7.64 (d, N*H*Z), 7.88 (d, N*H*Ac); ^{13}C NMR (50 MHz, Me_2SO-d_6): δ 23.0 (Me), 51.3 (C-6), 54.6 (C-2), 65.6 (Ph*C*H_2), 71.3 (C-4), 74.2 (C-5), 76.5 (C-3), 81.8 (C-l), 127.6–137.0 (Ph), 155.8 (NCO_2), 170.4 (C=O). Anal. Calcd for $C_{16}H_{21}N_5O_6$: C, 50.65; H, 5.58; N, 18.46. Found: C, 50.29; H, 5.70; N, 18.29.

*2-Acetamido-2,3-di-*O*-acetyl-6-(2′-*tert*-butoxycarbonylamino)benzamido-2,6-dideoxy-*N*-(benzyloxycarbonyl)-β-*D*-glucopyranosylamine* (**15**).—A solution of the azide **14** (540 mg, 1.42 mmol) and triphenylphosphine (560 mg, 2.13 mmol) in 7:1 THF–water (16 mL) was heated at reflux for 3 h. The mixture was cooled to room temperature and Boc-ABz-ODhbt [23] (655 mg, 1.71 mmol) was added. The mixture was stirred for 3 h and then was concentrated. The residue was dissolved in CH_2Cl_2 (20 mL) and treated with acetic anhydride (5 mL) and pyridine (5 mL) for 6 h at room temperature. MeOH (5 mL) was added, and the mixture was stirred for 20 min and then concentrated. The residue was dissolved in EtOAc and was washed successively with M HCl, water, saturated aqueous sodium bicarbonate and brine, dried ($MgSO_4$), filtered and concentrated. The residue was purified by flash chromatography (7:3 EtOAc–petroleum ether) to give the title compound **15** (850 mg, 91%) as needles: mp 216–218 °C (EtOH–diisopropyl ether); $[\alpha]_D$ +17° (*c* 1.5, $CHC1_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.50 (s, Me_3), 1.90, 2.04, 2.08 (3 s, Ac), 3.45 (ddd, A part of an ABMX system, $J_{6A,6B}$ 14.2, $J_{5,6A} \approx J_{6A,NH}$ = 6.0 Hz, H-6A) 3.64–3.72 (m, H-5), 3.76 (ddd, B part of an ABMX system, $J_{5,6b}$ 2.7, $J_{6b,NH}$ 6.2 Hz, H-6B) 4.10 (ddd, $J_{1,2}$ 9.0, $J_{2,3}$ 10.1, $J_{2,NH}$ 8.1 Hz, H-2), 4.86 (dd, $J_{1,NH}$ 8.1 Hz, H-l), 4.97 (dd, $J_{3,4} \approx J_{4,5}$ = 9.5 Hz, H-4), 5.04 (dd, H-3), 5.02–5.14 (m, 2 H, PhCH_2), 6.06 (d, N*H*Ac), 6.38 (d, N*H*Z), 6.61 (bdd, N*H*COAr), 6.29–8.36 (9 H, Ar), 10.02 (s, N*H*Boc); ^{13}C NMR (50 MHz, $CDCl_3$): 20.60, 20.65, 23.0 (4×CO*Me*), 28.3 (CMe_3), 39.5 (C-6), 52.9 (C-2), 67.3 (Ph*C*H_2), 69.2 (C-4), 73.2, 73.7 (C-3, 5), 80.1 (*C*Me_3), 82.2 (C-l), 119.6–140.2 (Ar), 153.0, 156.3 (NCO_2), 168.9–171.7 (C=O). DCI: *m/z* 657 ($M+H^+$, 6.57%), 674 ($M+NH_4^+$, 1.77%). Anal. Calcd for $C_{32}H_{40}N_4O_{11}$: C, 58.53; H, 6.14; N, 8.53. Found: C, 58.58; H, 6.27; N, 8.51.

N^{γ}-*[2-Acetamido-2,3-di-*O*-acetyl-6-(2′-tert-butoxycarbonylamino)benzamido-2,6-dideoxy-β-*D*-glucopyranosyl]-N^{α}-benzoyl-Asn-Tyr(NO_2)-OMe* (**3**).—A solution of the carbamate **15** (344 mg, 0.52 mmol) in EtOH (10 mL) was hydrogenated at room temperature and atmospheric pressure in the presence of 10% palladium-on-charcoal (30 mg) for 1 h. The mixture was then filtered (Celite) and concentrated to give the amine, 2-acetamido-2,3-di-*O*-acetyl-6-(2′-*tert*-butoxycarbonylamino)benzamido-2,6-dideoxy-β-D-glucopyranosylamine (**1**) (275 mg, 100%) as a white foam that was used without further purification. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.50 (s, Me_3), 1.8–2.10 (bs, NH_2), 1.95, 2.03, 2.09 (3 s, Ac), 3.36 (ddd, A part of an ABX system, $J_{6A,6B}$ 14.5, $J_{5,6A} \approx J_{6A,NH}$ = 5.8 Hz, H-6A), 3.59 (ddd, $J_{5,6B}$ 2.5, $J_{4,5}$ 9.0 Hz, H-S), 3.85 (ddd, B part of an ABX system, $J_{6B,NH}$ 7.0 Hz, H-6B), 3.97 (ddd, $J_{1,2}$ 9.5 $J_{2,3}$ $J_{2,NH}$ = 9.0 Hz, H-2), 4-13 (d, H-1), 4.95 (dd, $J_{3,4}$ 9.0 Hz, H-4), 5.05 (dd, H-3), 5.68 (d, N*H*Ac), 6.66 (bdd, N*H*COAr), 6.98–8.40 (4 H, Ar), 10.02 (s, N*H*Boc).

(a) Using DCC. The dipeptide Bz-Asp-Tyr(NO_2)-OMe (**2**, 74 mg, 0.16 mmol), DCC (33 mg, 0.16 mmol) and DhbtOH (26 mg, 0.16 mmol) were dissolved in DMF (0.5 mL) and stirred at room temperature for 20 min. The amine **1** (84 mg, 0.16 mmol) in DMF (0.5 mL) was then added, and the mixture stirred at room temperature overnight. The mixture was filtered, diluted with ethyl acetate, washed with water and brine, dried over sodium sulfate, and the solvent was removed in vacuo. Repeated flash chromatography (1:9 MeOH–CH_2Cl_2) gave the protected glycopeptide **3** (65 mg, 42%) as a yellow amorphous solid.

(b) Using EEDQ. EEDQ (99 mg, 0.40 mmol) was added to a solution of the amine **1** (155 mg, 0.29 mmol) and Bz-Asp-Tyr(NO_2)-OMe **2** (138 mg, 0.30 mmol) in DMF (5 mL). The mixture was stirred at room temperature under nitrogen for 4 days. The mixture was concentrated, and the residue was subjected to repeated flash chromatography (1:9 MeOH–CH_2Cl_2) to give the protected glycopeptide **3** (122 mg, 43%) as a yellow amorphous solid: $[\alpha]_D$ +4.8° (*c* 1.3, Me_2NCHO); ^{1}H NMR (400 MHz, Me_2SO-d_6): δ 1.47 (s, Me_3), 1.62, 1.87, 1.95 (3 s, Ac), 2.57, 2.62 (2 dd, AB part of an ABX system, J_{AB} 16.2, J_{AX} 8.5, J_{BX} 5.1 Hz, Asn β-H), 2.91, 2.99 (2 dd, AB part of an ABX system, J_{AB} 14.0, J_{AX} 8.7, J_{BX} 5.4 Hz, Tyr β-H), 3.35 (obscured by H_2O, H-6A), 3.51 (ddd, B part of an ABMX system, $J_{6A,6B}$ 14.2, $J_{5,6B}$ 5.5, $J_{6B,NH}$ 5.6 Hz, H-6B), 3.60 (s,

OMe), 3.77 (ddd, $J_{5,6a}$ 3.4, $J_{4,5}$ 9.7 Hz H-5) 3.92 (ddd $J_{2,3}$ 9.5, $J_{2,3}$ 9.8, $J_{2,NH}$ 9.2 Hz, H-2), 4.44 (ddd, $J_{H,NH}$ 7.7 Hz, Tyr α-H), 4.71 (dd, $J_{3,4}$ 9.8 Hz, H-4), 4.80 (ddd, $J_{H,NH}$ 8.0 Hz, Asn α-H) 5.03 (dd, H-3), 5.14 (dd, $J_{1,NH}$ 9.1 Hz, H-l), 6.92–8.18 (Ar), 7.84 (d, N*H*Ac), 8.25 (d, N*H*Tyr), 8.45 (d, N*H*Bz), 8.49 (d, Asn N$^{\gamma}$*H*), 8.65 (bdd, $J_{6,ANH}$ 5.6 Hz, C-6 NH) 10-44 (s, N*H*Boc); ^{13}C NMR (75 MHz, Me_2SO-d_6): δ 20.4, 20.6, 22.5 (CO*Me*), 27.9 (C*Me*$_3$), 35.0, 36.7 (C-β), 39.5 (C-6), 49.8, 52.0, 53.4 (4 C, C-α, C-2, OMe), 70.0 (C-4), 73.0, 73.8 (C-3, 5), 78.2 (C-l), 79.7 (CMe_3), 118.4–139.5 (17 C, Ar), 151.1, 152.1 (ArOH, NCO_2), 166.1–171.5 (8 C, C=O). LSIMS: *m/z* 964 ($M+H^+$, 0.5%); HRMS calcd for $C_{45}H_{54}N_7O_{17}$: 964.3576; found: 964.3592. Anal. Calcd for $C_{45}H_{53}N_7O_{17}\cdot H_2O$: C, 55.04; H, 5.64; N, 9.98. Found: C, 55.17; H, 5.58; N, 9.85.

N$^{\gamma}$-*[2-Acetamido-6-(2′-amino)benzamido-2,6-dideoxy-β*-D-*glucopyranosyll*-N$^{\alpha}$-D-*benzoyl-Asn-Tyr(NO$_2$)-OMe trifluoroacetic acid salt* (**4**).—The glycopeptide **3** (44 mg, 0.04 mmol) was suspended in MeOH (5 mL), the mixture was cooled to 0 °C, then saturated with ammonia and stirred overnight at 0 °C. The resulting orange solution was concentrated, and the residue was dissolved in 19:1 trifluoroacetic acid–water (2 mL) and stirred at room temperature for 10 min. Toluene (2×5 mL) was then added, and the mixture was concentrated to give the title compound **4** as a yellow, amorphous solid (40 mg). The product was approximately 92% pure as estimated by ^{1}H NMR and analytical reversed-phase HPLC/ESIMS. Final purification was achieved by semipreparative reversed-phase HPLC using a linear gradient of 30–80% solvent B in 20 min. $[\alpha]_D$ −37° (*c* 0.2 DMF); ^{1}H NMR (400 MHz, Me_2SO-d_6+D_2O): δ 1.64 (s, Ac), 2.52, 2.58 (2 dd, AB part of an ABX system, J_{AB} 17.1, J_{AX} 8.4, $J_{B}X$ 5.1 Hz, Asn β-H), 2.87, 2.98 (2 dd, AB part of an ABX system, J_{AB} 13.9, J_{AX} 8.6, J_{BX} 5.4 Hz, Tyr β-H), 3.01 (dd, $J_{3,4}\approx J_{4,5}=9.0$ Hz, H-4), 3.24–3.39 (m, 3H, H-3, 5, 6A), 3.52 (dd, $J_{1,2}$ 9.8, $J_{2,3}$ 9.9 Hz, H-2) 3.56 (s, OMe), 3.55–3.62 (m, H-6B), 4.43 (dd, Tyr α-H), 4.75 (dd, Asn α-H), 4.81 (d, H-l), 6.62–7.76 (Ar); ^{13}C NMR (75 MHz, Me_2SO-d_6): δ 22.7 (CO*Me*), 35.0, 36.8 (C-β), 39.5 (obscured by Me_2SO, C-6), 49.8 (C-α Asn), 52.0, 53.4, 54.5 (C-α Tyr, C-2, OMe), 72.3, 73.9, 76.2 (C-3, 4, 5), 79.1 (C-l), 115.3–150.9 (Ar), 158.2 (q, J_{CF} 33 Hz, F_3CCO_2), 166.1, 169.2, 169.8, 170.0, 171.4, 171.6 (C=O). LSIMS: *m/z* 780 (M, 4.7%) 802 ($M+Na^+$, 5.5%); HRMS Calcd for $C_{36}H_{42}N_7O_{13}$: 780.2841; found 780.2853.

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