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Solid-phase synthesis of an *O*-linked glycopeptide based on a benzyl-protected glycan approach

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Abstract

The solid-phase synthesis of asialo-[Ala[18]]-B-chain (**2**) of human α2HS glycoprotein is described. Disaccharide-linked serine unit **12**, carrying a benzyl protecting group, was synthesized via stereoselective glycosylation of **8** with **6**. Peptide synthesis was carried out by the Fmoc method utilizing an automated peptide synthesizer. A modified procedure using a mechanical shaker at the coupling step with **12** made easy the recovery of unreacted **12**. The benzylated glycopeptide thus synthesized was cleaved from the resin and hydrogenated to give **2**. © 1996 Elsevier Science Ltd.

Keywords: Solid-phase synthesis; *O*-Linked glycopeptide; Benzyl-protected glycan; B-chain of α2HS glycoprotein

1. Introduction

The chemical synthesis of glycoproteins has been a big challenge for organic chemists. Numerous efforts have been made to synthesize glycopeptides, fragments of a glycoprotein, using the combined technologies of carbohydrate and peptide chemistry. Among them, the solid-phase technique is a method of choice to obtain larger oligomers in a rapid and efficient fashion [1]. A number of fragments and mimics of biologically significant glycoproteins have been synthesized according to the solid-phase protocol based on the Fmoc method of peptide synthesis, together with the employment of acyl protection for hydroxyl groups of saccharide moieties [2,3]. In contrast, we have recently

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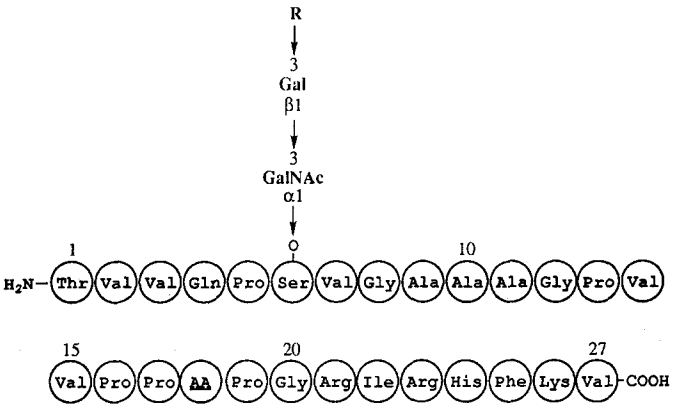


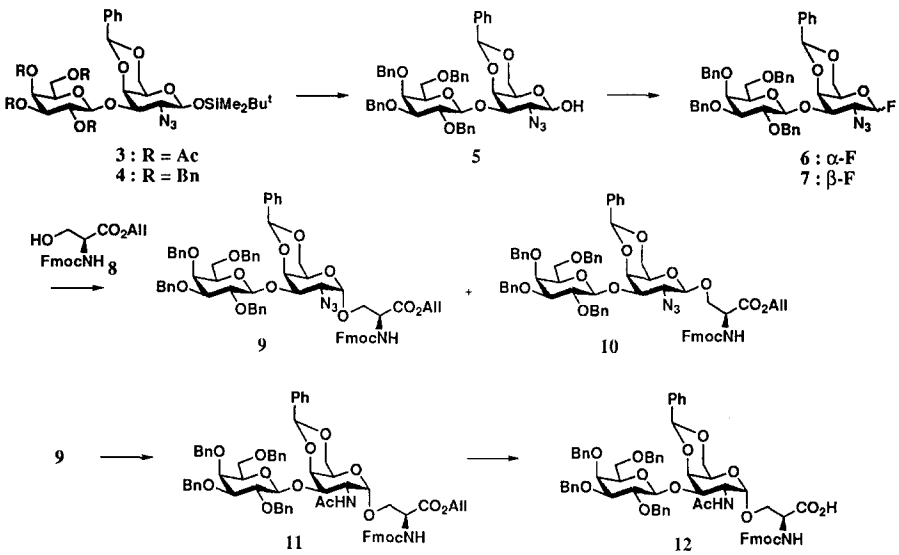
1 : R = NeuAc-α-2, AA = Cys
2 : R = H, AA = Ala

Scheme 1.

proposed an approach involving benzyl-protected oligosaccharides and succeeded in the solution-phase syntheses of sialyl saccharide-clustering fragments of human glycophorin A, a typical *O*-linked glycoprotein present in erythrocyte membrane [4]. In order to verify the suitability of our strategy as applied to solid-phase synthesis, we next chose as a synthetic target B-chain of human α2HS glycoprotein **1** [5], which is a normal plasma globulin associated with the biological functions both in bone mineralization and in the immune response [6,7]. It is notable that the use of a benzyl-protected *C*-glycosyl amino acid as a building block for the solid-phase synthesis of a stable analogue of a glycopeptide has been reported recently [8]. The B-chain has as a part of its structure many chemically labile moieties such as sialic acid, a cystein residue, and the basic amino acid residues located at the C-terminal reagion. Before synthesizing the complex molecule **1**, we studied the solid-phase synthesis of a model glycopeptide. Described herein is a synthesis of asialo-[Ala^{18}]-B-chain **2**, which was facilitated by utilizing an automated peptide synthesizer. (See Scheme 1.)

2. Results and discussion

Our study began with the preparation of protected Galβ1 → 3GalNAcα1 → Ser (**12**) (Thomsen–Friedenreich antigen, T-antigen) as a key glycosyl amino acid substrate for the Fmoc method. Known disaccharide **3** [4](d) was deacetylated and benzylated in the usual manner (1. NaOMe/MeOH, 2. BnBr/NaH/THF) to afford **4** in 87%, which was desilylated with Bu_4NF/AcOH/THF [9] to give a hemiacetal **5** (94%). On exposure to diethylaminosulfur trifluoride (DAST) in THF [10], **5** gave an α:β mixture of glycosyl fluorides **6** and **7** (**6**:**7** = 7:1) in 86%. Cp_2ZrCl_2–$AgClO_4$-promoted reaction [11] of **6** and Fmoc serine allyl ester **8** [12] produced glycosyl serine derivatives **9** (69%) and **10** (21%). Treatment of the α-glycoside **9** with AcSH/pyridine provided ready conversion



Scheme 2.

of the azide into the acetamide (**11**, 78%), which by Pd(0)-catalyzed deallylation [12](b) afforded the desired disaccharide-serine unit **12** in 99% yield. (See Scheme 2.)

With the key intermediate **12** in hand, we studied the solid-phase synthesis of the glycopeptide **2**. First, we practiced construction of the whole sequence of the glycopeptide with the synthesizer using the ready-made program, where the peptide chain was elongated on 4-hydroxymethylphenoxymethyl–copolystylene–1% divinylbenzene resin (HMP resin) by sequential addition of Fmoc amino acids activated with dicyclohexylcarbodiimide (DCC)-hydroxybenztriazole (HOBt) in *N*-methylpyrrolidone (NMP). For the side-chain protection, were employed triphenylmethyl (Trt, for Gln and His), *tert*-butoxycarbonyl (Boc, for Lys), and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc, for Arg) groups. Those were simultaneously removed in the trifluoroacetic acid (TFA)-cleavage process for releasing the peptide from the resin support. A henicosapeptide-linked resin (residues 7–27) was synthesized in 0.25-mmol scale with an efficiency of 87% (monitored by ninhydrin reaction). Condensation steps with **12** and further with the N-terminal five amino acid residues were carried out in a smaller scale (29 μmol). Three equivalents (86 μmol) of **12** was used for the condensation under a program of prolonged reaction time (165 min). After completing the peptide-chain elongation, cleavage of glycopeptide from the resin and deprotection of the side-chain functional groups were simultaneously performed by treatment with aq TFA in the presence of phenol, 1,2-ethanedithiol, and thioanisole. Reversed-phase chromatographic and mass-spectral analyses revealed that the product was comprised of non-glycosylated (missing Ser unit) peptide (56%), partly debenzylated (di- and tribenzyl) glycopeptides (37%), and tetrabenzyl glycopeptide (7%). Since this procedure was inconvenient for recovery of the valuable sample of unreacted **12**, we next examined a modified approach involving a non-automated step for coupling of **12**. The de-Fmoc henicosapeptide-resin

(14 μmol) was mechanically shaken with activated **12** (3 equiv) in NMP for 64 h, and filtered. The resin was transferred to the synthesizer and allowed to automatically react with the necessary five amino acids, while the unreacted **12** was readily isolated in a reasonable amount from the filtrate. Fig. 1A shows a chromatogram of the product released from the resin. The major peaks in the chromatogram were mass-spectrometrically assigned. A broad peak (1) at 7 min represents the serine unit-deleted peptide (M + 1: 2621.6) contaminated by small amounts of monobenzyl glycopeptides (M + 1: 3163.7). Peak 2 and the combined areas of peaks 3–6 were identified as dibenzyl glycopeptides (M + 1: 3254.6). The peaks 7–9 corresponded to the tribenzyl derivatives (M + 1: 3344.6), while peak 10 represents the tetrabenzyl glycopeptide (M + 1: 3434.8). The fractions containing di-, tri-, and tetrabenzyl glycopeptides were collected (55%) and hydrogenated with 20% $Pd(OH)_2/C$ in aq EtOH (aldehyde free) at room temperature for 5 days. An HPLC of the hydrogenated product is shown in Fig. 1B. The product was purified by gel-permeation and reversed-phase (C_{18}) chromatography to afford the target disaccharide-linked heptacosapeptide **2** in 26% yield (based on the henicosapeptide-linked resin). The structure was established by NMR spectroscopy (Fig. 2) and mass-spectrometry (M + 1: 3074.5, M + Na: 3095.7).

In this paper we have shown a synthesis of asialo-[Ala^{18}]-analogue of B-chain of a2HS glycoprotein that was achieved by the solid-phase method in combination with a benzyl-protected glycan strategy. TFA cleavage of the glycopeptide from the resin suppot resulted in concomitant partial debenzylation of the disaccharide side chain to afford a complex mixture of products. However, the more hydrophobic benzylated glycopeptides were readily separated from the mixture by reversed-phase chromatography, and hydrogenated to give the target glycopeptide **2**. Optimization of the conditions for coupling of a glycosyl amino acid unit and for cleavage of the glycopeptide from the resin, and application of this approach to the synthesis of natural glycopeptide **1** are in progress.

3. Experimental

General.—Optical rotations were determined with a Jasco DIP-370 polarimeter for solutions in $CHCl_3$, unless noted otherwise. Column chromatography was performed on Silica Gel-60 (E. Merck 70–230 mesh or 230–400 mesh). TLC and HPTLC were performed on Silica Gel 60 F_{254} (E. Merck). ^1H and ^{13}C NMR spectra were recorded with either a JEOL α600 [^1H (600 MHz)] or EX270 [^1H (270 MHz), ^{13}C (68 MHz)] spectrometer. Chemical shifts are expressed in ppm downfield from the signal for internal Me_4Si for solutions in $CDCl_3$. MALDI–TOF mass-spectra were obtained with a Bruker REFLEX (2,5-Dihydroxybenzoic acid was used as a matrix). Peptide synthesis was performed with an Applied Biosystems Model 431A peptide synthesizer. Fmoc Val-preloaded HMP resin, Fmoc amino acids in cartridges, and the reagents for the peptide synthesis were purchased from Applied Biosystems Inc.

tert-*Butyldiphenylsilyl 2,3,4,6-tetra*-O-*benzyl*-β-D-*galactopyranosyl-(1 → 3)-2-azido-4,6*-O-*benzylidene-2-deoxy*-β-D-*galactopyranoside* (**4**).—To a stirred solution of **3** (1.20 g, 1.39 mmol) in a mixture of MeOH (15 mL) and toluene (5 mL) was added 5 M

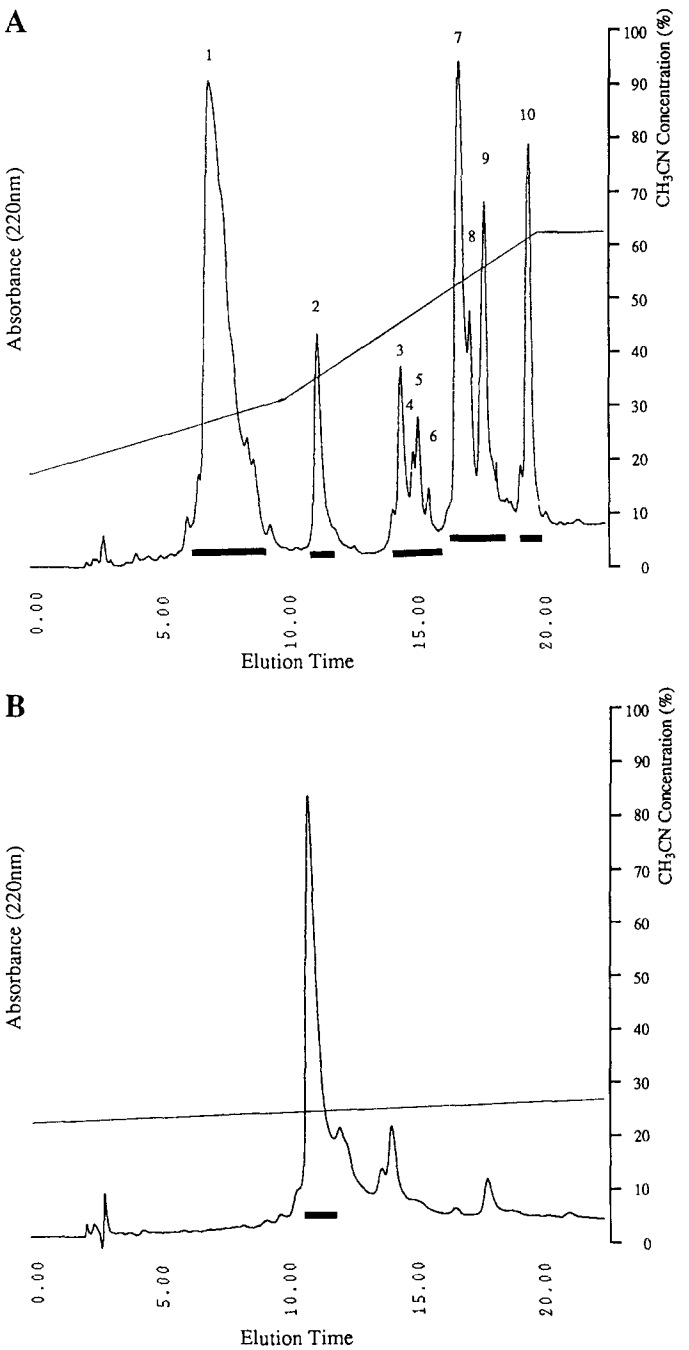


Fig. 1. (A) Reserved-phase HPLC of the synthetic glycopeptides cleaved from the resin. (B) Reversed-phase HPLC of the hydrogenated glycopeptide. The LiChrospher 100 RP-18 column (250-ϕ10) was eluted with a gradient of CH_3CN containing 0.1% CF_3CO_2H (flow rate 5.0 mL/min). The fractions indicated by the solid bars were collected and characterized.

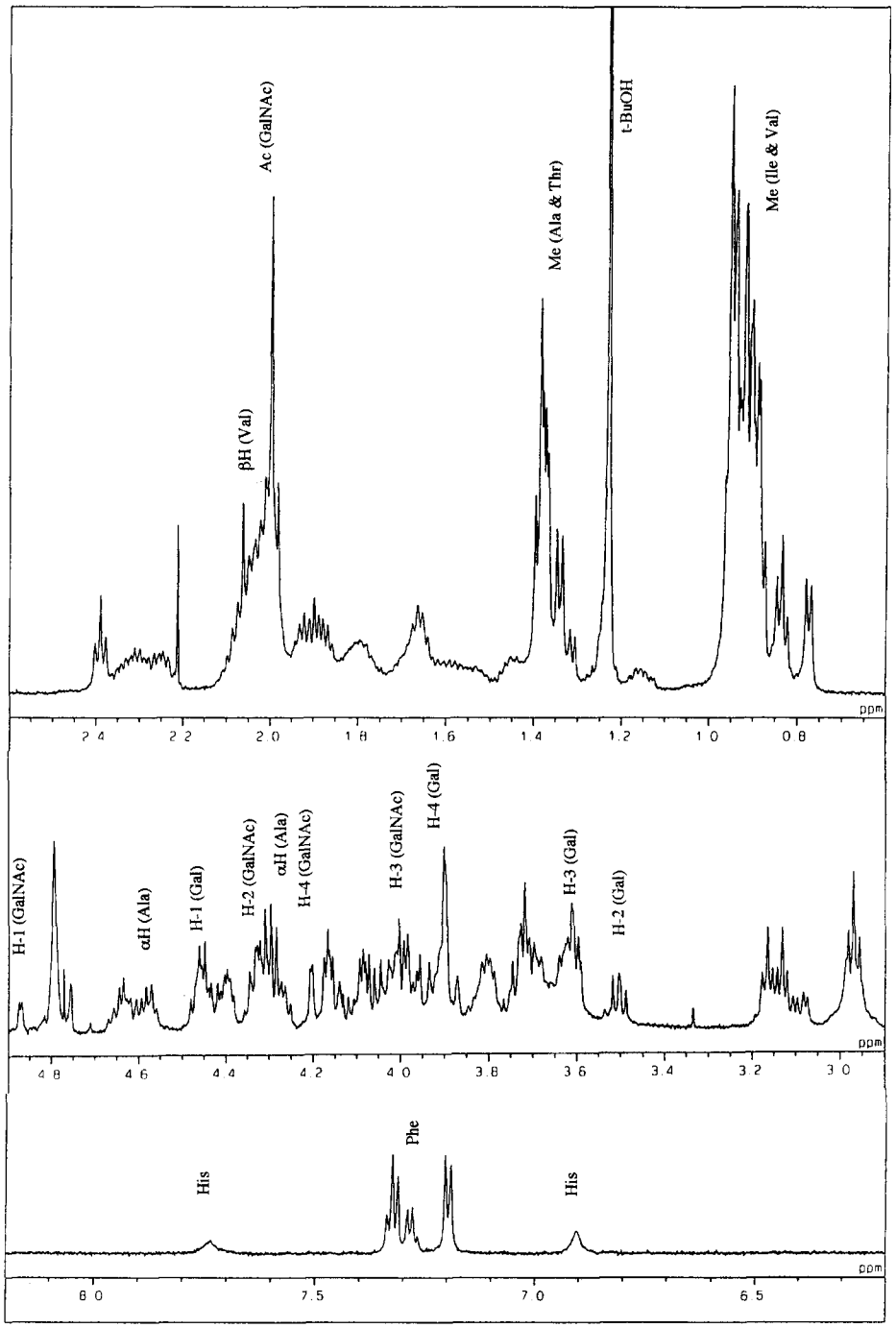


Fig. 2. ^{1}H NMR spectrum of asialo-[Ala^{18}]-B-chain of human α2HS glycoprotein D_2O, 600 MHz.

NaOMe–MeOH (0.2 mL, 1 mmol). The mixture was stirred at room temperature for 1 h, then concentrated in vacuo. MeOH remaining in the crude tetraol was removed by coevaporation three times with a mixture of dry THF and dry toluene. The residue was dissolved in dry THF (20 mL), and stirred with 60% NaH (0.50 g, 7.2 mmol) at 60 °C for 1 h. BnBr (1.3 mL, 10.9 mmol) was added to the mixture, which was stirred at 60 °C overnight. After cooling, the reaction was quenched by adding a few pieces of ice, and the mixture was concentrated in vacuo. The residue was extracted with 1:1 ether–EtOAc, washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. The crude product was chromatographed on silica gel with 19:1 toluene–EtOAc to give **4** (1.27 g, 87%): $[\alpha]_D$ +16.3° (c 2.2), R_f 0.55 (9:1 toluene–EtOAc). ^{1}H NMR: δ 7.79 (dd, 2 H, *J* 7.8 Hz, Ar), 7.71 (dd, 2 H, *J* 1.5, 7.9 Hz, Ar), 7.54 (m, 2 H, Ar), 7.4–7.2 (m, 29 H, Ar), 5.42 [s, 1 H, PhC*H*(O)$_2$], 4.98 and 4.77 (2d, 2 H, *J* 11.2 Hz, PhCH_2), 4.92 and 4.57 (2d, 2 H, *J* 11.6 Hz, PhCH_2), 4.80 and 4.70 (2d, 2 H, *J* 11.9 Hz, PhCH_2), 4.63 (d, 1 H, *J* 7.6 Hz, H-1a), 4.43 (d, 1 H, *J* 7.9 Hz, H-1b), 4.37 (brs, 2 H, PhCH_2), 4.09 (d, 1 H, *J* 3.0 Hz, H-4a), 2.83 (s, 1 H, H-5a), 1.13 (s, 9 H, *t*-Bu). Anal. Calcd for $C_{63}H_{67}N_3O_{10}Si$: C, 71.77; H, 6.41; N, 3.99. Found: C, 71.69; H, 6.45; N, 3.92.

2,3,4,6-Tetra-O-*benzyl*-β-D-*galactopyranosyl*-(*1* → *3*)-*2-azido-4,6*-O-*benzylidene-2-deoxy*-β-D-*galactopyranose* (**5**).—To a stirred mixture of **4** (1.20 g, 1.14 mmol) and AcOH (0.7 mL, 12 mmol) in dry THF (35 mL), was added 1 M Bu_4NF–THF (4.6 mL, 4.6 mmol) at 0 °C. Stirring was continued between 0 °C and room temperature for 1 day. The mixture was concentrated in vacuo, the residue was extracted with 1:1 ether–EtOAc, and the extract was washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. Column chromatography of the residue on silica gel with 13:7 toluene–EtOAc gave **5** (0.87 g, 94%): R_f 0.49 and 0.56 (1:1 toluene–EtOAc). ^{1}H NMR: δ 7.52 (m, 2 H, Ar), 7.4–7.2 (m, 23 H, Ar), 5.49 [s, 1 H, PhC*H*(O)$_2$], 5.44 (brt, 0.75 H, H-1aα). Anal. Calcd for $C_{47}H_{49}N_3O_{10}$: C, 69.19; H, 6.05; N, 5.15. Found: C, 69.17; H, 6.03; N, 4.89.

2,3,4,6-Tetra-O-*benzyl*-β-D-*galactopyranosyl*-(*1* → *3*)-*2-azido-4,6*-O-*benzylidene-2-deoxy*-α- (**6**) *and* -β-D-*galactopyranosyl fluoride* (**7**).—To a stirred solution of **5** (0.83 g, 1.02 mmol) in dry THF (10 mL) at 0 °C, was added diethylaminosulfur trifluoride (200 μL, 1.5 mmol). The mixture was stirred for 30 min, MeOH (0.5 mL) was added, and the mixture was concentrated in vacuo. The residue was extracted with 1:1 ether–EtOAc, and the extract was washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. The product was chromatographed on silica gel with 9:1–4:1 toluene–EtOAc to give **6** (0.61 g, 73%) and **7** (0.11 g, 13%).

Compound **6**: $[\alpha]_D$ +90.8° (c 2.4), R_f 0.59 (4:1 toluene–EtOAc). ^{1}H NMR: δ 7.50 (m, 2 H, Ar), 7.4–7.2 (m, 23 H, Ar), 5.81 (dd, 1 H, *J* 2.3, 53.1 Hz, H-1a), 5.51 [s, 1 H, PhC*H*(O)$_2$], 4.98 and 4.78 (2 d, 2 H, *J* 11.2 Hz, PhCH_2), 4.95 and 4.58 (2 d, 2 H, *J* 11.6 Hz, PhCH_2), 4.79 and 4.71 (2 d, 2 H, *J* 11.9 Hz, PhCH_2), 4.69 (d, 1 H, *J* 7.6 Hz, H-1b), 4.43 and 4.38 (2 d, 2 H, *J* 11.9 Hz, PhCH_2). Anal. Calcd for $C_{47}H_{48}FN_3O_9$: C, 69.02; H, 5.92; N, 5.14; F, 2.32. Found: C, 68.85; H, 5.92; N, 5.22; F, 2.26.

Compound **7**: $[\alpha]_D$ +32.7° (c 1.2), R_f 0.39 (4:1 toluene–EtOAc). ^{1}H NMR: δ 7.52 (m, 2 H, Ar), 7.4–7.2 (m, 23 H, Ar), 5.50 [s, 1 H, PhC*H*(O)$_2$], 5.08 (dd, 1 H, *J* 7.6, 52.8 Hz, H-1a),4.95 and 4.78 (2 d, 2 H, *J* 11.2 Hz, PhCH_2), 4.95 and 4.58 (2 d, 2 H, *J* 11.6 Hz, PhCH_2), 4.80 and 4.72 (2 d, 2 H, *J* 11.5 Hz, PhCH_2), 4.67 (d, 1 H, *J* 7.6 Hz,

H-1b), 4.43 and 4.37 (2 d, 2 H, *J* 11.9 Hz, PhCH_2). Anal. Found: C, 69.03; H, 5.95; N, 5.17; F, 2.07.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[2,3,4,6*-tetra-O-*benzyl-β*-D-*galactopyranosyl-(1 → 3)-2-azido-4,6*-O-*benzylidene-2-deoxy-α*- (**9**) *and -β*-D-*galactopyranosyl]*-L-*serine allyl ester* (**10**).—A mixture of Cp_2ZrCl_2 (210 mg, 0.72 mmol), $AgClO_4$ (150 mg, 0.72 mmol), and powdered molecular sieves 4A (2 g) in dry CH_2Cl_2 (13 mL) was stirred at room temperature for 30 min under Ar, then cooled on dry ice–CH_3CN bath (−40 °C). A solution of **6** (400 mg, 0.48 mmol) and **8** (185 mg, 0.50 mmol) in dry CH_2Cl_2 (17 mL) was added, and the mixture was stirred between −40 °C and room temperature overnight, then diluted with EtOAc, and filtered through Celite. The filtrate was washed with aq $NaHCO_3$, water, and brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was stirred with 1,1-dimethoxytoluene (200 μL) and *p*-TsOH (5 mg) in dry CH_3CN (20 mL) at room temperature for 30 min, then quenched with aq $NaHCO_3$, and concentrated in vacuo. The residue was extracted with EtOAc, and the extract was washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. Chromatography of the crude product on Bio-beads S x3 (300 mL) with 1:1 toluene–EtOAc gave a mixture of **9** and **10**, which was chromatographed on silica gel with 17:3 toluene–EtOAc to afford **9** (390 mg, 69%) and **10** (120 mg, 21%).

Compound **9**: $[\alpha]_D$ +90.5° (c 2.0), R_f 0.47 (4:1 toluene–EtOAc). ^{1}H NMR: δ 7.76 (d, 2 H, *J* 6.9 Hz, Ar), 7.60 (d 2 H, *J* 7.3 Hz, Ar), 7.50 (m, 2 H, Ar), 7.4–7.2 (m, 27 H, Ar), 5.98 (d, 1 H, *J* 8.6 Hz, N*H*), 5.90 (m, 1 H, C*H*=CH_2), 5.47 [s, 1 H, PhC*H*$(O)_2$], 5.34 (brd, 1 H, *J* 17.2 Hz, =C*H*2), 5.26 (dd, 1 H, *J* 1.0, 10.6 Hz, =CH_2), 5.02 (brs, 1 H, H-1a), 4.65 (d, 1 H, *J* 7.3 Hz, H-1b); ^{13}C NMR: δ 100.2 (C-1a), 100.4 [Ph*C*H$(O)_2$], 104.8 (C-1b), 155.9 (NH*C*O), 169.5 (*C*O_2All). Anal. Calcd for $C_{68}H_{68}N_3O_{14}$: C, 70.09; H, 5.88; N, 4.81. Found: C, 69.96; H, 5.86; N, 4.41.

Compound **10**: $[\alpha]_D$ +17.0° (c 1.5), R_f 0.23 (4:1 toluene–EtOAc). ^{1}H NMR: δ 7.74 (d, 2 H, *J* 7.4 Hz, Ar), 7.60 (d 2 H, *J* 7.3 Hz, Ar), 7.51 (m, 2 H, Ar), 7.4–7.2 (m, 27 H, Ar), 5.95 (d, 1 H, *J* 8.6 Hz, N*H*), 5.89 (m, 1 H, C*H*=CH_2), 5.48 [s, 1 H, PhC*H*$(O)_2$], 5.31 (brd, 1 H, *J* 17.2 Hz, =C*H*2), 5.17 (dd, 1 H, *J* 1.0, 10.2 Hz, =CH_2); ^{13}C NMR: δ 100.5 [Ph*C*H$(O)_2$], 102.7 (C-1a), 104.6 (C-1b), 155.9 (NH*C*O), 169.2 (*C*O_2All). Anal. Found: C, 70.08; H, 5.85; N, 4.73.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[2,3,4,6*-tetra-O-*benzyl-β*-D-*galactopyranosyl-(1 → 3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy-α*-D-*galactopyranosyl]*-L-*serine allyl ester* (**11**).—A mixture of **9** (310 mg, 0.26 mmol), AcSH (5 mL), and pyridine (2.5 mL) was stirred at room temperature overnight, then concentrated in vacuo, and the residue was chromatographed on silica gel with 3:2 toluene–EtOAc to give **11** (245 mg, 78%): $[\alpha]_D$ +76.3° (c 1.0), R_f 0.46 (1:1 toluene–EtOAc). ^{1}H NMR: δ 7.76 (d, 2 H, *J* 7.6 Hz, Ar), 7.59 (d 2 H, *J* 7.3 Hz, Ar), 7.53 (m, 2 H, Ar), 7.4–7.2 (m, 27 H, Ar), 5.91 (d, 1 H, *J* 8.9 Hz, N*H*), 5.87 (m, 1 H, C*H*=CH_2), 5.73 (d, 1 H, *J* 7.3 Hz, N*H*), 5.45 [s, 1 H, PhC*H*$(O)_2$], 5.32 (brd, 1 H, *J* 18.5 Hz, =C*H*2), 5.17 (brd, 1 H, *J* 10.6 Hz, =CH_2), 5.13 (d, 1 H, *J* 3.3 Hz, H-1a), 1.75 (s, 3 H, Ac). Anal. Calcd for $C_{70}H_{72}N_2O_{15}$: C, 71.17; H, 6.14; N, 2.37. Found: C, 71.42; H, 6.05; N, 2.26.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[2,3,4,6*-tetra-O-*benzyl-β*-D-*galactopyranosyl-(1 → 3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy-α*-D-*galactopyranosyl]*-L-*serine* (**12**).—A mixture of **11** (240 mg, 0.2 mmol), $Pd(PPh_3)_4$ (50 mg, 0.04 mmol), and *N*-methylaniline

(500 μL, 4.6 mmol) in dry THF (3.5 mL) was stirred at room temperature under Ar overnight, and then concentrated in vacuo. The residue was extracted with EtOAc, washed with 0.1 N HCl, water, and brine, dried (Na_2SO_4), and concentrated in vacuo. Chromatography of the crude product on silica gel with 193:6:1 $CHCl_3$–EtOH–AcOH gave **12** (230 mg, 99%): $[\alpha]_D$ +91.7° (c 1.1), R_f 0.47 (185:14:1 $CHCl_3$–EtOH–AcOH). ^{1}H NMR: δ 7.73 (d, 2 H, *J* 7.6 Hz, Ar), 7.6–7.5 (m, 4 H, Ar), 7.4–7.2 (m, 27 H, Ar), 6.28 (d, 1 H, *J* 6.9 Hz, N*H*), 5.98 (d, 1 H, *J* 7.6 Hz, N*H*), 5.43 [s, 1 H, PhC*H*$(O)_2$], 5.17 (brs, 1 H, H-1a), 1.71 (s, 3 H, Ac). Anal. Calcd for $C_{67}H_{68}N_2O_{15}$: C, 70.51; H, 6.01; N, 2.45. Found: C, 70.24; H, 6.03; N, 2.45.

L-*Threonyl*-L-*valyl*-L-*valyl*-L-*glutaminyl*-L-*prolyl*-L-[β-D-*galactopyranosyl*-(1 → 3)-2-*acetamido*-2-*deoxy*-α-D-*galactopyranosyl*]-L-*seryl*-L-*valyl*-L-*glycyl*-L-*alanyl*-L-*alanyl*-L-*alanyl*-L-*glycyl*-L-*prolyl*-L-*valyl*-L-*valyl*-L-*prolyl*-L-*prolyl*-L-*alanyl*-L-*prolyl*-L-*glycyl*-L-*arginyl*-L-*isoleucyl*-L-*arginyl*-L-*histidyl*-L-*phenylalanyl*-L-*lysyl*-L-*valine* (**2**).—*(a) Synthesis of the benzylated glycoheptacosapeptide:* An Fmoc-protected henicosapeptide-linked resin (883 mg) was synthesized after twenty cycles of the standard synthesizer program of condensation with the DCC–HOBt-activated Fmoc amino acids (1 mmol each) starting from FmocVal-preloaded HMP resin (357 mg, 250 mmol). Efficiency of the condensation at each step was monitored by utilizing ninhydrin test and the overall yield of the henicosapeptide was estimated as 87.4%.

A part of this resin (70 mg, 14 μmol) was treated with piperidine to remove the Fmoc group, while **12** (50 mg, 44 μmol) was activated by shaking with M DCC–NMP (0.25 mL, 250 μmol) and M HOBt–NMP (0.25 mL, 250 μmol) at room temperature for 1 h. The resin, dried after washing with dry CH_2Cl_2, and NMP (0.5 mL) were added to the activated **12**. The mixture was mechanically shaken at a rate of 200 rpm for 64 h at room temperature, and filtered. The resulting resin was washed with NMP and dry CH_2Cl_2, dried in vacuo, and transferred to the automated synthesizer. Pro, Gln, Val, Val, and Thr residues were sequentially condensed. After deprotection with piperidine, the glycopeptide-linked resin (85 mg) thus obtained was treated with a mixture of phenol (125 mg), 1,2-ethanedithiol (40 μL), thioansole (80 μL), water (80 μL), and TFA (1.7 mL) at room temperature for 1.5 h, then filtered, and washed with TFA and CH_2Cl_2. The combined filtrate and washings were concentrated in vacuo to a volume of ca. 1 mL, diluted with water (10 mL), and extracted with Et_2O. The separated aqueous layer was concentrated in vacuo to a residue, which was dissolved in 50% aq CH_3CN containing 0.1% TFA (1 mL) and passed through a cartridge of C_{18} reversed-phase media (Varian, Analytichem Bond Elut). The eluate was concentrated in vacuo, dissolved in water, and lyophilized to give crude glycopeptide (56 mg), which was chromatographed on a gel-permeation column (Pharmacia Biotech, Superdex peptide) with 30% aq CH_3CN containing 0.1% TFA as the eluent. The most mobile fractions were collected and concentrated in vacuo. The residue (41 mg) was then fractionated by preparative HPLC on C_{18} silica gel (E.Merck, LiChrosphere 100 RP-18, 250-φ10) with a gradient elution of aq CH_3CN containing 0.1% TFA (concentration of CH_3CN: 0–10 min; 24–32%, 10–20 min; 32–64%). The fractions including peaks 2–10 (see Fig. 1A) were collected and concentrated in vacuo to give a mixture of di-, tri-, and tetrabenzyl glycopeptides (27 mg). Concentration of the fraction corresponding to peak 1 gave Ser unit-deleted peptide as the major component (13 mg). On the other hand, unreacted **12**

(36 mg) was recovered from the filtrate. The filtrate was stirred with a large excess of water at room temperature for 3 h, and concentrated in vacuo. The residue was dissolved in EtOH, the insoluble material being filtered off, and concentrated in vacuo. The resulting crude material was purified by column chromatography on silica gel to give **12** (35 mg).

(b) Hydrogenation of the benzylated glycopeptide: The mixture of benzylated glycopeptides was hydrogenated with 20% $Pd(OH)_2$–C (15 mg) in 50% aq EtOH (aldehyde free) containing 0.05% TFA (50 mL) at room temperature for 5 days, then filtered through Celite, and concentrated in vacuo. The crude product was chromatographed on LiChrosphere 100 RP-18 with aq CH_3CN containing 0.1% TFA (concentration of CH_3CN: 0–30 min; 22–30%, see Fig. 1B). The major fraction was collected and further purified by the same HPLC to give **2** (11 mg, 26% based on the henicosapeptide-linked resin): $[\alpha]_D$ −63.3° (*c* 0.15, H_2O). ^1H NMR [D_2O, *t*-BuOH (δ 1.23)]: δ 7.73 (br, 1 H, His), 7.32 (brt, 2 H, Phe), 7.28 (t, 1 H, *J* 6.8 Hz, Phe), 7.20 (d, 2 H, *J* 8.8 Hz, Phe), 6.90 (br, 1 H, His), 4.87 (d, 1 H, *J* 2.9 Hz, H-1:GalNAc), 4.57 (m, 1 H, αH:Ala), 4.45 (d, 1 H, *J* 7.8 Hz, H-1:Gal), 4.20 (brs, 1 H, H-4:GalNAc), 3.50 (dd, 1 H, *J* 8.3, 9.8 Hz, H-2:Gal), 2.00 (s, 3 H, Ac:GalNAc), 1.40–1.37 (m) and 1.34 (d, 3 H, *J* 7.3 Hz) [Me: Ala and Thr], 0.96–0.87 (m), 0.84 (d, 3 H, *J* 6.8 Hz), 0.83 (d, 3 H, *J* 7.3 Hz), and 0.78 (d, 3 H, *J* 6.8 Hz) [Me: Ile and Val]. MALDI–TOF MS: *m/z* 3074.5 $[M + 1]^+$, 3095.7 $[M + Na]^+$.

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