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Solid-phase synthesis of the B-chain of human α2HS glycoprotein

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Abstract

The B-chain of human α2HS glycoprotein **1**, a heptacosapeptide carrying a trisaccharide (sialyl T) side chain, was synthesized. Prior to the Fmoc-based solid-phase synthesis of the glycopeptide, the benzyl-protected glycosyl serine building block **6** was prepared via β-stereoselective glycosylation of the 2-azido-2-deoxygalactosyl serine **11** with the sialyl galactosyl trichloroacetimidate **9**. An automated peptide synthesizer was efficiently used for the elongation of the entire peptide chain except for the coupling with **6**. The synthesized glycopeptide was cleaved from the resin by the TFA method. The resultant mixture of the benzylated glycopeptides was treated with TMSOTf–thioanisole in TFA and then with aq $NaHCO_3$ and 1,4-dithiothreitol to give **1**.

Keywords: α2HS glycoprotein; Glycopeptide synthesis; Solid-phase synthesis

1. Introduction

There has been a growing demand for chemically or chemoenzymatically synthesized samples of N- and O-glycans, and their peptide conjugates as useful probes to obtain insight into the glycoprotein-mediated biologial reactions [1]. Despite the current developments of the technologies in this field [2], there are only a few papers reporting the synthesis of large oligopeptides carrying a complex carbohydrate side chain [3]. We have been studying syntheses of glycopeptides, especially those possessing sialic acid as a component of the oligosaccharide side chains, and we have accomplished the synthesis of a sialooligosaccharide-clustering fragment of human glycophorin Am, utilizing solution-phase Fmoc peptide chemistry based on the benzyl protecting-group strategy [4]. Since it was of great interest to apply the strategy to the solid-phase synthesis of a glycopeptide with a longer peptide back bone, B-chain of α2HS-glycoprotein was next chosen as a synthetic target.

α2HS-Glycoprotein is a normal human plasma globulin involved in a variety of significant biological events, such as bone mineralization, endocytosis,

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and opsonization [5]. Decreases in its plasma concentration are frequently correlated with malignant diseases. The glycoprotein is composed of two polypeptide subunits, the structures of which have been well established and include the inter- and the intra-chain disulfide linkages as well as the attached N- and O-linked oligosaccharide side-chains [6].

B-Chain, the minor subunit split from α2HS-glycoprotein, was characterized as a heptacosapeptide carrying a sialotrisaccharide (**1**) as depicted in Fig. 1. In a preliminary investigation using the solid-phase technique, we have synthesized the model glycopeptide of B-chain, an asialo-[Ala^{18}]-analog, in which we omitted the labile cysteine and sialic acid residues [7]. The successful utilization of solid-phase synthesis led us to adopt similar tactics for the native B-chain of α2HS-glycoprotein. This paper describes the full details of the investigation. Preliminary accounts of this work have appeared earlier [8].

2. Results and discussion

In the course of the studies on the oligosaccharide fragment of glycophorin AM, we have synthesized as an intermediate a trisaccharide-linked serine derivative **2** [9] that was thought to be readily converted into the building block **6** suitable for the solid-phase synthesis of the B-chain glycopeptide. Benzylidenation of **2**, followed by reductive conversion of the azide functionality into acetamide with thioacetic acid–pyridine [10], gave **4** (72%, 2 steps), which was deallylated by catalysis

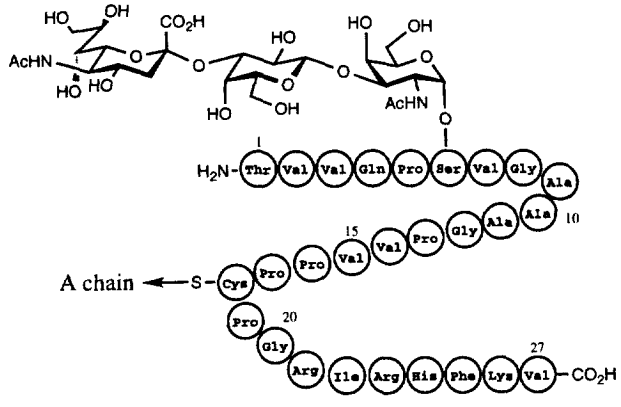

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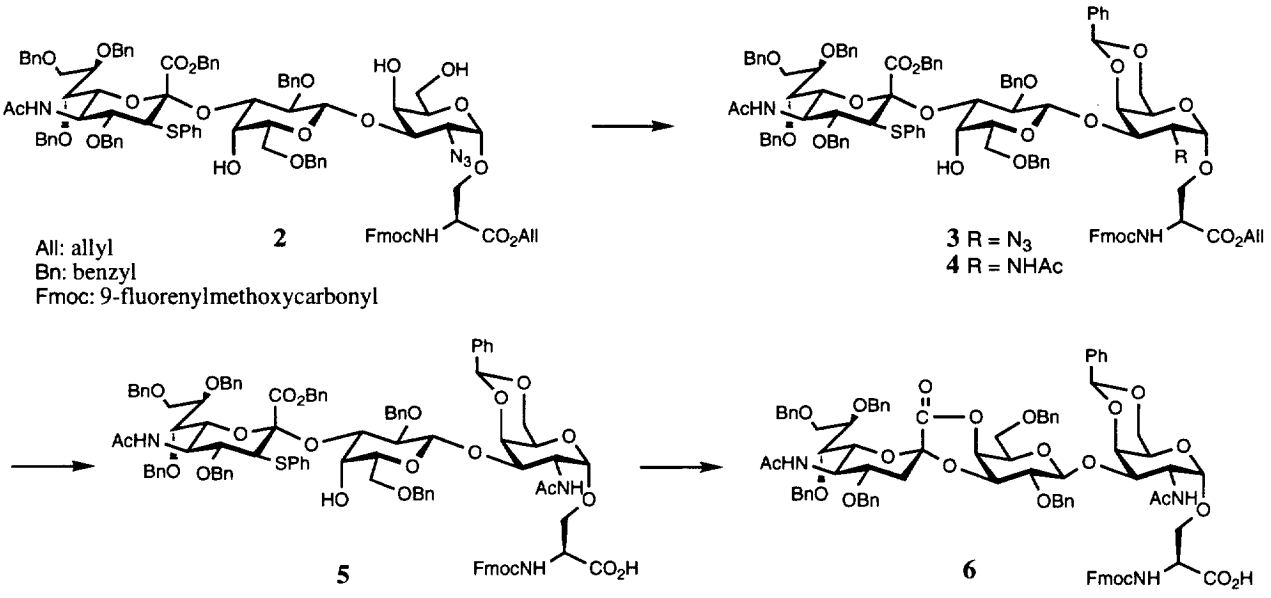
Fig. 1. Structure of the B-chain of human α2HS glycoprotein.

of Pd(0) [11] to a carboxylic acid **5** (95%). Desulfurization of **5** was performed according to the previously established procedure using Ph_3SnH in refluxing benzene [12]. Under these conditions, compound **5** was readily lactonized, but not smoothly desulfurized. The desired product **6** was isolated only in 28% yield due to formation of the polar byproducts.

This unsatisfactory result led us to search for a more practical synthetic route to the compound **6**. To this end, we investigated a new route involving glycosylation of a 2-azidogalactosyl serine derivative with a sialyl galactose unit, since compound **7** related to the latter had previously been synthesized through a smooth desulfurization of the corresponding 3-phenylthio derivative followed by lactonization [4]. Oxidative cleavage of 4-methoxyphenyl group from **7** with CAN (ceric ammonium nitrate) afforded an anomeric mixture of hemiacetal **8** (77%), which was converted into the corresponding trichloroacetimidate **9** by treatment with trichloroacetonitrile and DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in 1,2-dichloroethane. The imidate **9** was thus obtained as an α:β mixture (α:β = 3:1) in 97% yield. On the other hand, compound **10** [13] and its sterically less-hindered analogue **11** were chosen as the glycosyl acceptors, the latter being prepared in two steps (1. debenzylidenation, 2. regioselective silylation, 88%) from **10**. Glycosylation of **11** (3 equiv) with **9** (α:β = 3:1) was promoted by $BF_3{\cdot}OEt_2$ (0.3 equiv) at −15°–5 °C in 2:1 toluene–CH_2Cl_2 to give the β-glycoside **12** (53%) and the α-isomer **13** (16%). A glycosyl fluoride **14** (α:β = 1:1) was also produced in 10% yield. The 1:3 α:β-isomeric ratio of the glycosylation products seemed to suggest that the glycosylation might proceed via inversion of the stereoisomeric trichloroacetimidate [14]. However, the α-isomer **13** (7%) was formed (α:β = 1:8), even when the same reaction was carried out with the pure α-trichloroacetimidate **9**. The use of TMSOTf as an alternative promoter for the above reaction was also examined in toluene–CH_2Cl_2 or in CH_3CN, but no coupling product was produced. The compound **12** was desilylated with 80% aq CF_3CO_2H (**15**, 88%) and benzylidenated with 1,1-dimethoxytoluene and *p*-TsOH in acetonitrile to give **16** (95%).

In contrast, the reaction of α-**9** and **10** (3 equiv) predominated the formation of α-glycoside **18** (α:β = 4.6:1) probably due to a mismatch of the substrate pair for β-glycosidation [15].



Scheme 1.

Treatment of **16** with thioacetic acid–pyridine (**17**, 77%) and Pd(0)-catalyzed cleavage of the allyl ester afforded the desired compound **6** (91%).

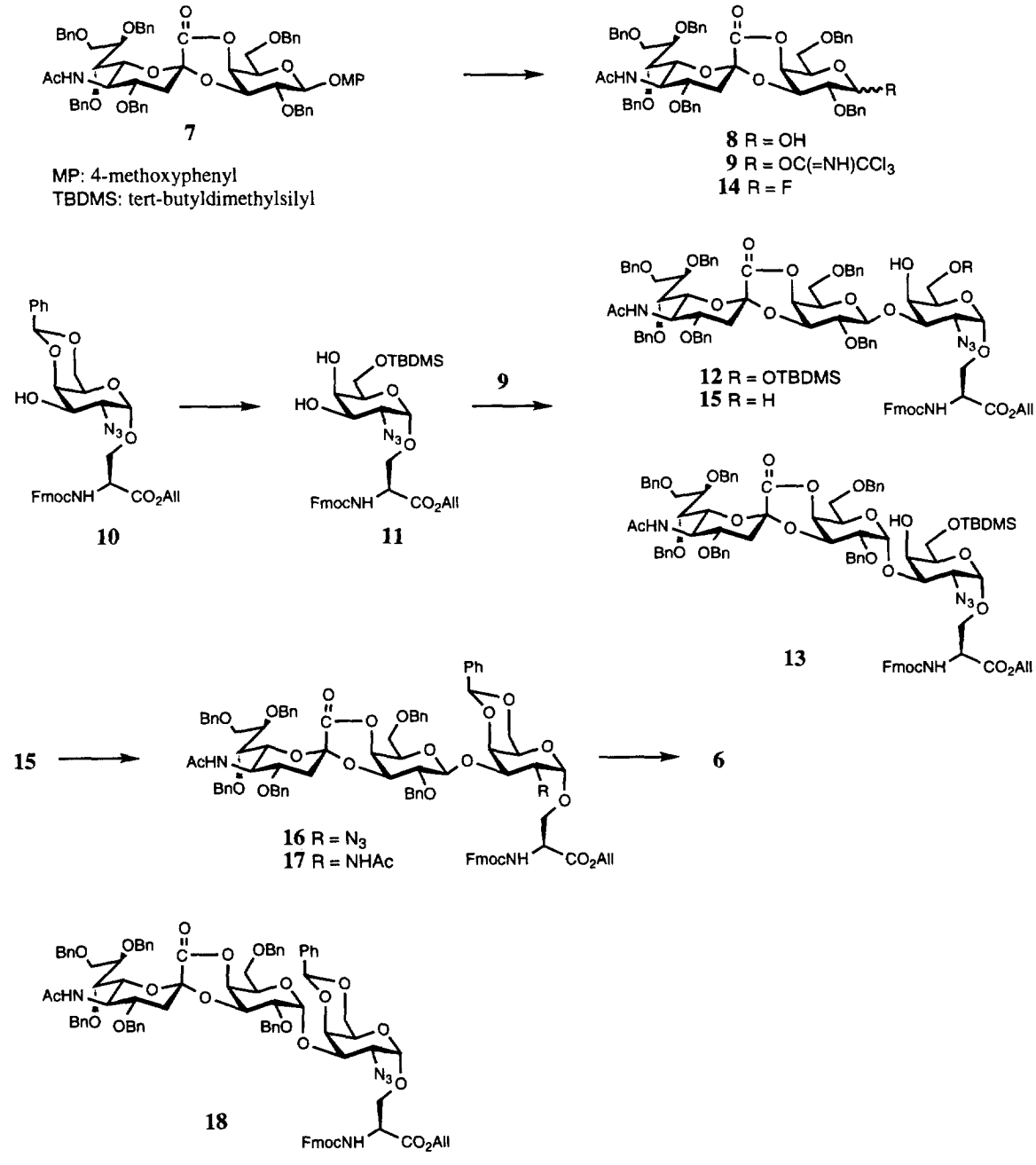
Solid-phase synthesis of the glycopeptide based on the strategy employed in a previous study was performed [7]. According to the ready-made protocol, an Fmoc protected henicosapeptide (residues 7–27) was synthesized on HMP resin (4-hydroxymethylphenoxymethyl-copolystylene-1% divinylbenzene) with an automated peptide synthesizer. In the program the Fmoc-amino acids were activated with DCC (dicyclohexylcarbodiimide)–HOBt (hydroxybenztriazole) in NMP (*N*-methylpyrrolidone) before condensation, while N-terminal Fmoc group was removed with piperidine in NMP. The side-chain functional groups of the amino acids were masked with Trt (triphenylmethyl) groups for cysteine, glutamine, and histidine, Boc (*tert*-butoxycarbonyl) group for lysine, and Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl) group for arginine. These protecting groups were concomitantly removed at the stage of TFA treatment.

The henicosapeptide-linked resin was obtained after twenty condensations. The efficiency of condensation in each step was monitored by ninhydrin test, and the overall yield thus estimated was 88%.

In a model study, we employed a mechanical shaker only at the coupling step with the disaccharide–serine, to facilitate the recovery of unreacted glycoserine [7]. However, the glycoserine unit-deleting hexacosapeptide was eventually formed in a substantial quantity (45%), indicating that the coupling was incomplete even after 64 h of shaking. The unreacted glycoserine was recovered from the reaction mixture in a reasonable amount. Based on this result, we were convinced that more efficient mixing of the resin with the activated glycoserine would be necessary to increase the coupling yield.

In this study, we utilized a vortexing tube-mixer which provided more vigorous mixing. The peptide-resin (14.9 μmol) was treated with piperidine to remove the Fmoc group and reacted with the activated trisaccharide–serine (**6**, 38.3 μmol) in NMP using the mixer. The reaction was run for 24 h, the resin was then washed and transfered to the automated peptide synthesizer to complete the peptide chain with the five N-terminal amino acid residues. The resulting glycopeptide was cleaved from the resin with a solution of aq TFA containing alkyl cation scavengers. Reversed-phase HPLC showed that the glycopeptide thus obtained consisted of three major products (in 14, 17, and 52%). MALDI-TOF mass spectra indicated the third product to be the glycopeptide with expected molecular weight ($(M+1)^+$, 3920), whereas the other two were its mono-debenzylated congeners ($(M+1)^+$, 3830). In the minor fractions were also detected di-debenzylated glycopeptides (5%).

In contrast to the previous experiment [7] little of the non-glycosylated peptide (hexacosapeptide)



Scheme 2.

was produced. The combined benzylated glycopeptides were treated with 1 M TMSOTf/TFA [16] in the presence of thioanisole at 0 °C for 1.5 h and then with aq ammonium fluoride to realize the complete deprotection, although hydrogenolytic deprotection was unsuccessful because of simultaneous desulfurization of the cysteine residue. The debenzylated products were separated as two fractions (58:42) by gel-permeation chromatography. The first fraction consisted of the dimeric product with the molecular ion of 6760 ($[M+1]^+$, calcd 6756). The second fraction was the monomer (3363 $[M+1]^+$, calcd 3380). Those fractions were further purified by reversed-phase HPLC. The NMR spectrum of the dimeric product exhibited, not only the characteristic lower field (δ 5.30 ppm) signal for the 4-O-acylated (lactonized) Gal H-4, but also the two doublet-of-doublet signals for the equatorial protons of NeuAc H-3 at δ 2.58 and 2.75 ppm (ratio about 1:1). Therefore, it was concluded that 50% of the molecule kept the lactonic linkage, but the other half had been hydrolyzed

during a series of deprotective procedures. On the other hand, the monomeric fraction had no such signals characteristic for the lactone structure, and was the target compound **1**. The dimer was treated with $NaHCO_3$ in D_2O (pH 7.5) for 3 days, then with dithiothreitol overnight, and chromatographed (gel-permeation) to afford the compound **1** (85% yield for deprotection). The structure was established by ^1H NMR and ESI mass spectral data (see Experimental section). It is noteworthy that the glycopeptide **1** is readily oxidized to form the dimer in the absence of antioxidizing agent.

In conclusion, total synthesis of B-chain of α2HS-glycoprotein was achieved by solid-phase synthesis. TFA-based conditions for cleavage of the synthesized glycopeptide from the resin was accompanied by partial debenzylation (~30%). Full deprotection was successfully performed with TMSOTf–thioanisole in TFA. The lactone hydrolysis and reduction with dithiothreitol afforded the target compound **1** in good yield.

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 (using 2,5-dihydroxybenzoic acid as matrix). ESI mass spectra were measured with a Finnigan MAT TSQ 700. 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.

N-*(9-Fluorenylmethoxycarbonyl)*-O-{*[benzyl (5-acetamido-4,7,8,9-tetra*-O-*benzyl-5-deoxy-3*-S-*phenyl-3-thio*-D-erythro-α-L-gluco-*2-nonulopyranosyl)onate]-(2→3)-(2,6-di*-O-*benzyl*-β-D-*galactopyranosyl)-(1→3)-2-azido-4,6*-O-*benzylidene-2-deoxy*-α-D-*galactopyranosyl*}-L-*serine allyl ester* (**3**). A mixture of **2** (107 mg, 61 μmol), 1,1-dimethoxytoluene (100 mL) and *p*-TsOH (cat.) in dry CH_3CN (5 mL) was stirred at room temperature for 1 h. The reaction was quenched with aq $NaHCO_3$, 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 7:3 toluene–EtOAc to give **3** (95 mg, 85%). R_f 0.47 (7:3 toluene–EtOAc); $[\alpha]_D$ +69.9° (*c* 1.0); ^1H NMR (270 MHz): δ 7.7–7.1 (m, 53 H, Ar), 6.01 (d, 1 H, *J* 8.2 Hz, NH), 5.88 (m, 1 H, -C*H*=CH_2), 5.38 [s, 1 H, PhC*H*$(O)_2$], 5.31 (brd, 1 H, *J* 17.0 Hz, =CH_2), 5.23 (brd, 1H, *J* 10.4 Hz, =CH_2), 5.19 and 5.01 (2d, 2 H, *J* 12.2 Hz, -CO_2CH_2Ph), 4.99 (d, 1 H, *J* 3.1 Hz, H-1a), 4.85 (d, 1 H, *J* 11.3 Hz, PhCH_2), 3.35 (d, 1 H, *J* 8.9 Hz, H-3c), 1.57 (s, 3 H, Ac); Anal. Calcd for $C_{106}H_{107}N_5O_{22}S{\cdot}H_2O$: C, 68.70; H, 5.93; N, 3.78. Found: C, 68.50; H, 5.83; N, 3.51.

N-*(9-Fluorenylmethoxycarbonyl)*-O-{*[benzyl (5-acetamido-4,7,8,9-tetra*-O-*benzyl-5-deoxy-3*-S-*phenyl-3-thio*-D-erythro-α-L-gluco-*2-nonulopyranosyl)onate]-(2→3)-(2,6-di*-O-*benzyl*-β-D-*galactopyranosyl)-(1→3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy*-α-D-*galactopyranosyl*}-L-*serine allyl ester* (**4**). To a solution of **3** (84 mg, 46 μmol) in pyridine (1.2 mL) was added freshly distilled AcSH (2.4 mL). The mixture was stirred at room temperature for 1 day, then concentrated in vacuo, and the residue was chromatographed on silica gel with 3:2 toluene–EtOAc to give **4** (72 mg, 85%). R_f 0.40 (1:1 toluene–EtOAc); $[\alpha]_D$ +57.9° (*c* 1.0); ^1H NMR (270 MHz): δ 7.7–7.1 (m, 53 H, Ar), 6.07 (d, 1 H, *J* 8.3 Hz, NH), 5.86 (m, 1 H, -C*H*=CH_2), 5.57 (d, 1 H, *J* 6.9 Hz, NH), 5.38 [s, 1 H, PhC*H*$(O)_2$], 5.29 (brd, 1 H, *J* 18.2 Hz, =CH_2), 5.23 (brd, ^1H, *J* 10.6 Hz, =CH_2), 5.15 (brs, 1 H, H-1a), 5.12 and 5.00 (2d, 2 H, *J* 11.9 Hz, -CO_2CH_2Ph), 3.39 (d, 1 H, *J* 8.3 Hz, H-3c), 1.59 and 1.37 (2s, 6 H, 2 Ac); Anal. Calcd for $C_{108}H_{111}N_3O_{23}S$: C, 70.08; H, 6.04; N, 2.27. Found: C, 69.70; H, 6.05; N, 2.17.

N-*(9-Fluorenylmethoxycarbonyl)*-O-{*[benzyl (5-acetamido-4,7,8,9-tetra*-O-*benzyl-5-deoxy-3*-S-*phenyl-3-thio*-D-erythro-α-L-gluco-*2-nonulopyranosyl)onate]-(2→3)-(2,6-di*-O-*benzyl*-β-D-*galactopyranosyl)-(1→3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy*-α-D-*galactopyranosyl*}-L-*serine* (**5**). A mixture of **4** (67 mg, 36 μmol), $Pd(PPh_3)_4$ (22 mg, 19 μmol), and *N*-methylaniline (220 μL, 2 mmol) in dry THF (1.5 mL) was stirred under Ar at room temperature for 1 day and concentrated in vacuo. The residue was extracted with EtOAc, washed with 0.1 N aq

HCl at pH 3, water, and brine, dried (Na_2SO_4), and concentrated in vacuo. The crude product was chromatographed on silica gel with 96.5:3:0.5 $CHCl_3$–EtOH–AcOH to give **5** (62 mg, 95%). R_f 0.26 (92.5:7:0.5 $CHCl_3$–EtOH–AcOH); $[\alpha]_D$ +55.3° (*c* 0.8); ^{1}H NMR (270 MHz): δ 7.7–7.1 (m, 53 H, Ar), 6.07 (br, 2 H, 2 NH), 5.30 [s, 1 H, PhC*H*(O)$_2$], 5.13 (brs, 1 H, H-1a), 5.13 and 5.00 (2d, 2 H, *J* 11.9 Hz, -CO_2CH_2Ph), 3.39 (d, 1 H, *J* 7.9 Hz, H-3c), 1.58 and 1.45 (2s, 6 H, 2 Ac); Anal. Calcd for $C_{105}H_{107}N_3O_{23}S$: C, 69.64; H, 5.96; N, 2.32. Found: C, 69.58; H, 5.92; N, 2.06.

5-Acetamido-4,7,8,9-tetra-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid-(2→3)-2,6-di*-O-*benzyl*-D-*galactopyranose-(1b→4a)-lactone* (**8**). A mixture of **7** (235 mg, 0.21 mmol) and CAN (1.2 g, 2.19 mmol) in 3:4:3 toluene–CH_3CN–H_2O (54 mL) was stirred at room temperature for 2.5 h. The organic layer was separated, the aqueous layer was diluted with water and extracted with EtOAc, the organic layer and the extract were combined, washed successively with water, dil aq $NaHCO_3$, and brine, dried (Na_2SO_4), and concentrated in vacuo. Chromatography of the crude product on silica gel with 4:1–3:2 toluene–EtOAc gave **8** (163 mg, 77%) as an anomeric mixture. R_f 0.28 and 0.26 (7:3 toluene–EtOAc); ^{1}H NMR (270 MHz): δ 7.4–7.1 (m, 30 H, Ar), 5.26 [d, 0.6 H, *J* 4.0 Hz, H-1a (α)], 5.16 (d, 1 H, *J* 3.6 Hz, H-4a), 2.35 [dd, 0.6 H, *J* 5.3, 13.5 Hz, H-3b eq (α)], 2.26 [dd, 0.4 H, *J* 5.0, 13.2 Hz, H-3b eq (β)], 1.80 (m, 1 H, H-3b ax), 1.74 (α) and 1.72 (β) (2s, 3 H, Ac); Anal. Calcd for $C_{59}H_{63}NO_{13}\cdot 0.5H_2O$: C, 70.64; H, 6.43; N, 1.40. Found: C, 70.82; H, 6.34; N, 1.33.

5-Acetamido-4,7,8,9-tetra-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid-(2→3)-2,6-di*-O-*benzyl*-α- *and* β-D-*galactopyranosyl trichloroacetimidate-(1b→4a)-lactone* (**9**). To a stirred mixture of **8** (160 mg, 0.16 mmol) and DBU (2.8 μL, 18.7 μmol) in dry 1,2-dichloroethane (2.8 mL), was added CCl_3CN (170 μL, 1.7 mmol) at 0 °C. After stirring for 2 h, the mixture was chromatographed on silica gel with 9:1–17:3 toluene–EtOAc to give **9** as three fractions of pure α-imidate, α:β-mixture and β-imidate (100, 60, and 17 mg, respectively, 97%). α-**9**; R_f 0.47 (7:3 toluene–EtOAc); $[\alpha]_D$ +44.6° (*c* 1.1); ^{1}H NMR (270 MHz): δ 8.61 (s, 1 H, =NH), 7.4–7.1 (m, 30 H, Ar), 6.46 (d, 1 H, *J* 3.6 Hz, H-1a), 5.41 (d, 1 H, *J* 3.0 Hz, H-4a), 2.34 (dd, 1 H, *J* 4.9, 13.5 Hz, H-3b eq), 1.84 (dd, 1 H, *J* 10.5, 13.5 Hz, H-3b ax), 1.75 (s, 3 H, Ac). β-**9**; R_f 0.43 (7:3 toluene–EtOAc); $[\alpha]_D$ +23.5° (*c* 1.2); ^{1}H NMR (270 MHz): δ 8.70 (s, 1 H, =NH), 7.4–7.1 (m, 30 H, Ar), 5.59 (d, 1 H, *J* 7.6 Hz, H-1a), 5.28 (d, 1 H, *J* 4.0 Hz, H-4a), 2.27 (dd, 1 H, *J* 5.3, 13.5 Hz, H-3b eq), 1.74 (s, 3 H, Ac). Anal. (α:β-imidate) Calcd for $C_{61}H_{63}Cl_3N_2O_{13}$: C, 64.35; H, 5.58; N, 2.46. Found: C, 64.10; H, 5.63; N, 2.41.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*(2-azido-6*-O-tert-*butyldimethylsilyl-2-deoxy*-α-D-*galactopyranosyl)*-L-*serine allyl ester* (**11**). A mixture of **10** (265 mg, 0.41 mmol), 80% aq CF_3CO_2H (3.5 mL), and CH_2Cl_2 (1 mL) was stirred at 0 °C for 3 h, diluted with water and toluene, and concentrated in vacuo. The residue was extracted with EtOAc, washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. The crude product was purified on a short column of silica gel with 19:1 $CHCl_3$–MeOH to afford a triol (222 mg) that was dissolved in dry DMF (3.8 mL) and stirred with *tert*-$BuMe_2SiCl$ (78 mg, 0.52 mmol) and imidazole (70 mg, 1.04 mmol) at room temperature for 1 h. The reaction was quenched with water 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 product was chromatographed on silica gel with 13:7 toulene EtOAc to give **11** (244 mg, 88%). R_f 0.53 (1:1 toluene–EtOAc); $[\alpha]_D$ +74.3° (*c* 1.9); ^{1}H NMR (270 MHz): δ 7.76 (d, 2 H, Ar), 7.58 (m, 2 H, Ar), 7.40 (t, 2 H, *J* 7.3 Hz, Ar), 7.31 (t, 2 H, *J* 7.3 Hz, Ar), 5.93 (m, 1 H, -C*H*=CH_2), 5.89 (d, 1 H, *J* 7.9 Hz, NH), 5.35 (brd, 1 H, *J* 17.2 Hz, =CH_2), 5.27 (brd, 1 H, *J* 10.6 Hz, =CH_2), 4.91 (d, 1 H, *J* 3.3 Hz, H-1), 3.53 (dd, 1 H, *J* 3.3, 10.2 Hz, H-2), 0.87 (s, 9 H, t-Bu) , 0.06 (s, 6 H, 2 Me). Combustion analysis of **11** did not give the correct CHN values because of decomposition at 80 °C.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[(5-acetamido-4,7,8,9-tetra*-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(2,6-di*-O-*benzyl*-β- *and* α-D-*galactopyranosyl)-(1→3)-2-azido-6*-O-tert-*butyldimethylsilyl-2-deoxy*-α-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*serine allyl ester* (**12**) *and its a anomer* (**13**). A mixture of **9** (α:β = 3:1, 47 mg, 41.3 μmol), **11** (92 mg, 137.6 μmol, 3.3 eq), and dried molecular sieves (AW 400 powder, 0.7 g) in dry 2:1 toluene–CH_2Cl_2 (3 mL) was stirred under Ar at room temperature for 1 h, and then cooled on an ice–MeOH bath. To the mixture was added 0.8 M $BF_3\cdot OEt_2$–CH_2Cl_2 (17 μL,

13.6 μmol) at −15 °C, and stirring was continued at −15 to −5 °C for 1.5 h. The mixture was diluted with EtOAc, and filtered through Celite. The filtrate was washed with dil aq $NaHCO_3$, water, and brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was chromatographed on Bio-beads S×1 with 1:1 toluene–EtOAc to afford a fraction (55 mg) containing **12** and **13**. Further elution gave a fraction (82 mg) containing unreacted **11** and **14**. The former fraction was purified by preparative TLC developed 6 times with 3:2 hexane EtOAc to give **13** (11 mg, 16%) and **12** (36 mg, 53%). From the latter, fluoride **14** (α:β = 1:1, 4 mg, 10%) and **11** (72 mg, 78% recovery) were obtained. Compound **12**: R_f 0.43 (7:3 toluene–EtOAc); $[\alpha]_D$ +58.7° (*c* 2.2); ^{1}H NMR (270 MHz): δ 7.75 (d, 2 H, *J* 7.3 Hz, Ar), 7.61 (d, 2 H, *J* 7.3 Hz, Ar), 7.4–7.1 (m, 34 H, Ar), 5.93 (m, 1 H, -C*H*=CH_2), 5.83 (d, 1 H, *J* 8.6 Hz, NH), 5.34 (brd, 1 H, *J* 17.2 Hz, =CH_2), 5.26 (brd, 1 H, *J* 10.2 Hz, =CH_2), 5.21 (d, 1 H, *J* 4.0 Hz, H-4b), 4.95 (d, 1 H, *J* 3.3 Hz, H-1a), 2.18 (dd, 1 H, *J* 4.6, 11.9 Hz, H-3c eq), 1.71 (s, 3 H, Ac), 0.85 (s, 9 H, t-Bu), 0.03 and 0.02 (2 s, 6 H, 2 Me); ^{13}C NMR (68 MHz): δ 103.0 (C-1b), 99.4 (C-1a), 95.3 (C-2c); Anal. Calcd for $C_{92}H_{105}N_5O_{21}Si\cdot H_2O$: C, 66.48; H, 6.43. Found: C, 66.52; H, 6.32.

Compound **13**: R_f 0.45 (7:3 toluene–EtOAc); ^{1}H NMR (270 MHz): δ 7.75 (d, 2 H, *J* 7.3 Hz, Ar), 7.59 (d, 2 H, *J* 7.3 Hz, Ar), 7.4–7.1 (m, 34 H, Ar), 5.90 (m, 1 H, -C*H*=CH_2), 5.80 (d, 1 H, *J* 8.6 Hz, NH), 5.36 (d, 1 H, *J* 4.0 Hz, H-4b), 5.32 (brd, 1 H, *J* 17.2 Hz, =CH_2), 5.23 (brd, 1 H, *J* 10.2 Hz, =CH_2), 4.92 and 4.91 (2 d, 2 H, *J* 3.0 Hz, H-1a and H-1b), 2.38 (dd, 1 H, *J* 4.6, 13.2 Hz, H-3c eq), 1.85 (dd, 1 H, *J* 10.2, 13.0 Hz, H-3c ax), 1.78 (s, 3 H, Ac), 0.88 (s, 9 H, t-Bu), 0.05 and 0.04 (2 s, 6 H, 2 Me).Compound **14**: R_f 0.22 (4:1 toluene–EtOAc); ^{1}H NMR (270 MHz): δ 7.4–7.1 (m, 30 H, Ar), 5.50 [dd, 0.5 H, *J* 2.3, 52.5 Hz, H-1a (α)], 5.37 [d, 0.5 H, *J* 3.3 Hz, H-4a (α)], 5.23 [br, 0.5 H, H-4a (β)], 4.96 [dd, 0.5 H, *J* 6.9, 52.1 Hz, H-1a (β)], 2.37 [dd, 0.5 H, *J* 4.6, 12.9 Hz, H-3b eq (α)], 2.32 [dd, 0.5 H, *J* 4.4, 12.5 Hz, H-3b eq (β)], 1.81 [m, 1 H, H-3b ax (α and β)], 1.70 and 1.69 (2 s, 3 H, Ac).

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[(5-acetamido-4,7,8,9-tetra*-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(2,6-di*-O-*benzyl*-β-D-*galactopyranosyl)-(1→3)-2-azido-2-deoxy*-α-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*serine allyl ester* (**15**). To an ice-cooled soln of **12** (29 mg, 17.6 mmol) in CH_2Cl_2 (0.5 mL) was added 80% aq CF_3CO_2H (0.5 mL). The mixture was stirred at 0 °C for 45 min, then diluted with water, neutralized with $NaHCO_3$, and extracted with EtOAc. The extract was washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. Chromatography of the residue on silica gel with 3:2 toluene–EtOAc gave **15** (24 mg, 88%). R_f 0.18 (7:3 toluene–EtOAc); $[\alpha]_D$ +72.6° (c 1.0); ^{1}H NMR (270 MHz): δ 7.76 (d, 2 H, *J* 7.5 Hz, Ar), 7.61 (d, 2 H, *J* 6.9 Hz, Ar), 7.4–7.1 (m, 34 H, Ar), 6.06 (d, 1 H, *J* 8.3 Hz, NH), 5.92 (m, 1 H, -C*H*=CH_2), 5.34 (brd, 1 H, *J* 16.8 Hz, =CH_2), 5.26 (brd, 1 H, *J* 10.2 Hz, =CH_2), 5.19 (d, 1 H, *J* 4.0 Hz, H-4b), 4.94 (d, 1 H, *J* 3.3 Hz, H-1a), 2.19 (dd, 1 H, *J* 4.6, 13.5 Hz, H-3c eq), 1.68 (s, 3 H, Ac). Combustion analysis of azide **15** did not give the correct CHN values because of partial decomposition on vacuum drying at 90 °C.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[(5-acetamido-4,7,8,9-tetra*-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(2,6-di*-O-*benzyl*-β-D-*galactopyranosyl)-(1→3)-2-azido-4,6*-O-*benzylidene-2-deoxy*-α-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*serine allyl ester* (**16**). A mixture of **15** (65 mg, 42.5 μmol), α,α-dimethoxytoluene (55 μL, 0.37 mmol), and *p*-TsOH (cat.) in CH_3CN (2.5 mL) was stirred at room temperature for 0.5 h, and the reaction was quenched with a few drops of pyridine before concentration in vacuo. The residue was chromatographed on silica gel with 4:1 toluene–EtOAc to give **16** (65 mg, 95%). R_f 0.42 (7:3 toluene–EtOAc); $[\alpha]_D$ +91.4° (c 1.0); ^{1}H NMR (270 MHz): δ 7.76 (d, 2 H, *J* 7.2 Hz, Ar), 7.59 (d, 2 H, *J* 7.6 Hz, Ar), 7.53 (brd, 2 H, *J* 7.3 Hz, Ar), 7.4–7.1 (m, 37 H, Ar), 6.01 (d, 1 H, *J* 8.2 Hz, NH), 5.90 (m, 1 H, -C*H*=CH_2), 5.44 [s, 1 H, PhC*H*$(O)_2$], 5.35 (brd, 1 H, *J* 17.2 Hz, =CH_2), 5.27 (brd, 1 H, *J* 10.6 Hz, =CH_2), 5.17 (d, 1 H, *J* 4.0 Hz, H-4b), 5.04 (d, 1 H, *J* 3.3 Hz, H-1a), 2.14 (brd, 1 H, *J* 12.2 Hz, H-3c eq), 1.69 (s, 3 H, Ac); Anal. Calcd for $C_{93}H_{95}N_5O_{21}$: C, 69.00; H, 5.92; N, 4.33. Found: C, 69.33; H, 5.97; N, 4.28.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[(5-Acetamido-4,7,8,9-tetra*-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(2,6-di*-O-*benzyl*-β-D-*galactopyranosyl)-(1→3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy*-α-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*serine allyl ester* (**17**). Compound **16** (62 mg, 38.3 μmol) was treated with AcSH in pyridine as described for the synthesis of **4**. The crude product was chromatographed on silica gel with 1:1 toluene–EtOAc to give **17** (48 mg, 77%). R_f 0.23 (1:1 toluene–EtOAc);

$[\alpha]_D$ +78.2° (*c* 1.3); ^{1}H NMR (270 MHz): δ 7.76 (d, 2 H, *J* 7.6 Hz, Ar), 7.56 (m, 4 H, Ar), 7.4–7.1 (m, 37 H, Ar), 5.88 (m, 2 H, NH and -C*H*=CH_2), 5.65 (d, 1 H, NH), 5.45 [s, 1 H, PhC*H*(O)$_2$], 5.33 (brd, 1 H, *J* 17.3 Hz, =CH_2), 5.28 (dd, 1 H, *J* 1.0, 10.2 Hz, =CH_2), 5.16 (d, 1 H, *J* 4.0 Hz, H-4b), 5.00 (brs, 1 H, H-1a), 2.13 (dd, 1 H, *J* 5.0, 12.9 Hz, H-3c eq), 1.77 and 1.71 (2 s, 6 H, 2 Ac); Anal. Calcd for $C_{95}H_{99}N_3O_{22}$·0.5H_2O: C, 69.41; H, 6.13; N, 2.56. Found: C, 69.38; H, 6.07; N, 2.48.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[(5-acetamido-4,7,8,9-tetra*-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(2,6-di*-O-*benzyl*-α-D-*galactopyranosyl)-(1→3)-2-azido-4,6*-O-*benzylidene-2-deoxy*-α-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*serine allyl ester* (**18**). (a) Glycosylation of **10** with **9**: Reaction of **10** (43 mg, 66.9 μmol) and **9** (α-isomer, 25 mg, 22 μmol) was performed in a similar manner as described for **12**. The crude product was chromatographed on Bio-beads S×1 and then on silica gel to give **18** (24 mg, 68%) as an α:β mixture (α:β = 4.6 : 1).

Conversion of **13**.—According to the procedure described for the synthesis of **16**, compound **13** (10 mg, 6.1 μmol) was desilylated and benzylidenated to afford **18** (8 mg, 86%). R_f 0.38 (7:3 toluene–EtOAc); $[\alpha]_D$ +84.9° (*c* 0.5); ^{1}H NMR (270 MHz): δ 7.76 (d, 2 H, *J* 7.3 Hz, Ar), 7.56 (d, 2 H, *J* 7.6 Hz, Ar), 7.4–7.1 (m, 39 H, Ar), 5.88 (d, 1 H, *J* 8.2 Hz, NH), 5.86 (m, 1 H, -C*H*=CH_2), 5.34–5.20 [m, 4 H, PhC*H*(O)$_2$, =CH_2, and H-4b], 5.13 and 5.01 (2 d, 2 H, *J* 3.3 Hz, H-1a and H-1b), 2.39 (dd, 1 H, *J* 4.6, 13.5 Hz, H-3c eq), 1.87 (dd, 1 H, *J* 10.6, 13.5 Hz, H-3c ax), 1.75 (s, 3 H, Ac). Combustion analysis of azide **18** did not give the correct CHN values because of partial decomposition on vacuum drying at 90 °C.

N-*(9-Fluorenylmethoxycarbonyl)*-O-*[(5-acetamido-4,7,8,9-tetra*-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(2,6-di*-O-*benzyl*-β-D-*galactopyranosyl)-(1→3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy*-α-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*serine* (**6**). (a) Desulfurization of **5**: A mixture of **5** (60 mg, 33.1 μmol), M Ph_3SnH-benzene (1.5 mL, 1.5 mmol), and 5% α,α′-azobis(isobutyronitrile) (AIBN)–benzene (0.2 mL, 60.9 μmol) in dry benzene (1.5 mL) was heated under reflux in an atmosphere of Ar for 13 h. During this period, 5% AIBN solution (0.1 mL each) was repeatedly (every two hours) added to the mixture. After cooling, the precipitate was filtered off and washed with EtOAc. The combined filtrate and washings were concentrated in vacuo. The residue was chromatographed on silica gel with 96.5:3:0.5–92:7:1 $CHCl_3$–EtOH–AcOH to afford **6** (15 mg, 28%). (b) Deallylation of **17**: Compound **17** (72 mg, 44.0 μmol) was deallylated as described for the synthesis of **5**. The crude product was chromatographed on silica gel with 93.5:6:0.5 $CHCl_3$–EtOH–AcOH, and then on a C_{18} reversed-phase column with 95% aq CH_3CN containing AcOH (0.1%) to afford **6** (64 mg, 91%). R_f 0.33 (92.5:7:0.5 $CHCl_3$–EtOH–AcOH); $[\alpha]_D$ +91.2° (*c* 0.9); ^{1}H NMR (270 MHz): δ 7.72 (d, 2 H, *J* 7.6 Hz, Ar), 7.54 (m, 4 H, Ar), 7.4–7.1 (m, 37 H, Ar), 6.12 (brd, 1 H, *J* 6.9 Hz, NH), 5.93 (d, 1 H, *J* 7.3 Hz, NH), 5.39 [brs, 1 H, PhC*H*(O)$_2$], 5.12 (d, 1 H, *J* 3.6 Hz, H-4b), 5.04 (brs, 1 H, H-1a), 2.13 (brd, 1 H, *J* 13.0 Hz, H-3c eq), 1.82 and 1.72 (2 s, 6 H, 2 Ac); Anal. Calcd for $C_{92}H_{95}N_3O_{22}$: C, 69.29; H, 6.00; N, 2.63. Found: C, 69.58; H, 6.08; N, 2.67.

L-*Threonyl*-L-*valyl*-L-*valyl*-L-*glutaminyl*-L-*prolyl-[(5-Acetamido-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(*β-D-*galacto-pyranosyl)-(1→3)-2-acetamido-2-deoxy*-α-D-*galacto-pyranosyl]*-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-*cysteinyl*-L-*prolyl*-L-*glycyl*-L-*arginyl*-L-*isoleucyl*-L-*arginyl*-L-*histidyl*-L-*phenylalanyl*-L-*lysyl*-L-*valine* (**1**). 1. *Synthesis of the benzylated glycoheptacosapeptide.* Starting from commercial Fmoc-Val-preloaded HMP resin (357 mg, 250 μmol), an Fmoc-protected henicosapeptide-linked resin (953 mg) was obtained after twenty cycles of the standard synthesizer program of condensation with the DCC–HOBt-activated Fmoc amino acids (1 mmol each) in NMP. A cycle of the program involved a coupling step for 71 min and a deprotection step for 21 min. Efficiency of the condensation at each step was monitored by utilizing the ninhydrin test, and the overall yield of the henicosapeptide was estimated as 88.0%.

A part of this resin (68 mg, 14.9 μmol) was stirred with 20% piperidine–NMP (2 mL) for 45 min, washed with dry CH_2Cl_2, and dried to give Fmoc deprotected peptide-resin (63 mg), while **6** (61 mg, 38.3 μmol) was activated with DCC–NMP (1 M, 0.25 mL, 250 μmol) and HOBt–NMP (1 M, 0.25 mL, 250 μmol) in a polypropylene test tube using a vortexing test tube mixer at room temperature for 1 h. The resin and NMP (0.3 mL) were added to the activated **6**. Vortex mixing of the resultant suspension was continued for 24 h at

room temperature. The resin was removed by filtration, washed with NMP and dry CH_2Cl_2, and dried in vacuo (83 mg), before being transfered to the automated synthesizer. Pro, Gln, Val, Val, and Thr residues were sequentially coupled, and finally the N-terminal Fmoc group was removed according to the small-scale program (37 min for coupling and 14 min for deprotection) to afford glycoheptacosapeptide-resin (86 mg, 94%).

2. *Cleavage from the resin*: The above glycoheptacosapeptide-resin (25.9 mg, 4.2 μmol) was stirred with a mixture of TFA (0.85 mL), deionized H_2O (40 μL), thioanisole (40 μL), 1,2-ethanedithiol (40 μL), and phenol (65 mg) at room temperature for 1.5 h. The mixture was filtered and the resin was washed with 80% aq TFA (0.5 mL). The combined filtrate and washings were diluted with water (20 mL) and extracted three times with ether (20 mL). The separated aqueous layer was concentrated in vacuo below 40 °C. The residue was dissolved in 30% aq CH_3CN and the insoluble material was filtered off through a membrane filter before chromatography on a gel-permeation column (Pharmacia Biotech. Superdex peptide HR 10/30) with 30% aq CH_3CN containing 0.1% TFA as the eluent. The most mobile fractions were collected and concentrated in vacuo. The residue (15.8 mg) was then fractionated by preparative HPLC on C_{18} silica gel (Kanto Chemical Co, Mightysil RP-18, 250–10 (5 μm)) 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 1,2,4,5, and 6 were collected and concentrated in vacuo to give a mixture of desired glycopeptide, mono-, and di-debenzylated products (14.4 mg, 3.7 μmol, 88%).

3. *Deprotection*: The mixture of the benzylated glycopeptides (5.2 mg, 1.3 μmol) was stirred with TMSOTf–TFA solution (1 M, 200 μL, 200 μmol) and thioanisole (20 μL, 169 μmol) at room temperature for 1.5 h. The mixture was added dropwise into dry ether (7 mL) to precipitate glycopeptidic substances, and ethereal layer was pipetted out after centrifugation. The precipitate was washed twice with ether, dryed in vacuo, and then stirred with NH_4F (5 mg) in H_2O (0.5 mL) at room temperature. The reaction mixture was chromatographed on a gel-permeation column (Pharmacia Biotech. Hi-load 26/60 superdex 30 pg) with 30% aq CH_3CN containing 0.1% TFA to give two major fractions. The first fraction (2.9 mg) mainly consisted of the dimerized glycopeptide (TOF MS M + 1; 6760, calcd 6756), which kept lactonic structure partly (ca. 50%). The second (2.1 mg) was the monomeric glycopeptide fraction (M + 1: 3363, calcd 3379), which possessed no lactone. Both fractions were further purified on C_{18} silica gel with a gradient elution of aq CH_3CN containing 0.1% TFA (concentration of CH_3CN: 0→20 min; 16→32%, 20→30 min; 32%) to give the dimer (2.8 mg) and the monomer (**1**: 1.0 mg, 0.3 μmol), respectively. The dimer fraction was dissolved in D_2O (0.7 mL), and to the solution was added 0.2 M $NaHCO_3$–D_2O (0.15 mL, 30 μmol). The mixture (pH 7.5) was left at room temperature for 3 days, the progress of lactone hydrolysis being monitored by NMR. Then a solution of 1,4-dithiothreitol (1.2 mg) in D_2O was added to the mixture, which was allowed to stand overnight. The mixture was chromatographed on the Superdex 30 pg column in the same manner to give **1** (2.8 mg, 0.8 μmol, total yield 85%). The isolated monomer was prone to dimerize easily in the solution without antioxidant. ESIMS; *m/z* 1133.1 $[(M+3)/3]^{3+}$, ^1H NMR [600 MHz; D_2O, 25 °C (or 60 °C), *t*-BuOH (δ 1.23)]: *d* 7.62 (brs, 1 H, His), 7.31 (brt, 2 H, Phe), 7.27 (brt, 1 H, Phe), 7.19 (d, 2 H, *J* 7.3 Hz, Phe), 6.85 (brs, 1 H, His), 4.89 (d, 1 H, *J* 2.4 Hz, H-1:GalNAc, 60 °C), 4.45 (d, 1 H, *J* 7.3 Hz, H-1:Gal), 4.19 (brd, 1 H, *J* 2.4 Hz, H-4:GalNAc), 3.51 (brt, 1 H, H-2:Gal), 2.74 (dd, 1 H, *J* 4.3, 12.2 Hz, H-3eq: NeuAc), 2.02 and 1.99 (2s, 6 H, 2Ac:GalNAc and NeuAc), 1.77 (t, 1 H, *J* 12.2 Hz, H-3ax: NeuAc).

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