Biosci. Biotechnol. Biochem., **69** (8), 1575–1583, 2005

Solid-Phase Synthesis of Core 8 *O*-Glycan-Linked MUC5AC Glycopeptide

Makoto MAEMURA, Atsushi OHGAKI, Yuko NAKAHARA, Hironobu HOJO,† and Yoshiaki NAKAHARA†

Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University, Kitakaname 1117, Hiratsuka, Kanagawa 259-1292, Japan

Received April 1, 2005; Accepted May 16, 2005

The benzyl-protected disaccharide building blocks of core 8 *O*-glycan (15a/15b) for glycopeptide were stereoselectively synthesized by two glycosidation reactions with the glycosyl fluoride method. The building blocks were utilized in the solid-phase synthesis of a glycopeptide carrying two *O*-glycans with the consensus sequence of the tandem-repeat domain of MUC5AC. The synthetic glycopeptide was detached from the resin with reagent K, and subsequent debenzylation under conditions of low-acidity TfOH afforded glycopeptide 2. The synthetic sample will be used as a suitable standard in studies of the physicochemical or immunochemical characterization of mucin glycoforms.

Key words: core 8 *O*-glycan; glycopeptide; solid-phase synthesis; MUC5AC

O-Glycans are important types of post-translationally modified proteins. Most abundant *O*-glycans with the common structure of α-D-GalNAc-(1→3)-L-Ser/Thr are grouped into eight core classes on the basis of glycosyl substitution at the GalNAc residue.[1] Mucin glycoproteins are densely *O*-glycosylated by a variety of heterogeneous oligosaccharides with or without negatively charged sialic acid or sulfate, and are assumed to form a barrier to protect the epithelial cell surfaces from desiccation and invading pathogenic microbes. On the other hand, altered glycoforms that express mucins have been found as the result of malignant transformation, thereby being potential immunotherapeutic targets.[2,3] However, studies on the analyses of intact glycoproteins as well as on the functions of *O*-glycan have been obstructed due to difficulties in the purification of natural mucin molecules of huge molecular mass. The whole molecule of such a glycoprotein, although difficult to synthesize, can be partly mimicked by chemically synthesized pure glycopeptides which will act as useful probes in studies on the immunological and viscoelastic properties of native mucins. These samples will also be used as a standard in developing the technique for higher-ordered MS analyses of the mucin fragments.

To this end, we have been studying the solid-phase synthesis of glycopeptides especially by taking advantage of the benzyl group for consistent protection of the carbohydrate moiety. The discovery of appropriate acidic conditions for cleavage of the benzyl groups have made our syntheses of several glycopeptides carrying core 1[4–6] and core 2 *O*-glycans[7–9] successful. Our synthetic studies were then directed toward the other core class glycan displayed on the mucin peptide backbone. The rare core 8 glycan [α-D-Gal-(1→3)-D-GalNAc-] was the latest characterized in a rather simple trisaccharide form (**1**) amongst the complex oligosaccharides isolated from human respiratory mucin collected from a patient suffering from chronic bronchitis.[10] There has been a report on the synthesis of the related α-D-Gal-(1→3)-D-GalNAc analog with a lipophilic tag,[11] but no extensive study on the synthesis of the core 8 *O*-glycan-linked glycopeptide has previously appeared. From the easy access to α glycoside with the fully benzylated glycosyl donor and its compatibility with our strategy in solid-phase synthesis, we decided to synthesize model glycopeptides **2** carrying duplicate *O*-glycans that will be a useful standard in elaborating a facile physicochemical or immunological method for identification of this rare glycan structure. Since the backbone amino acid sequence has not been identified for the natural core 8 glycan, we designated an octapeptide (TTSTTSAP) corresponding to the consensus sequence of MUC5AC, a tracheobronchial mucin, as a putative scaffold.[12]

The target octapeptide contains a *C*-terminal proline residue which reduces the total yield of the synthetic peptide by generating an undesired diketopiperazine in the early stage of Fmoc solid-phase synthesis. Sakamoto *et al.* have recently reported an effective procedure to prevent this diketopiperazine formation by a procedure

† To whom correspondence should be addressed. Tel: +81-463-50-2075; Fax: +81-463-50-2012; E-mail: yonak@keyaki.cc.u-tokai.ac.jp; hojo@keyaki.cc.u-tokai.ac.jp

Abbreviations: DAST, diethylaminosulfur trifluoride; DIEA, *N*,*N*-diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MS, mass spectrometry; NMP, 1-methyl-2-pyrolidinone; Pfp, pentafluorophenyl; TBAF, tetra-*n*-butylammonium fluoride; TFFH, fluoro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate; TfOH, trifluoromethanesulfonic acid; Tsoc, triisopropylsilyloxycarbonyl

that involved fluoride ion-catalyzed cleavage of *N*-triisopropylsilyloxycarbonyl group on the resin-bound dipeptide in the presence of the third Fmoc amino acid activated as its acid fluoride.[13] In order to enhance this procedure that can be applied to glycopeptide synthesis, we designed our solid-phase synthesis so as to use a glycoamino acid fluoride.

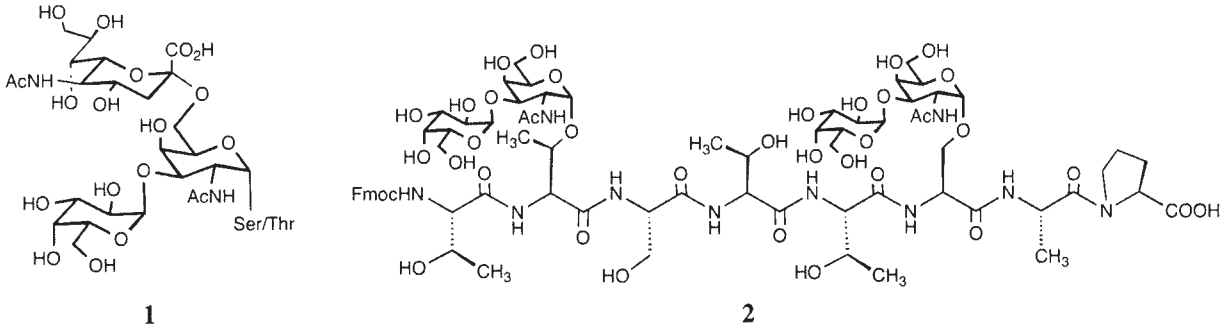


Fig. 1. Structures of Naturally Occurring Core 8 *O*-Glycan and MUC5AC Glycopeptide.

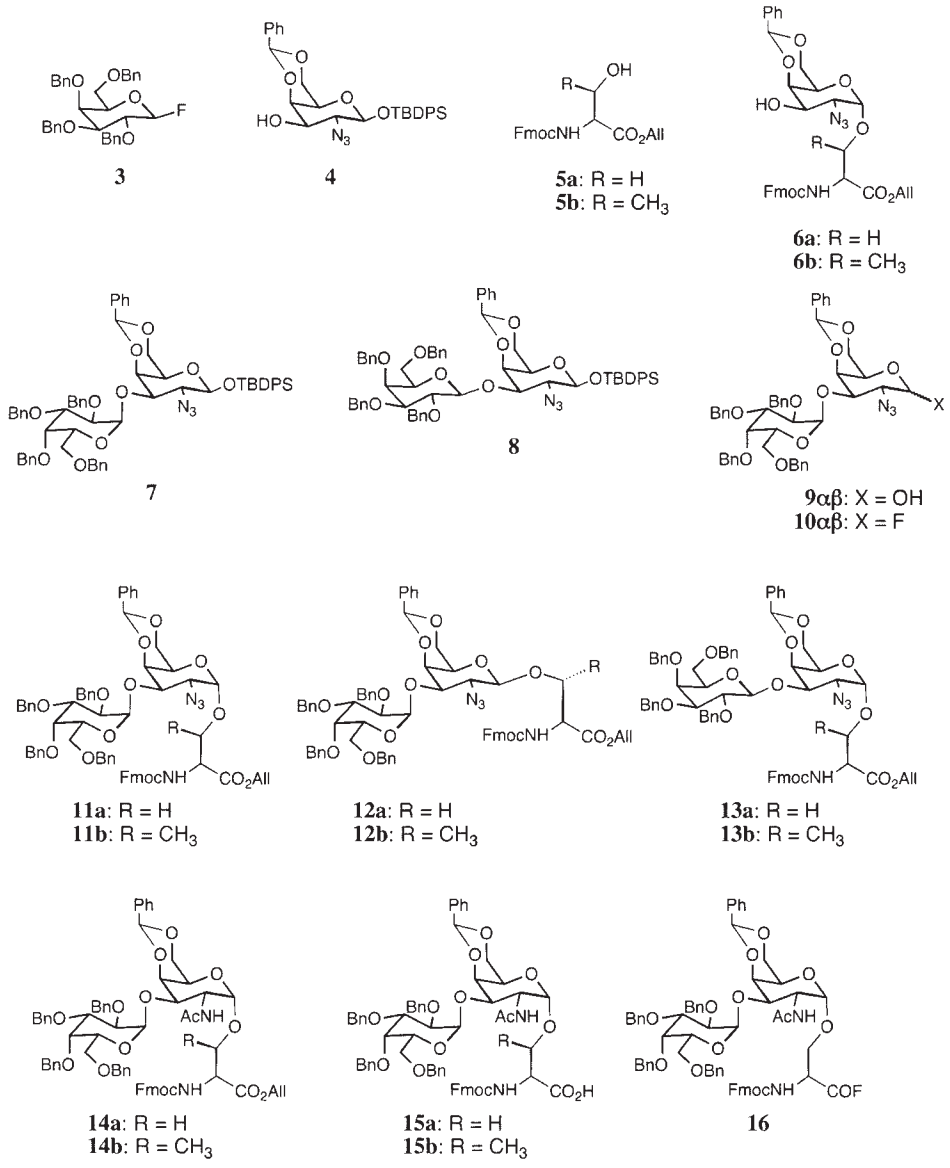
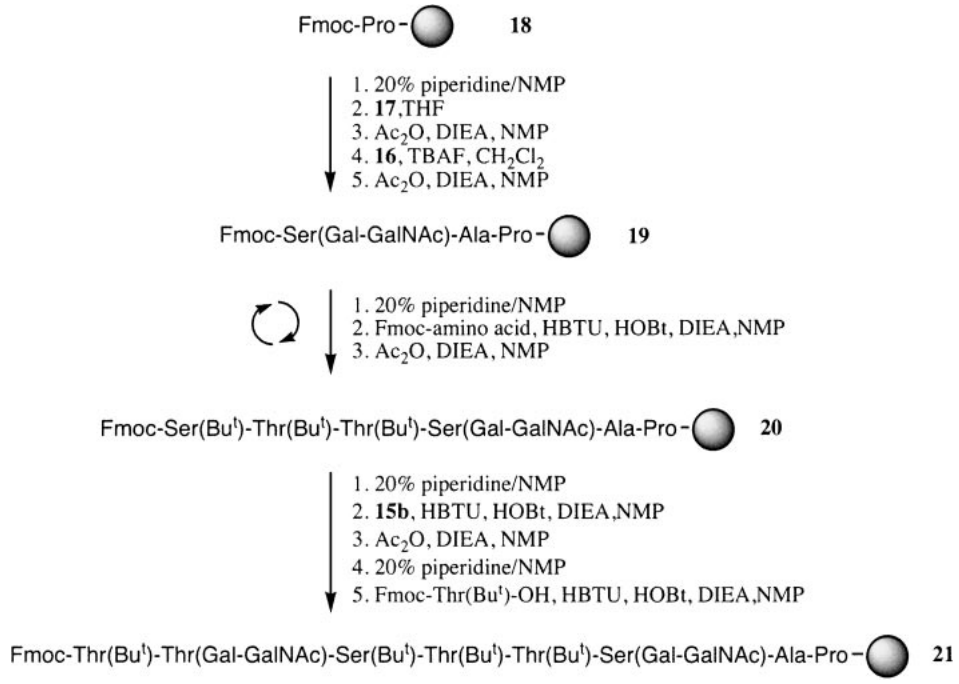


Fig. 2. Core 8 *O*-Glycan Building Blocks and Synthetic Intermediates.



Scheme 1. Solid-Phase Synthesis of the Glycopeptide.

Results and Discussion

*Synthesis of building blocks **15a** and **15b***

Synthesis of the disaccharide building blocks was carried out *via* two complementary routes, using the glycosyl fluoride method with known compounds **3**,[14] **4**,[15,16] **5a**,[17,18] **5b**,[17,18] **6a**,[19,20] and **6b**.[21,22] Glycosylation of **4** with fluoride **3** ($\alpha/\beta = 1/2$) was promoted by Cp_2ZrCl_2–$AgClO_4$[23] in CH_2Cl_2 to form an α/β mixture of disaccharides ($\alpha/\beta = 3.7/1$) in a 93% yield. Minor β-isomer **8** was identical with the known disaccharide reported in the synthesis of core 1 *O*-glycan.[24] Separated α-isomer **7** was desilylated with TBAF-AcOH in THF, and resulting hemiacetal **9** was treated with DAST in THF to afford α- and β-fluoride **10** in an 81% yield in two steps. The reaction between serine derivative **5a** and **10** proceeded under the above mentioned glycosidation conditions to furnish coupling product **11a** (62%) and its β-isomer **12a** (16%). Analogously, threonine **5b** was glycosylated with **10** to afford **11b** (52%) and isomer **12b** (17%). Disaccharyl amino acids **11a** and **11b** were alternatively obtained by glycosylation of **6a** and **6b** with **3** in 62% and 63% yields, respectively. Corresponding β-isomers **13a** and **13b**, the known core 1 derivatives,[24,25] were also generated as minor products in 10% and 8% yields, respectively. The azide compounds were susceptible to reduction with Zn–AcOH and subsequent *N*-acetylation with Ac_2O to give acetamides **14a** (88%) and **14b** (92%). Cleavage of the allyl esters with $Pd(Ph_3P)_4$-dimedone completed the preparation of building blocks **15a** and **15b**, both in a quantitative yield.

Solid-phase synthesis of the glycopeptide and deprotection

The synthesis of glycopeptide **2** was commenced with commercial Fmoc-Pro-CLEAR acid resin **18** (9 μmol) as shown in Scheme 1. Solid-phase reactions were manually operated in a polypropylene tube with vigorous stirring by a vortex mixer. The Fmoc group was removed with 20% piperidine/NMP. The second amino acid residue was condensed to the *N*-deprotected proline on the resin in THF twice with Tsoc-Ala-OPfp **17** (5 equiv.). The unreacted proline was blocked by acetylation. A solution of glycoamino acid fluoride **16** (2 equiv.), prepared from **15a** and TFFH in CH_2Cl_2, was then added to the resin, and the mixture was stirred for 12 h after adding a catalytic amount of TBAF. This coupling procedure was repeated with another 2 equiv. of **16**. After capping with Ac_2O, obtained resin **19** was *N*-deprotected and reacted with Fmoc-Thr(Bu^t)-OH (4 equiv.) by activation with HBTU, HOBt and DIEA in NMP at 50 °C for 1 h. Analogouly, the peptide was consecutively elongated with Fmoc-Thr(Bu^t)-OH and Fmoc-Ser(Bu^t)-OH to give **20**. The second *O*-glycan was introduced by *N*-deprotection of the resin, this being followed by coupling with glycothreonine **15b** (2 equiv.) under similar conditions. The condensation procedure was duplicated with another two equivalents of **15b**. Finally, the *N*-terminal threonine was attached to complete the solid-phase synthesis. The synthesized glycopeptide was released from resin **21** by a treatment with reagent K.[26] The acid-labile Bu^t groups, benzylidene groups and part of the benzyl groups were removed by this treatment. The crude product, which was precipitated from ether, was subsequently debenzylated

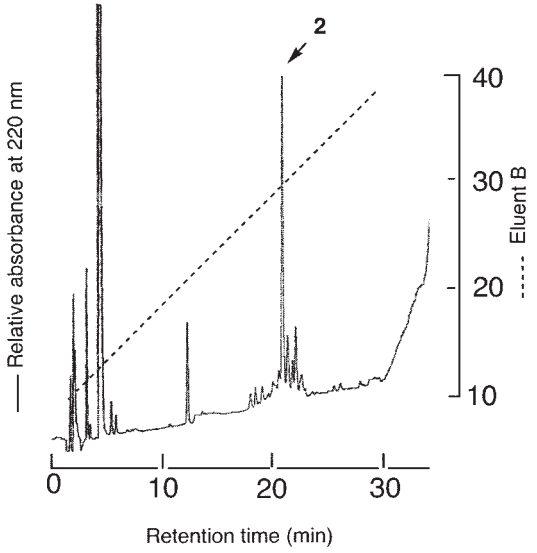


Fig. 3. HPLC Profile of the Synthesized Glycopeptide.
Column: Mightysil RP-18 (150 × 4.6 mm). eluent A: distilled water containing 0.1% TFA, B: acetonitrile containing 0.1% TFA; flow rate: 1 ml/min.

under the low-acidity TfOH conditions reported previously.[7] The resulting mixture was analyzed by reversed-phase HPLC with a C18 column. Figure 3 presents a chromatogram of the product, in which the major peak corresponds to glycopeptide **2**. The structure was identified by MALDI TOF MS of the separated sample (*m/z* 1739.73). MS analysis of the less-mobile fraction containing minor peaks indicated the presence of a by-product missing one galactose residue that had probably been generated during the debenzylation process. The overall yield of **2** was estimated to be 11% by the amino acid analysis, after an acid hydrolysis of part of the separated sample. The *N*-terminal Fmoc group, which could be removed with piperidine, was retained in synthetic sample **2** in anticipation of ready characterization by HPLC in further studies such as enzymatic glycosyltransfer.

In conclusion, core 8 *O*-linked disaccharide building blocks **15a** and **15b** were stereoselectively synthesized *via* two complementary synthetic pathways. The benzyl-protected disaccharides were successfully introduced into the MUC5AC octapeptide, a putative scaffold of core 8 glycans, by the Fmoc solid-phase synthesis with an application of Sakamoto's procedure. The benzyl-protecting groups in the synthetic glycopeptide were finally removed with minimum loss of glycosidic linkages by a combination of hard TfOH and soft nucleophiles to furnish title compound **2**. Synthesis of the glycopeptide carrying native sialoglycan **1** is currently being investigated.

Experimental

Optical rotation values were determined with a Jasco DIP-370 polarimeter at $20 \pm 2\,^{\circ}C$ for solutions in $CHCl_3$, unless noted otherwise. Column chromatography was performed on silica gel PSQ 100B (Fuji Silysia). TLC and HPTLC were performed on silica gel 60 F_{254} (E. Merck). ^1H and ^{13}C NMR spectra were recorded with a Jeol AL400 spectrometer [^1H (400 MHz) and ^{13}C (100 MHz)]. 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 PerSeptive Voyager-DE PRO spectrometer (2,5-dihydroxybenzoic acid was used as a matrix). High-resolution Fab mass spectra were measured with Jeol JMS HX-110 spectrometer (2,5-dihydroxybenzoic acid was used as a matrix). All solid-phase reactions were performed at room temperature in capped polypropylene test tubes equipped with a filter and three-way stopcock by stirring on an EYELA CM-1000 vortex mixer. HPLC was performed with Mightysil RP-8 and RP-18 (150 × 4.6 mm for analysis and 250 × 10 mm for preparation, Kanto Chemical Co.). Amino acids were analyzed by a Hitachi L-8500 amino acid analyzer. Fmoc-Pro-CLEAR-acid resin was purchased from Peptide International Inc.

tert-Butyldiphenylsilyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (7). A mixture of **4** (505 mg, 0.95 mmol), Cp_2ZrCl_2 (416 mg, 1.42 mmol), $AgClO_4$ (295 mg, 1.42 mmol), and dried 4A molecular sieves (3 g) in anhydrous CH_2Cl_2 (20 ml) was stirred at $-15\,^{\circ}C$ under Ar for 1 h. A solution of **3** (759 mg, 1.43 mmol) in anhydrous CH_2Cl_2 (20 ml) was then added to the stirred mixture. The mixture was stirred at $-15\,^{\circ}C$-room temperature for 14 h, before the reaction was quenched with aq. $NaHCO_3$. The mixture was diluted with $CHCl_3$ and filtered through Celite. The filtrate was successively washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The product was chromatographed on silica gel with toluene–EtOAc (9:1) to afford **7** (730 mg, 73%) and **8** (196 mg, 20%). Compound **7**: $[\alpha]_D = +35.9^{\circ}$ (c 1). R_f 0.41 (9:1 toluene–EtOAc). ^1H-NMR δ: 7.79–7.77 (m, 2H, Ar), 7.73–7.71 (m, 2H, Ar), 7.52–7.49 (m, 2H, Ar), 7.42–7.10 (m, 27H, Ar), 7.09–7.02 (m, 2H, Ar), 5.41 [s, 1H, PhC*H*(O–)$_2$], 5.17 (d, 1H, $J = 2.2$ Hz, H-1b), 4.93 (d, 1H, $J = 11.5$ Hz, –CH_2Ph), 4.85 (d, 1H, $J = 11.8$ Hz, –CH_2Ph), 4.71 (d, 1H, $J = 11.7$ Hz, –CH_2Ph), 4.56 (d, 1H, $J = 11.5$ Hz, –CH_2Ph), 4.54 (d, 1H, $J = 12.0$ Hz, –CH_2Ph), 4.50 (d, 1H, $J = 11.7$ Hz, –CH_2Ph), 4.43 (brs, 2H, –CH_2Ph), 4.40 (d, 1H, $J = 7.8$ Hz, H-1a), 4.12–4.06 (m, 4H, H-4a, H-2b, H-3b, H-5b), 4.02 (dd, 1H, $J = 7.8$, 10.5 Hz, H-2a), 3.92 (brs, 1H, H-4b), 3.87 (dd, 1H, $J = 1.2$, 12.2 Hz, H-6a), 3.77 (dd, 1H, $J = 1.7$, 12.2 Hz, H-6a), 3.54 (dd, 1H, $J = 6.9$, 10.0 Hz, H-6b), 3.50 (dd, 1H, $J = 3.4$, 10.5 Hz, H-3a), 3.44 (dd, 1H, $J = 5.8$, 10.0 Hz, H-6b), 2.75 (brs, 1H, H-5a), 1.13 (s, 9H, *t*-Bu). *Anal*. Found: C, 71.74; H, 6.51; N, 3.87%. Calcd. for $C_{63}H_{67}N_3O_{10}Si$: C, 71.77; H, 6.41; N, 3.99%.

*2,3,4,6-Tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-D-galactopyranose (**9**).* To a mixture of **7** (497 mg, 0.46 mmol) and AcOH (0.28 ml, 4.60 mmol) in freshly distilled THF (20 ml) was added 1 M *n*-Bu_4NF/THF (2.76 ml, 2.76 mmol). The mixture was stirred overnight at room temperature and then concentrated *in vacuo*. The residue was extracted with $CHCl_3$, successively washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was chromatographed on silica gel with toluene–EtOAc (3:2) to give **9** (351 mg, 91%). Rf 0.21 (4:1 toluene–EtOAc). ^{1}H-NMR δ: 7.49–7.46 (m, 3H, Ar), 7.48–7.20 (m, 14H, Ar), 7.19–7.05 (m, 8H, Ar), 5.49 and 5.46 [2s, 1H, PhC*H*(O–)$_2$], 5.42 and 5.28 (2d, 1H, $J = 3.2$ and 2.2 Hz, H-1b), 5.20 (brs, 0.5H, H-1aα). *Anal*. Found: C, 69.16; H, 6.07; N, 4.96%. Calcd. for $C_{47}H_{49}N_3O_{10}$: C, 69.19; H, 6.05; N, 5.15%.

*2,3,4,6-Tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-α and β-D-galactopyranosyl fluoride (**10**).* To a stirred solution of **9** (273 mg, 0.35 mmol) in freshly distilled THF (10 ml) was added Et_2NSF_3 (0.19 ml, 1.41 mmol) at 0 °C. The mixture was stirred for 40 min before the reaction was quenched with MeOH, and the mixture concentrated *in vacuo*. The residue was extracted with EtOAc, successively washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was chromatographed on silica gel with toluene–EtOAc (4:1) to give **10α** (195 mg, 66%) and **10β** (65 mg 22%). Compound **10α**: $[\alpha]_D = +68.6°$ (c 1). Rf 0.65 (4:1 toluene–EtOAc). ^{1}H-NMR δ: 7.49–7.46 (m, 2H, Ar), 7.36–7.25 (m, 19H, Ar), 7.19–7.07 (m, 4H, Ar), 5.77 (dd, 1H, $J = 2.4$, 52.7 Hz, H-1a), 5.47 [s, 1H, PhC*H*(O–)$_2$], 5.28 (d, 1H, $J = 2.3$ Hz, H-1b), 4.95 (d, 1H, $J = 11.4$ Hz, –CH_2Ph), 4.82 (d, 1H, $J = 11.3$ Hz, –CH_2Ph), 4.69 (d, 1H, $J = 11.3$ Hz, –CH_2Ph), 4.59 (brs, 2H, –CH_2Ph), 4.57 (d, 1H, $J = 11.5$ Hz, –CH_2Ph), 4.50–4.43 (m, 3H, –CH_2Ph, H-4a), 4.26 (dd, 1H, $J = 1.4$, 12.7 Hz, H-6a), 4.19 (dd, 1H, $J = 2.7$, 9.5 Hz, H-3a), 4.17–4.06 (m, 4H, H-2a, H-2b, H-3b, H-5b), 4.00–3.97 (m, 2H, H-4b, H-6a), 3.75 (brs, 1H, H-5a), 3.60 (dd, 1H, $J = 6.4$, 9.5 Hz, H-6b), 3.52 (dd, 1H, $J = 6.3$, 9.5 Hz, H-6b). *Anal*. Found: C, 69.02; H, 6.01; N, 4.60%. Calcd. for $C_{47}H_{48}FN_3O_9$: C, 69.02; H, 5.92; N, 5.14%.

Compound **10β**: $[\alpha]_D = +58.9°$ (c 1). Rf 0.45 (4:1 toluene–EtOAc). ^{1}H-NMR δ: 7.52–7.49 (m, 2H, Ar), 7.37–7.23 (m, 19H, Ar), 7.18–7.05 (m, 4H, Ar), 5.50 [s, 1H, PhC*H*(O–)$_2$], 5.21 (d, 1H, $J = 3.4$ Hz, H-1b), 4.95 (d, 1H, $J = 11.4$ Hz, –CH_2Ph), 4.91 (dd, 1H, $J = 7.6$, 52.5 Hz, H-1a), 4.84 (d, 1H, $J = 11.5$ Hz, –CH_2Ph), 4.71 (d, 1H, $J = 11.7$ Hz, –CH_2Ph), 4.58 (d, 1H, $J = 10.5$ Hz, –CH_2Ph), 4.57 (brs, 2H, –CH_2Ph), 4.46 (brs, 2H, –CH_2Ph), 4.32 (dd, 1H, $J = 1.4$, 12.6 Hz, H-6a), 4.26 (br, 1H, H-4a), 4.11–3.99 (m, 5H, H-2a, H-6a, H-2b, H-3b, H-5b), 3.94 (brd, 1H, $J = 1.5$ Hz, H-4b), 3.67 (dd, 1H, $J = 2.9$, 10.5 Hz, H-3a), 3.60 (dd, 1H, $J = 7.1$, 9.7 Hz, H-6b), 3.44 (dd, 1H, $J = 5.4$, 9.7 Hz, H-6b), 3.33 (brs, 1H, H-5a). *Anal*. Found: C, 68.42; H, 5.91; N, 5.00%. Calcd. for $C_{47}H_{48}FN_3O_9 \cdot 0.3H_2O$: C, 68.57; H, 5.95; N, 5.10%.

*N-(9-Fluorenylmethoxycarbonyl)-O-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-azido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-serine allyl ester (**11a**).*

Procedure A (*reaction of **5a** and **10***): A mixture of **5a** (45 mg, 0.12 mmol), Cp_2ZrCl_2 (36 mg, 0.12 mmol), $AgClO_4$ (26 mg, 0.12 mmol), and dried 4A molecular sieves (0.2 g) in anhydrous CH_2Cl_2 (3 ml) was stirred at −15 °C under Ar for 1 h. A solution of **10** (68 mg, 0.08 mmol) in anhydrous CH_2Cl_2 (1 ml) was then added to the stirred mixture. The mixture was stirred at −15 °C-room temperature for 16 h, before the reaction was quenched with aq. $NaHCO_3$. The mixture was diluted with $CHCl_3$ and filtered through Celite. The filtrate was successively washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The product was chromatographed on Bio-beads S × 3 with toluene and then on silica gel with toluene–EtOAc (4:1) to afford **11a** (60 mg, 62%) and **12a** (15 mg, 16%). Compound **11a**: $[\alpha]_D = +101.9°$ (c 1). Rf 0.40 (4:1 toluene–EtOAc). ^{1}H-NMR δ: 7.76–7.72 (m, 2H, Ar), 7.58–7.54 (m, 2H, Ar), 7.49–7.46 (m, 2H, Ar), 7.40–7.22 (m, 21H, Ar), 7.18–7.08 (m, 6H, Ar), 5.91 (d, 1H, $J = 7.3$ Hz, –N*H*), 5.88 (m, 1H, –C*H*=CH$_2$), 5.38 [s, 1H, PhC*H*(O–)$_2$], 5.32 (brd, $J = 17.1$ Hz, –CH=CH_2), 5.25–5.23 (m, 2H, –CH=CH_2, H-1b), 4.99 (d, 1H, $J = 3.2$ Hz, H-1a), 4.93 (d, 1H, $J = 11.2$ Hz, –CH_2Ph), 4.82 (d, 1H, $J = 12.0$ Hz, –CH_2Ph), 4.70 (d, 1H, $J = 11.7$ Hz, –CH_2Ph), 4.66 (m, 2H, –CH_2CH=CH$_2$), 4.59 (brs, 2H, –CH_2Ph), 4.55–4.45 (m, 3H, –CH_2Ph × 2, Ser-αH), 4.42 (d, 1H, $J = 12.0$ Hz, –CH_2Ph), 4.38–4.29 (m, 3H, –OCH_2C*H*Ar$_2$), 4.22–4.05 (m, 6H, Ser-βH, H-3a, H-4a, H-6a, H-2b, H-5b), 3.98–3.94 (m, 3H, H-2a, H-4b, Ser-βH), 3.86 (brd, 1H, $J = 12.2$ Hz, H-6a), 3.64–3.52 (m, 3H, H-5a, H-6b × 2). *Anal*. Found: C, 70.17; H, 5.95; N, 4.23%. Calcd. for $C_{68}H_{68}N_4O_{14}$: C, 70.09; H, 5.88; N, 4.81%.

Compound **12a**: $[\alpha]_D = +38.4°$ (c 1). Rf 0.24 (4:1 toluene–EtOAc). ^{1}H-NMR δ; 7.74 (brd, 2H, $J = 7.6$ Hz, Ar), 7.62–7.58 (m, 2H, Ar), 7.50–7.48 (m, 2H, Ar), 7.38–7.04 (m, 27H, Ar), 5.97 (d, 1H, $J = 8.3$ Hz, N*H*), 5.90 (m, 1H, –C*H*=CH$_2$), 5.49 [s, 1H, PhC*H*(O–)$_2$], 5.34 (brd, 1H, $J = 17.3$ Hz, –CH=CH_2), 5.20–5.17 (m, 2H, H-1b, –CH=CH_2), 4.94 (d, 1H, $J = 11.7$ Hz, –CH_2Ph), 4.86 (d, 1H, $J = 11.7$ Hz, –CH_2Ph), 4.73 (d, 1H, $J = 11.7$ Hz, –CH_2Ph), 4.71–4.68 (m, 2H, –CH_2CH=CH$_2$), 4.65–4.52 (m, 4H, –CH_2Ph × 3, Ser-αH), 4.45–4.20 (m, 8H, Ser-βH, –CH_2Ph × 2, –OCH_2C*H*Ar$_2$, H-4a, H-6a), 4.18 (d, 1H, $J = 8.0$ Hz, H-1a), 4.12–4.04 (m, 3H, H-2b, H-3b, H-5b), 4.00–3.92 (m, 3H, H-2a, H-6a, H-4b), 3.87 (dd, 1H, $J = 3.0$, 10.0 Hz, Ser-βH), 3.61 (dd, 1H, $J = 3.2$, 10.5 Hz, H-3a), 3.58 (dd, 1H, $J = 6.8$, 9.7 Hz, H-6b), 3.39 (dd, 1H, $J = 5.3$, 9.7 Hz, H-6b). *Anal*. Found: C, 69.94; H, 5.99;

N, 4.53%. Calcd. for $C_{68}H_{68}N_4O_{14}$: C, 70.09; H, 5.88; N, 4.81%.

*Procedure B (reaction of **3** and **6a**)*: A mixture of Cp_2ZrCl_2 (67 mg, 0.23 mmol), $AgClO_4$ (94 mg, 0.45 mmol), and dried 4A molecular sieves (0.5 g) in anhydrous CH_2Cl_2 (2 ml) was stirred at −15 °C under Ar for 1 h. A mixture of **3** (82 mg, 0.15 mmol) and **6a** (152 mg, 0.24 mmol) in anhydrous CH_2Cl_2 (1 ml) was then added to this mixture. The mixture was stirred at −15 °C-room temperature for 16 h, before the reaction was quenched with aq. $NaHCO_3$. The mixture was diluted with $CHCl_3$ and filtered through Celite. The filtrate was successively washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The product was chromatographed on Bio-beads S × 3 with toluene and then on silica gel with toluene–EtOAc (4:1) to afford **11a** (108 mg, 62%) and **13a** (17 mg, 10%). The latter was identical in all respects with the sample previously prepared in this laboratory.

*N-(9-Fluorenylmethoxycarbonyl)-O-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-azido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-threonine allyl ester (**11b**).*

*Procedure A (reaction of **5b** and **10**)*: Glycosylation of **5b** (116 mg, 0.36 mmol) with **10** (200 mg, 0.24 mmol) was conducted with Cp_2ZrCl_2 (105 mg, 0.36 mmol), $AgClO_4$ (74 mg, 0.36 mmol), and dried 4A molecular sieves (0.4 g) in CH_2Cl_2 (12 ml) as described for **11a**. The product was chromatographed on Bio-beads S × 3 with toluene and then on silica gel with toluene–EtOAc (4:1) to afford **11b** (149 mg, 52%) and **12b** (50 mg, 17%). Compound **11b**: $[\alpha]_D = +91.4°$ (c 1). Rf 0.47 (4:1 toluene–EtOAc). ^{1}H-NMR δ: 7.76–7.74 (m, 2H, Ar), 7.60–7.59 (m, 2H, Ar), 7.49–7.47 (m, 2H, Ar), 7.38–7.20 (m, 21H, Ar), 7.19–6.99 (m, 6H, Ar), 5.91 (m, 1H, –C*H*=CH$_2$), 5.82 (d, 1H, J = 7.3 Hz, –N*H*), 5.43 [s, 1H, PhC*H*(O–)$_2$], 5.33 (dd, 1H, J = 1.2, 17.1 Hz, –CH=C*H*$_2$), 5.28 (d, 1H, J = 2.4 Hz, H-1b), 5.24 (brd, 1H, J = 10.2 Hz, –CH=C*H*$_2$), 5.05 (d, 1H, J = 3.4 Hz, H-1a), 4.92 (d, 1H, J = 11.5 Hz, –C*H*$_2$Ph), 4.82 (d, 1H, J = 11.7 Hz, –C*H*$_2$Ph), 4.74–4.62 (m, 3H, –C*H*$_2$Ph, –C*H*$_2$CH=CH$_2$), 4.59 (brs, 2H, –C*H*$_2$Ph), 4.54 (d, 1H, J = 11.4 Hz, –C*H*$_2$Ph), 4.47–4.36 (m, 7H, H-4a, –C*H*$_2$Ph, –OC*H*$_2$CHAr$_2$, Thr-αH, Thr-βH), 4.28 (dd, 1H, J = 7.4, 10.3 Hz, –OCH$_2$C*H*Ar$_2$), 4.23–4.06 (m, 5H, H-3a, H-6a, H-2b, H-3b, H-5b), 4.02–3.96 (m, 3H, H-2a, H-6a, H-4b), 3.63 (brs, 1H, H-5a), 3.59 (dd, 1H, J = 6.6, 9.6 Hz, H-6b), 3.50 (dd, 1H, J = 6.6, 9.3 Hz, H-6b), 1.25 (d, 3H, J = 6.6 Hz, Thr-γH). *Anal*. Found: C, 70.08; H, 6.05; N, 4.47%. Calcd. for $C_{69}H_{70}N_4O_{14}$: C, 70.27; H, 5.98; N, 4.75%.

Compound **12b**: $[\alpha]_D = +26.1°$ (c 1). Rf 0.29 (4:1 toluene–EtOAc). ^{1}H-NMR δ: 7.74 (brd, 2H, J = 7.6 Hz, Ar), 7.62 (m, 2H, Ar), 7.49 (brd, 2H, J = 6.6 Hz, Ar), 7.47–7.23 (m, 21H, Ar), 7.18–7.04 (m, 6H, Ar), 5.87 (m, 1H, –C*H*=CH$_2$), 5.79 (d, 1H, J = 9.6 Hz, N*H*), 5.49 [s, 1H, PhC*H*(O–)$_2$], 5.26 (dd, 1H, J = 1.5, 17.1 Hz, –CH=C*H*$_2$), 5.19 (d, 1H, J = 2.7 Hz, H-1b), 5.11 (brd, 1H, J = 10.5 Hz, –CH=C*H*$_2$), 4.95 (d, 1H, J = 11.5 Hz, –C*H*$_2$Ph), 4.86 (d, 1H, J = 11.7 Hz, –C*H*$_2$Ph), 4.73 (d, 1H, J = 11.5 Hz, –C*H*$_2$Ph), 4.65–4.63 (m, 2H, –C*H*$_2$CH=CH$_2$), 4.60–4.55 (m, 4H, –C*H*$_2$Ph × 3, Thr-βH), 4.50–4.43 (m, 3H, –C*H*$_2$Ph × 2, Thr-αH), 4.41–4.22 (m, 6H, –OC*H*$_2$CHAr$_2$, –OCH$_2$C*H*Ar$_2$, H-1a, H-4a, H-6a), 4.15 (brt, 1H, J = 6.4 Hz, H-5b), 4.12–4.05 (m, 2H, H-2b, H-3b), 3.99–3.96 (m, 2H, H-6a, H-4b), 3.62–3.56 (m, 2H, H-3a, H-6b), 3.46 (dd, 1H, J = 5.6, 9.8 Hz, H-6b), 3.16 (brs, 1H, H-5a), 1.34 (d, 1H, J = 6.4 Hz, Thr-γH). *Anal*. Found: C, 69.90; H, 6.03; N, 4.52%. Calcd. for $C_{69}H_{70}N_4O_{14}\cdot 0.5H_2O$: C, 69.74; H, 6.02; N, 4.71%.

*Procedure B (reaction of **3** and **6b**)*: As described for **11a**, disaccharyl threonine **11b** was prepared by glycosidation of **3** (60 mg, 0.11 mmol) and **6b** (90 mg, 0.14 mmol) with Cp_2ZrCl_2 (48 mg, 0.16 mmol), $AgClO_4$ (69 mg, 0.33 mmol), and dried 4A molecular sieves (0.5 g) in CH_2Cl_2 (5 ml). The product was chromatographed on Bio-beads S × 3 with toluene and then on silica gel with toluene–EtOAc (4:1) to afford **11b** (82 mg, 63%) and the **13b** (10 mg, 8%). The latter was identical in all respects with the sample previously prepared in this laboratory.

*N-(9-Fluorenylmethoxycarbonyl)-O-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-acetamido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-serine allyl ester (**14a**).* A mixture of **11a** (56 mg, 48 μmol), AcOH (39 μl, 0.68 mmol) and powdered Zn (195 mg, 2.98 mmol) in CH_2Cl_2 (1 ml) was stirred for 3 h. The mixture was filtered through Celite, and the filtrate was concentrated with toluene *in vacuo*. The residue dissolved in a mixture of MeOH (3 ml) and CH_2Cl_2 (2 ml) was stirred with Ac_2O (69 μl, 0.68 mmol) for 1 h. The mixture was concentrated *in vacuo*. The product was extracted with EtOAc, successively washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. Chromatography of the crude product on silica gel with toluene–EtOAc (1:1) afforded **14a** (50 mg, 88%). $[\alpha]_D = +92.3°$ (c 1). Rf 0.43 (1:1 toluene–EtOAc). ^{1}H-NMR δ: 7.76 (brd, 2H, J = 7.3 Hz, Ar), 7.57 (m, 2H, Ar), 7.47 (m, 2H, Ar), 7.39 (brt, 2H, J = 7.3 Hz, Ar), 7.31–7.11 (m, 25H, Ar), 5.89–5.85 (m, 2H, –C*H*=CH$_2$, –N*H*), 5.33 (brd, 1H, J = 17.6 Hz, –CH=C*H*$_2$), 5.28 (brd, 1H, J = 11.2 Hz, –CH=C*H*$_2$), 5.24 [s, 1H, PhC*H*(O–)$_2$], 5.04 (d, 1H, J = 2.9 Hz, H-1b), 5.02 (d, 1H, J = 3.0 Hz, H-1a), 3.59 (brs, 1H, H-5a), 3.52 (brt, J = 7.5 Hz, H-6b), 3.30 (dd, 1H, J = 4.1, 9.8 Hz, H-6b), 1.85 (s, 3H, Ac). *Anal*. Found: C, 70.53; H, 6.09; N, 2.31%. Calcd. for $C_{70}H_{72}N_2O_{15}\cdot 0.5H_2O$: C, 70.63; H, 6.18; N, 2.35%.

*N-(9-Fluorenylmethoxycarbonyl)-O-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-acetamido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-threonine allyl ester (**14b**).* Azide **11b** (137 mg, 0.12 mmol) was

reduced with Zn and AcOH, and then treated with Ac_2O as described for **14a**. The crude product was purified by chromatography on silica gel with toluene–EtOAc (1:1) to give **14b** (128 mg, 92%). $[\alpha]_D = +87.9^\circ$ (c 1.2). Rf 0.47 (1:1 toluene–EtOAc). ^{1}H-NMR δ: 7.75–7.73 (m, 2H, Ar), 7.59–7.54 (m, 2H, Ar), 7.47–7.45 (m, 3H, Ar), 7.38–7.23 (m, 24H, Ar), 7.20–7.11 (m, 2H, Ar), 5.94 (d, 1H, $J = 8.8$ Hz, –N*H*), 5.87 (m, 1H, –C*H*=CH$_2$), 5.59 (d, 1H, $J = 9.8$ Hz, –N*H*), 5.33 (brd, 1H, $J = 17.3$ Hz, –CH=C*H*$_2$), 5.28 (brd, 1H, $J = 10.5$ Hz, –CH=C*H*$_2$), 5.22 [s, 1H, PhC*H*(O–)$_2$], 5.06 (d, 1H, $J = 3.2$ Hz, H-1b), 5.01 (d, 1H, $J = 3.2$ Hz, H-1a), 3.80 (brs, 1H, H-4b), 3.59 (brs, 1H, H-5a), 3.50 (brt, 1H, $J = 7.6$ Hz, H-6b), 3.30 (dd, 1H, $J = 4.1$, 9.6 Hz, H-6b), 1.99 (s, 3H, Ac), 1.25 (d, 3H, $J = 4.8$ Hz, Thr-γH). *Anal*. Found: C, 70.67; H, 6.29; N, 2.18%. Calcd. for $C_{71}H_{74}N_2O_{15}\cdot 0.5H_2O$: C, 70.81; H, 6.28; N, 2.33%.

*N-(9-Fluorenylmethoxycarbonyl)-O-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-acetamido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-serine (**15a**).* A mixture of **14a** (62 mg, 52 µmol), Pd(Ph$_3$P)$_4$ (6 mg, 5 µmol), and 5,5-dimethyl-1,3-cyclohexanedione (146 mg, 1.04 mmol) in freshly distilled THF (1 ml) was stirred at room temperature for 1.5 h under Ar. The mixture was concentrated *in vacuo*, and the resulting residue was chromatographed on silica gel with $CHCl_3$–MeOH–AcOH (38:2:1). The product was further purified in a column of Biobeads S × 3 with toluene–EtOAc (1:1) to afford **15a** (60 mg, quant.). $[\alpha]_D = +127.7^\circ$ (c 1.1). Rf 0.29 (9:1 $CHCl_3$–MeOH). ^{1}H-NMR (DMSO-d_6) δ: 7.99 (brd, 2H, $J = 7.6$ Hz, Ar), 7.72–7.69 (m, 3H, Ar, AcN*H*), 7.43–7.20 (m, 29H, Ar), 7.12 (d, 1H, $J = 9.5$ Hz, Ser-N*H*), 5.36 [s, 1H, PhC*H*(O–)$_2$], 5.22 (d, 1H, $J = 2.9$ Hz, H-1b), 4.81 (d, 1H, $J = 3.5$ Hz, H-1a), 4.78 (d, 1H, $J = 11.2$ Hz, –C*H*$_2$Ph), 4.73 (d, 1H, $J = 11.9$ Hz, –C*H*$_2$Ph), 4.69 (d, 1H, $J = 11.9$ Hz, –C*H*$_2$Ph), 4.61 (d, 1H, $J = 11.7$ Hz, –C*H*$_2$Ph), 4.57 (d, 1H, $J = 11.7$ Hz, –C*H*$_2$Ph), 4.51 (d, 1H, $J = 12.2$ Hz, –C*H*$_2$Ph), 4.45 (d, 1H, $J = 11.2$ Hz, –C*H*$_2$Ph), 4.43 (d, 1H, $J = 12.0$ Hz, –C*H*$_2$Ph), 4.39–4.34 (m, 2H, Ser-βH, –OC*H*$_2$CHAr$_2$), 4.30–4.22 (m, 4H, –OC*H*$_2$CHAr$_2$, –OCH$_2$C*H*Ar$_2$, Ser-αH, H-2a), 4.05–3.78 (m, 9H, Ser-βH, H-3a, H-4a, H-6a × 2, H-2b, H-3b, H-4b, H-5b), 3.66 (brs, 1H, H-5a), 3.51 (m, 2H, H-6b × 2), 1.80 (s, 3H, Ac). *Anal*. Found: C, 69.76; H, 5.98; N, 2.31%. Calcd. for $C_{67}H_{68}N_2O_{15}\cdot 0.5H_2O$: C, 69.96; H, 6.04; N, 2.44%.

*N-(9-Fluorenylmethoxycarbonyl)-O-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-acetamido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-threonine (**15b**).* Compound **14b** (262 mg, 0.22 mmol) was deallylated with Pd(Ph$_3$P)$_4$ (26 mg, 22 µmol) and 5,5-dimethyl-1,3-cyclohexanedione (617 mg, 4.40 mmol) in THF (10 ml) as described for **15a**. Chromatography on silica gel and then on Biobeads afforded **15b** (252 mg, quant.). $[\alpha]_D = +106.5^\circ$ (c 1.1). Rf 0.36 (9:1 $CHCl_3$–MeOH). ^{1}H-NMR δ: 7.90–7.88 (m, 2H, Ar), 7.75–7.73 (m, 2H, Ar), 7.68–7.67 (m, 1H, Ar), 7.54–7.16 (m, 28H, Ar), 5.20 (d, 1H, $J = 2.0$ Hz, H-1b), 5.15 [s, 1H, PhC*H*(O–)$_2$], 4.78 (d, 1H, $J = 3.7$ Hz, H-1a), 1.77 (s, 3H, Ac), 1.14 (d, 3H, $J = 6.3$ Hz, Thr-γH). *Anal*. Found: C, 70.13; H, 6.07; N, 2.32%. Calcd. for $C_{68}H_{70}N_2O_{15}\cdot 0.5H_2O$: C, 70.15; H, 6.15; N, 2.41%.

*N-(9-Fluorenylmethoxycarbonyl)-O-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)-2-acetamido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-seryl fluoride (**16**).* A mixture of compound **15a** (23 mg, 20 µmol), TFFH (5 mg, 19 µmol), and DIEA (4.7 µl, 36 µmol) in anhydrous CH_2Cl_2 (2.5 ml) was stirred at room temperature for 2 h. This mixture was used for the coupling reaction without isolating **16**.

*L-Threonyl-O-[α-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyl]-L-threonyl-L-seryl-L-threonyl-L-threonyl-O-[α-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyl]-L-seryl-L-alanyl-L-proline (**2**).* Commercial Fmoc-Pro-CLEAR-acid resin (31 mg, 9 µmol) was stirred with 20% piperidine/NMP (2 ml) by a vortex mixer for 2 min, and then filtered. The resin was again stirred with 20% piperidine/NMP (2.5 ml) for 3 min to complete *N*-deprotection. After filtration, the resin was washed with NMP (2.5 ml). This washing was repeated five more times with the same amount of NMP. The resin was then stirred with Tsoc-Ala-OPfp (21 mg, 45 µmol) in freshly distilled THF (2.5 ml) for 15 min and filtered. Tsoc-Ala-OPfp (21 mg, 45 µmol) in THF (2.5 ml) was again added to the resin, and the mixture was stirred for a further 15 min. The resin was washed with NMP (2.5 ml) and treated with 10% Ac_2O–5% DIEA/NMP (2.5 ml) for 5 min to acetylate the unreacted amino moiety. The resin was successively washed with NMP (2.5 ml) and CH_2Cl_2 (2.5 ml). To the resin were added a mixture of **16** prepared as already described, 1 M TBAF/THF (5 µl, 0.1 equiv.) and DIEA (5 µl). The mixture was stirred for 12 h, before successively washing with CH_2Cl_2 and NMP. The unreacted amino terminal was masked by acetylation, and the resin was washed with NMP. The resulting glycopeptide-loaded resin was *N*-deprotected with 20% piperidine/NMP (2.5 ml) for 5 min and again with the same amount of piperidine for 15 min. After washing six times with NMP (2.5 ml each) for 1 min, a mixture of Fmoc-Thr(Bu^t)-OH (40 mg, 0.1 mmol), 0.45 M TBTU/NMP (200 µl, 0.09 mmol), and DIEA (24 µl, 0.18 mmol) was added to the resin, which was then stirred at 50 °C for 1 h. The resin was washed and *N*-deprotected before being subjected to further condensation. After two amino-acid-elongation with Fmoc-Thr(Bu^t)-OH and Fmoc-Ser(Bu^t)-OH, glycothreonine **15b** (23 mg, 0.02 mmol) was analogously condensed in the presence of 0.45 M TBTU/NMP (40 µl, 18 µmol) and DIEA (5 µl, 36 µmol) in NMP (2.5 ml). The latter condensation procedure was repeated with the same amount of **15b**. Finally, Fmoc-Thr(Bu^t)-OH was con-

densed to complete the desired sequence. The resin (44 mg) was successively washed with NMP and CH_2Cl_2, before being stirred with reagent K [TFA-phenol-deionized water-thioanisole-1,2-ethanedithiol (82.5:5:5:5:2.5), 440 µl] at room temperature for 1 h to cleave the synthetic glycopeptide from the resin. Blowing in a nitrogen stream evaporated the volatile materials in the mixture. Ether was added to the resulting mixture to precipitate the crude product, which was separated by centrifugation. The precipitate was further washed with ether before being dissolved in a mixture of dimethyl sulfide (132 µl), m-cresole (44 µl), and TFA (220 µl), and the magnetically stirred mixture was cooled at −15 °C. TfOH (44 µl) was added to the mixture, which was stirred for 2 h at −15 °C. The reaction was terminated by precipitating with cold (−80 °C) ether. The ethereal layer was removed by decantation after centrifuging the mixture. The resulting precipitate was again washed with cold ether before being purified by reversed-phase HPLC on C-18 silica gel with gradient elution by CH_3CN/H_2O containing 0.1% TFA as depicted in Fig. 3. Glycopeptide **2** was collected, and part of the sample was subjected to an amino acid analysis in order to estimate the total efficiency (11%). MALDI TOF MS m/z ($M^+ + Na$): calcd. for $C_{73}H_{108}N_{10}O_{37}{\cdot}Na$, 1739.68; found, 1739.73.

Acknowledgments

We thank Dr. T. Chihara and his staff at Riken for the combustion analyses. This work was supported by NEDO (New Energy and Industrial Technology Development Organization, Japan) and in part by grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We also thank Tokai University for support by grant-aid for high-technology research.

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