Pergamon

Tetrahedron 56 (2000) 6235–6243

TETRAHEDRON

A Facile Silyl Linker Strategy for the Solid-Phase Synthesis of Protected Glycopeptide: Synthesis of an N-Terminal Fragment of IL-2 (1–10)

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Received 19 May 2000; accepted 30 June 2000

Abstract—An N-terminal glycodecapeptide fragment of interleukin 2 (**1**) was synthesized by solid-phase method utilizing a new silyl linker. The *O*-silylated Fmoc–Thr–OAll was attached to the commercial HMP-resin and peptide chain elongation was performed by Fmoc protocol to produce a protected heptapeptide (3–10), which was cleaved from the resin by fluoridolysis and used as the amino component for further condensation on the solid support. On the other hand, 6-hydroxyl group of an Fmoc–Thr(GalNAc)–OAll derivative was silylated with the linker and attached to the resin. Deallylation, block condensation with the heptapeptide (3–10), and elongation at N-terminal with two amino acids were performed on the resin. Fluoride ion-mediated cleavage released the *N*- and *C*-protected glycopeptide from the solid support in good efficiency. Fully deprotected glycopeptide was also synthesized through on-resin deallylation and acidic cleavage of the silyl ether linkage.

Introduction

Solid-phase synthesis has been the principal technology in the peptide chemistry since the first introduction by Merrifield.[1] In order to facilitate the multistep synthesis on resin and isolation of the products from the solid supports, various anchoring groups have been designed on the basis of peptide chemistry used. For example, *p*-alkoxybenzyl ester type linkers are widely used to pursue Fmoc chemistry.[2] The synthesized peptides are released under acidic conditions which allow simultaneous deprotection of the most side chain functional groups. On the other hand, the specific linkers involving such a very acid-labile linkage as chlorotrityl ester have been utilized to synthesize partially protected peptides. Recently, we have reported a novel silyl ether-type linker which binds a side chain hydroxyl group of (glyco-)peptides to the solid support.[3] This approach has several advantages by combination with the use of Fmoc and allyl ester protecting groups: (1) The peptide chain assembly is possible at both the N- and C-terminal after selective removal of the protecting groups.[4] (2) The orthogonal conditions for Si–O bond cleavage by fluoridolysis allow to liberate the *N*- and/or *C*-protected (glyco)peptides, which provide the useful synthetic intermediates for further transformation into the large oligopeptides (Fig. 1).

As an extension of this work, we planned to apply this strategy to the synthesis of larger glycooligopeptides and study the on-resin key reactions in detail. By selecting a fragment of human interleukin 2[5] (1–10) **1** as a suitable synthetic model, the solid-phase synthesis was undertaken.[6] The results are described below. The synthetic route to **1** was designed so as to use this silyl linker protocol twice, one for the synthesis of peptide block (4–10), another for on-resin block coupling at Thr (3) and the subsequent condensation with the N-terminal amino acid units. The necessary glycosyl threonine **9** was prepared in six steps from the known compound **2**.[7] Reduction of the azide with Zn–AcOH was followed by acetylation with Ac_2O in MeOH to quantitatively give the corresponding acetamide **3**, which on debenzylidenation with aq. CF_3CO_2H afforded a sparingly soluble amide-triol **4** in 95% yield. Regioselective silyl-etherification of **4** with the silyl chloride **5** (1.5 equiv.) was achieved to give **6** (67%), when the reaction was conducted in the presence of NaI and *N*-methylmorpholine in DMF.[3] A trace amount of disilylated product was also formed. To diminish the difficulty in handling less soluble **6**, the vicinal diol was masked by isopropylidenation to give **7** (95%). Alternatively, the compound **2** was first debenzylidenated to the triol **8** and regioselectively silylated with

Keywords: glycopeptides; solid-phase synthesis; silicon halides.
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0040–4020/00/$ - see front matter
PII: S0040-4020(00)00588-3

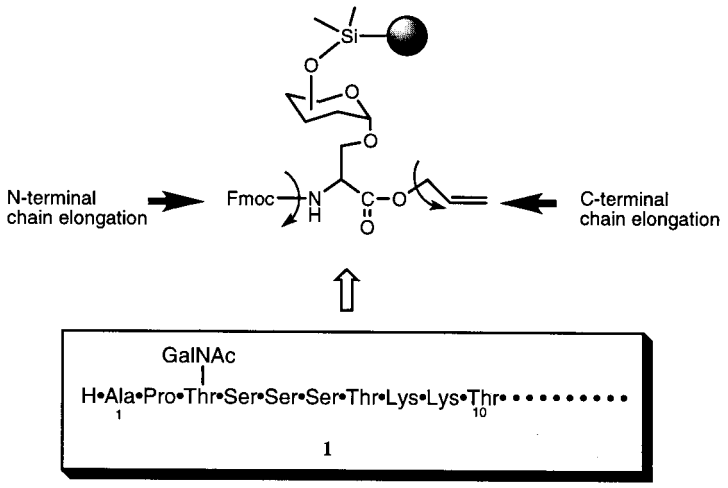
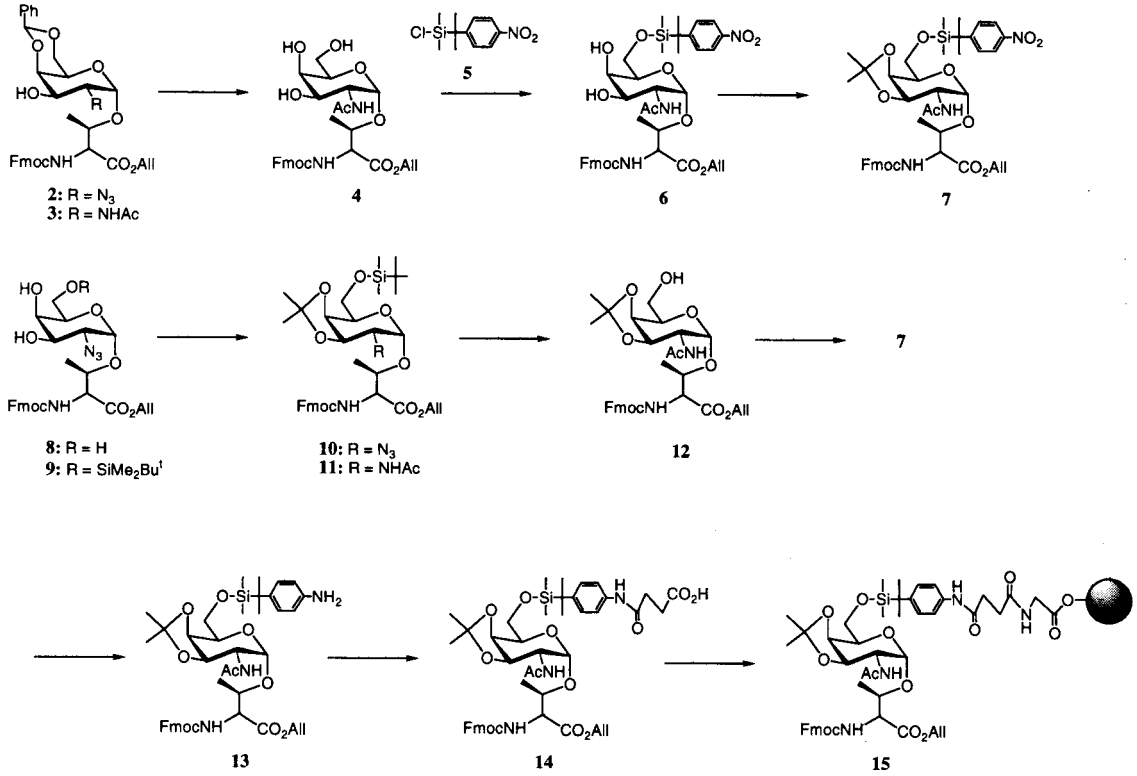


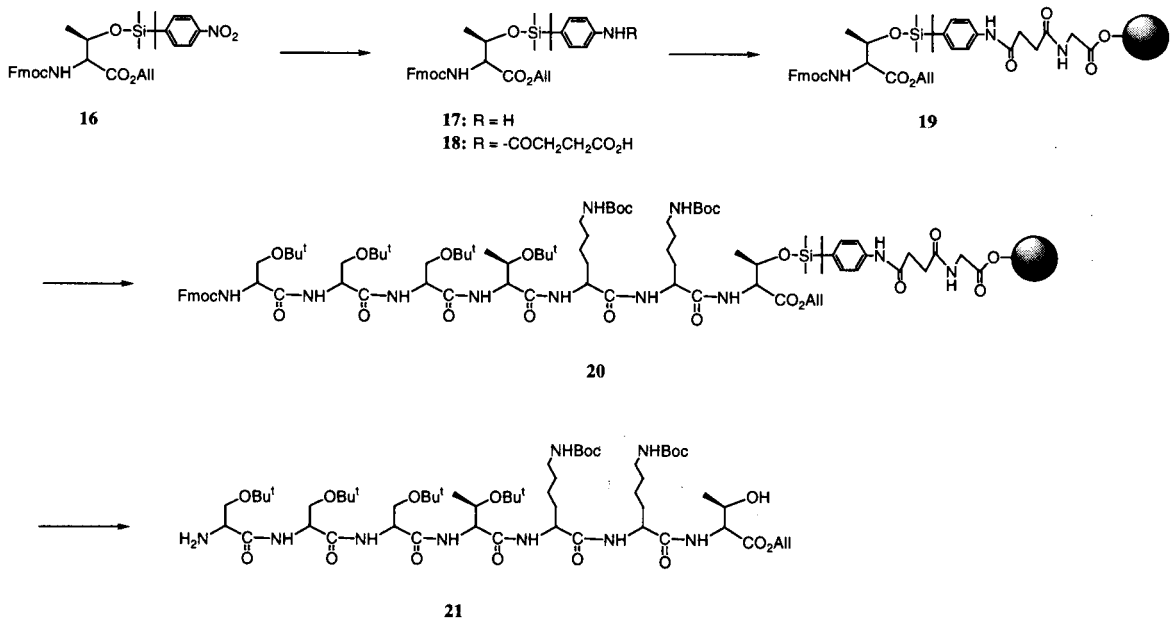
Figure 1.

t-butylchlorodimethylsilane to produce the 6-*O*-mono-silylated deivative **9** (97%).[7] 3,4-*O*-Isopropylidenation afforded **10** (84%). At this stage, the azide was transformed into acetamide **11** (90%). Removal of the *t*-butyldimethylsilyl group followed by re-silylation with **5** produced **7** in 89% yield. Though involved two steps more than the former route, the latter allowed the easier access to compound **7**.

The nitro group was reduced by treatment with Zn–AcOH in THF (94%) to give an aniline derivative **13**. Conversion of **13** into succinanilic acid **14** was performed by the reaction with succinic anhydride and *N*-methylmorpholine in CH_2Cl_2 (82%). The carboxylic acid **14** (2.5 equiv.) was attached to the H–Gly–HMP resin, derived from the commercial Fmoc–Gly–HMP-resin (0.78 mmol/g), in the presence of DCC, HOBt, and $^{i}Pr_2NEt$ in NMP using vortex tube mixer. The loading of **14** was ascertained by amino acid analysis. Comparison of threonine content with glycine in the cleavage mixture revealed that efficiency of the loading was 36%. Repeating of the loading procedure (double coupling) with another 1.2 equiv. of **14**, however, gave rise to only a slight increase in the total yield (41%). It is noteworthy that the unreacted glycosyl threonine was readily recovered from the reaction mixture. After concentration of the separated liquid layer followed by gel filtration, desilylation of the crude glycosyl threonine derivatives



Scheme 1.



Scheme 2.

with *n*-Bu_4NF–AcOH gave regenerated **12** in a reasonable amount.

On the other hand, the resin was treated with Ac_2O to cap the unreacted amine on the resin (Schemes 1 and 2).

In a similar manner, the Thr (10) residue was attached to the resin for the synthesis of heptapeptide block.

Fmoc–threonine allyl ester was silylated,[3] and the *p*-nitrobenzylsilyl ether **16** was reduced to give aniline **17** quantitatively. Treatment of **17** with succinic anhydride exclusively gave another key intermediate **18**. Installation of **18** (2 equiv.) to the H–Gly–HMP resin proceeded in a 68% of efficiency on the basis of amino acid analysis. Peptide synthesis was conducted with Fmoc–Lys(Boc)–OH, Fmoc–Thr(^{t}Bu)–OH, and Fmoc–Ser(^{t}Bu)–OH utilizing an automated synthesizer under the ready-made program (FastMoc). After *N*-deprotection, the heptapeptide was detached from the resin by fluoridolysis with CsF–AcOH in DMF. Fig. 2a shows a chromatograms of the released product. The major component was the desired peptide and the structure was confirmed by mass spectrometry and amino acid analysis. Thus the side chain- and *C*-protected peptide **21** was isolated in a good yield by preparative HPLC (Fig. 2b) and used for the on-resin block condensation.The crucial deallylation and block condensation were next investigated. The glycosyl threonine-bound resin **15** was treated overnight with $Pd(PPh_3)_4$ (0.5 equiv.) and *N*-methylaniline[8] in THF using a vortex mixer. Then the efficiency was monitored by HPLC and MS analyses of the acidic cleavage mixture from the resin sample. Since the incomplete conversion was evidenced by the analysis, the resin was submitted again to the

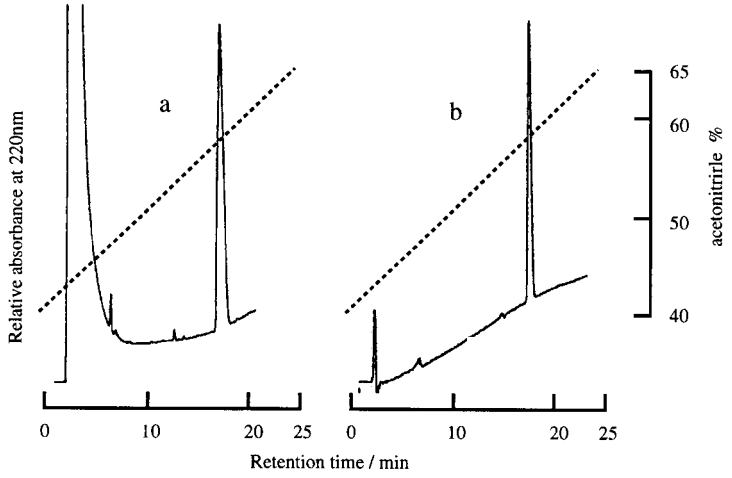


Figure 2. HPL chromatogram of **21** on Mightysil RP-18 (4.6×150 mm^2) with eluent A (distilled water containing 0.1% TFA) and B (acetonitrile containing 0.1% TFA): (a) the crude product released from the resin; (b) the purified **21**.

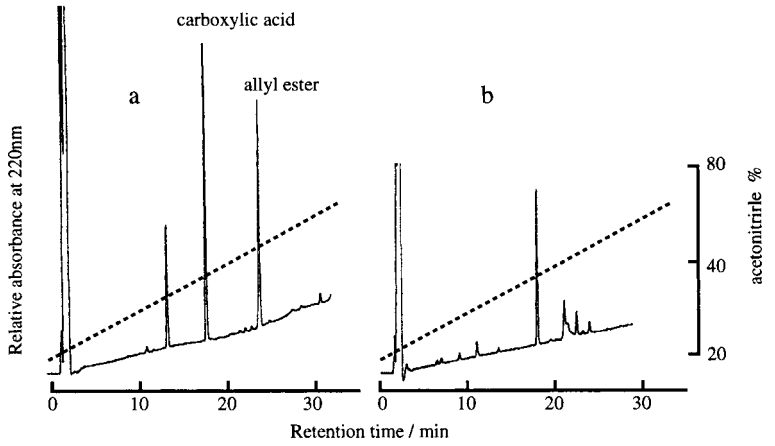


Figure 3. HPL chromatogram of the acid-cleavage products from **22**: (a) the sample derived after first run of on-resin deallylation; (b) the sample released after three repetitive deallylation.

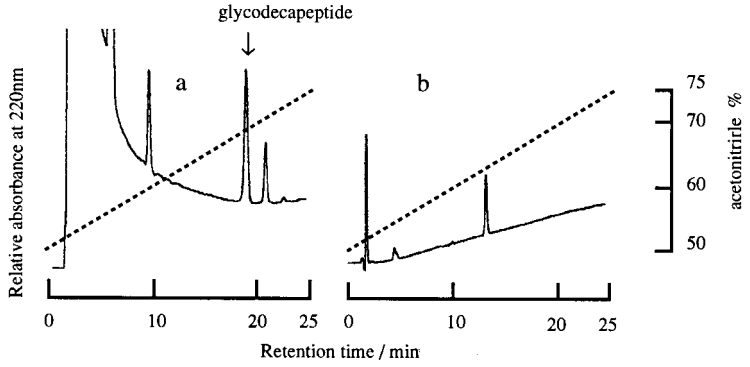
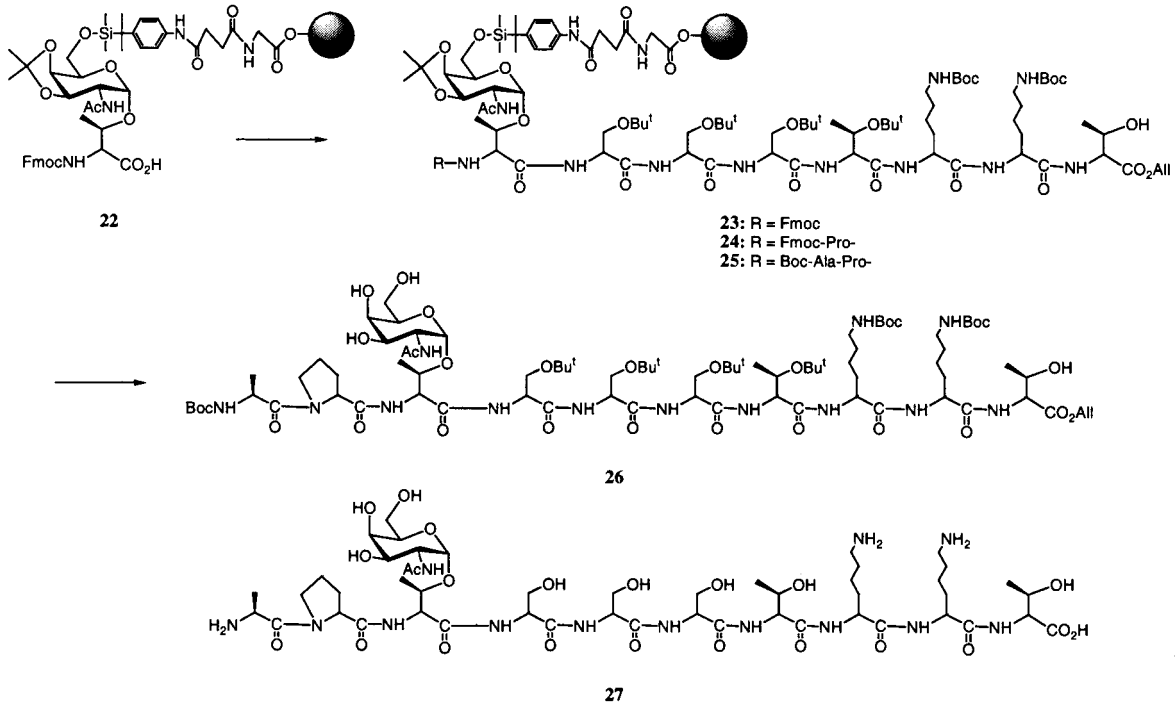


Figure 4. HPL chromatogram of the glycodecapeptides: (a) the crude product released from resin **25** by fluoridolysis; (b) isolated **26**.



Scheme 3.

deallylation conditions. In order to complete the deallylation, another repetition of this procedure was necessary as shown in Fig. 3.

On-resin block condensation with the heptapeptide fragment **21** (1.3 equiv.) was achieved in the presence of HBTU, HOBt, and $^{i}Pr_2NEt$ in DMF/NMP, whereas the use of DCC in place of HBTU was a little efficient for this coupling reaction. HPLC analysis indicated not only complete disappearance of the glycosyl threonine component but also formation of a new glycooctapeptide. The resin-bound peptide was then *N*-deprotected with 10% piperidine in NMP and coupled with Fmoc–Pro–OH using HBTU/ HOBt conditions. Similarly, the N-terminal alanine residue was introduced using Boc–Ala–OH.

Finally, the glycopeptide thus synthesized was released from the resin by fluoridolysis with CsF–AcOH (Fig. 4a). The crude product was purified by preparative HPLC. The isopropylidene group on GalNAc residue was cleaved during concentration of the TFA-containing eluate of the product to give the *N*-, *C*- and peptide side chain-protected fragment **26** in 86% yield from **15** (Fig. 4b). On the other hand, the fully deprotected glycopeptide **27** was obtained from **25** by on-resin deallylation and acidic cleavage. The structures of the purified products were established by NMR and mass spectra. The physical data of **27** was in good accordance with those reported (Scheme 3).[6]

In conclusion, we have demonstrated the potential of the newly developed synthetic strategy to the protected glycopeptide. Human interleukin 2 fragment (1–10) was chosen as the model compound and sucessfully synthesized through both C- and N-terminal peptide coupling on the resin. The key deallylation on the resin and block condensation were efficiently monitored by the use of HPLC and MS analyses. We could also demonstrate here that the new silyl linker is compatible with the automated procedure of Fmoc peptide synthesis.

Experimental

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 a JEOL AL400 [^1H (400 MHz), ^{13}C (100 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 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). Peptide synthesis for the fragment (4–10) was run with an Applied Biosystems Model 433A peptide synthesizer. All other solid-phase reactions were performed at room temperature in the capped polypropylene test tubes with stirring on a vortex tube-mixer. Fmoc–Gly–preloaded HMP resin and the reagents for the peptide synthesis were purchased from Perkin–Elmer Applied Biosystems, Div. HPLC was performed using Mightysil RP-18 (4.6×150 mm^2 for analysis and 10×250 mm^2 for preparation, Kanto Chemical Co.). Amino acids were analyzed on a Hitachi L-8500 amino acid analyzer.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-threonine allyl ester 3.** A mixture of compound **2** (860 mg, 1.33 mmol), AcOH (10.7 ml, 3.30 mmol), powdered Zn (870 mg, 13.3 mmol) in CH_2Cl_2 (12 ml) was stirred at room temperature for 20 min and filtered through Celite. The filtrate was concentrated with toluene. The residue was dissolved in MeOH (5 ml) and stirred with Ac_2O (0.7 ml, 6.7 mmol) at room temperature for 30 min. MeOH was evaporated with toluene and the product was extracted with EtOAc. The extract was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel with toluene–EtOAc (1:2) to give **3** (868 mg, quant.), R_f 0.44 (toluene–EtOAc, 1:2), $[\alpha]_D$=+70.2° (*c* 0.5). ^1H NMR: δ 7.66 (d, 2H, *J*=6.6 Hz, Ar), 7.50 (d, 2H, *J*=7.3 Hz, Ar), 7.39–7.14 (m, 9H, Ar), 6.11 (d, 1H, *J*=8.5 Hz, AcN*H*), 5.76 (m, 1H, –CH=CH2), 5.54 (d, 1H. *J*=9.5 Hz, Thr–N*H*), 5.42 [s, 1H, PhC*H*$(O)_2$], 5.20 (d, 1H, *J*=10.9 Hz, –CH=CH_2), 5.17 (d, 1H, *J*=10.5 Hz, –CH=CH_2), 4.80 (d, 1H, *J*=3.1 Hz, H-1), 4.51 (brt, 2H, –CH_2CH=CH_2), 3.56 (brs, 1H, H-4), 3.35 (d, 1H, *J*=10.0 Hz, H-3), 1.95 (s, 3H, Ac), 1.16 (brs, 3H, Thr–γH). MALDI TOF·MS *m/z* Calcd for $C_{37}H_{41}N_2O_{10}$ $(M+H)^+$: 673.27, found: 673.62. Anal. Calcd for $C_{37}H_{40}N_2O_{10}·H_2O$: C, 64.62; H, 5.72; N, 4.07%. Found: C, 64.56; H, 5.99; N, 4.11%.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine allyl ester 4.** A solution of **3** (197 mg, 0.92 mmol) in 80% aq TFA (4 ml) was stirred at 0°C for 3 h. The reaction mixture was concentrated with toluene and water. The residue was chromatographed on silica gel with $CHCl_3$–EtOH 19:1 –CHCl3–EtOH–AcOH 19:1:0.1) to afford **4** (141 mg, 83%), R_f 0.52 ($CHCl_3$–EtOH, 1:1), $[\alpha]_D$=+45.5° (*c* 0.3). ^1H NMR: δ 7.76 (brd, 2H, *J*=7.4 Hz, Ar), 7.71 (brd, 2H, *J*=7.1 Hz, Ar), 7.39 (brd, 2H, *J*=7.0 Hz, Ar), 7.31 (brd, 2H, *J*=7.1 Hz, Ar), 6.75 (brs, 1H, AcNH), 5.91–5.81 (m, 2H, Thr–NH, –CH=CH2), 5.31 (d, 1H, *J*=16.3 Hz, –CH=CH2), 5.26 (d, 1H, *J*=10.3 Hz, –CH=CH2), 4.85 (brs, 1H, H-1), 4.69–4.56 (m, 3H, –CH2CH=CH2, Ar2CH-), 4.48 (brs, 1H, Thr–αH), 4.42 (d, 2H, *J*=6.4 Hz, Ar_2CHCH_2–), 4.23 (m, 3H, H-2, H-6, Thr–βH), 4.15 (brs, 1H, H-4), 3.89 (m, 3H, H-3, H-5, H-6), 2.10 (s, 3H, Ac), 1.28 (d, 3H, *J*=6.6 Hz, Thr–γH). MALDI TOF·MS *m/z* Calcd for C30H36N2O10Na (M+Na)+: 607.22, found: 607.73.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-[2-acetamido-2-deoxy-6-*O*-(α,α-dimethyl-4-nitrobenzyl)dimethylsilyl-α-D-galactopyranosyl]-L-threonine allyl ester 6.** A mixture of **4** (30 mg, 0.05 mmol), **5** (15.5 mg, 0.06 mmol), NaI (22.5 mg, 0.15 mmol), and 4-methylmorpholine (11 μl, 0.10 mmol) in dry DMF (2 ml) was stirred at −20°C under Ar for 2 h. The mixture was diluted with EtOAc, washed with water and brine, dried over Na_2SO_4, and

concentrated in vacuo. The crude product was purified by preparative t.l.c. with $CHCl_3$–EtOH (9:1) to give **6** (28 mg, 68%), R_f 0.31 ($CHCl_3$–EtOH, 9:1), $[\alpha]_D$=+28.6° (*c* 0.5). ^{1}H NMR: δ 8.09 (m, 2H, Ar), 7.74 (m, 2H, Ar), 7.59–7.24 (m, 8H, Ar), 6.55 (d, 1H, *J*=8.1 Hz, AcN*H*), 5.84 (m, 1H, –C*H*=CH_2), 5.75 (d, 1H, *J*=9.5 Hz, Thr–N*H*), 5.30 (d, 1H, *J*=17.3 Hz, –CH=C*H*$_2$), 5.25 (d, 1H, *J*=10.3 Hz, –CH=C*H*$_2$), 4.78 (d, 1H. *J*=3.4 Hz, H-1), 4.15 (m, 1H, Thr–βH), 3.90 (brs, 1H, H-4), 3.48 (brs, 1H, OH), 2.07 (s, 3H, Ac), 1.39 (s, 6H, –C*Me*$_2$Ar), 1.28 (m, 3H, Thr–γH), 0.01 (s, 6H, –Si*Me*$_2$–). MALDI TOF·MS *m/z* Calcd for $C_{41}H_{51}N_3O_{12}SiNa$ $(M+Na)^+$: 828.31, found: 828.70. Anal. Calcd for $C_{41}H_{51}N_3O_{12}Si\cdot 1/2H_2O$: C, 60.42; H, 6.43; N, 5.16%. Found: C, 60.37; H, 6.39; N, 4.86%.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2-azido-2-deoxy-3,4-*O*-isopropylidene-6-*O*-*tert*-butyldimethylsilyl-α-D-galactopyranosyl)-L-threonine allyl ester 10.** A mixture of **9** (340 mg, 0.50 mmol), 2,2-dimethoxypropane (1.22 ml, 9.96 mmol), and camphorsulfonic acid (57.0 mg, 0.25 mmol) in dry CH_3CN (6 ml) was stirred at room temperature for 5 min. The mixture was diluted with EtOAc, washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel with toluene–EtOAc (7:3) to give **10** (365 mg, quant.). R_f 0.64 (toluene–EtOAc, 1:1), $[\alpha]_D$= +73.9° (*c* 0.7). ^{1}H NMR: δ 7.75 (d, 2H, *J*=7.3 Hz, Ar), 7.61 (brd, 2H, Ar), 7.40–7.28 (m, 4H, Ar), 5.93 (m, 1H, –C*H*=CH_2), 5.61 (d, 1H. *J*=9.5 Hz, Thr–N*H*), 5.35 (d, 1H, *J*=15.9 Hz, –CH=C*H*$_2$), 5.25 (d, 1H, *J*=10.3 Hz, –CH=C*H*$_2$), 4.87 (d, 1H, *J*=3.7 Hz, H-1), 4.68 (d, 2H, *J*=5.9 Hz, –C*H*$_2$CH=CH_2), 4.11 (ddd, 1H, *J*=2.4, 6.4, 8.8 Hz, H-5), 3.35 (dd, 1H, *J*=3.7, 8.3 Hz, H-2), 1.50 [s, 3H, –(O)$_2$C*Me*$_2$], 1.35 [br, 6H, –(O)$_2$C*Me*$_2$ and Thr–γH], 0.89 (s, 9H, *t*-Bu), 0.07 (s, 6H, –Si*Me*$_2$). MALDI TOF·MS *m/z* Calcd for $C_{37}H_{50}N_4O_9SiNa$ $(M+Na)^+$: 745.32, found: 745.71. Anal. Calcd for $C_{37}H_{50}N_4O_9Si$: C, 61.47; H, 6.97; N, 7.75%. Found: C, 61.58; H, 7.02; N, 7.28%.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2-acetamido-2-deoxy-3,4-*O*-isopropylidene-6-*O*-*tert*-butyldimethylsilyl-α-D-galactopyranosyl)-L-threonine allyl ester 11.** The azide **10** (365 mg, 0.51 mmol) was treated with Zn and AcOH in CH_2Cl_2, and then acetylated as described for **3**. The acetamide **11** (336 mg, 90%) was isolated by column chromatography on silica gel with $CHCl_3$–EtOH (19:1). R_f 0.29 (toluene–EtOH, 1:1), $[\alpha]_D$=+61.7° (*c* 0.5).^{1}H NMR: δ 7.75 (d, 2H, *J*=7.6 Hz, Ar), 7.59 (d, 2H, *J*=4.9 Hz, Ar), 7.40–7.28 (m, 4H, Ar), 5.85 (m, 1H, –C*H*=CH_2), 5.74 (d, 1H. *J*=9.5 Hz, Thr–N*H*), 5.42 (d, 1H, *J*=9.5 Hz, AcN*H*), 5.32 (d, 1H, *J*=17.1 Hz, –CH=C*H*$_2$), 5.27 (d, 1H, *J*=10.5 Hz, –CH=C*H*$_2$), 4.69 (d, 1H, *J*=3.2 Hz, H-1), 4.65 (brd, 1H, –C*H*$_2$CH=CH_2), 4.55 (brd, 1H, –C*H*$_2$CH=CH_2), 4.46 (brd, 2H, –COOC*H*$_2$CHAr$_2$), 4.36 (d, 1H, *J*=9.8 Hz, Thr–αH), 4.05 (brd, 1H, H-5), 2.01 (s, 3H, Ac), 1.55 [s, 3H, –(O)$_2$C*Me*$_2$], 1.30 [br, 6H, –(O)$_2$C*Me*$_2$ and Thr–γH], 0.88 (s, 9H, *t*-Bu), 0.06 (s, 6H, –Si*Me*$_2$). MALDI TOF·MS *m/z* Calcd for $C_{39}H_{54}N_2O_{10}SiNa$ $(M+Na)^+$: 761.34, found: 761.75. Anal. Calcd for $C_{39}H_{54}N_2O_{10}Si$: C, 63.39; H, 7.37; N, 3.79%. Found: C, 63.21; H, 7.34; N, 3.75%.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2-acetamido-2-deoxy-3,4-*O*-isopropylidene-α-D-galactopyranosyl)-L-threonine allyl ester 12.** To a stirred mixture of **11** (330 mg, 0.45 mmol) and AcOH (0.24 ml, 4.55 mmol) in dry THF (10 ml) was added 1 M Bu_4NF/THF (1.82 ml, 1.82 mmol) at 0°C under Ar. After stirring for 4 h at 0°C–room temperature, the mixture was concentrated to the half volume and extracted with $CHCl_3$. The extract was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The product was chromatographed on silica gel with $CHCl_3$–MeOH (97:3) to give **12** (257 mg, 92%), R_f 0.50 ($CHCl_3$–EtOH 9:1), $[\alpha]_D$=+74.0° (*c* 0.2). ^{1}H NMR: δ 7.78 (d, 2H, *J*=7.6 Hz, Ar), 7.61 (d, 2H, *J*=7.1 Hz, Ar), 7.43–7.30 (m, 4H, Ar), 5.91–5.82 (m, 2H, –C*H*=CH_2, AcN*H*), 5.48 (d, 1H. *J*=9.5 Hz, Thr–N*H*), 5.32 (brd, 2H, –CH=C*H*$_2$), 4.77 (d, 1H, *J*=3.4 Hz, H-1), 4.67 (dd, 1H, *J*=5.9, 12.9 Hz, –C*H*$_2$CH=CH_2), 4.58 (brd, 1H, *J*=6.1, 12.9 Hz, –C*H*$_2$CH=CH_2), 4.50 (brd, 2H, –COOC*H*$_2$ CHAr$_2$), 4.39 (d, 1H, *J*=9.5 Hz, Thr–αH), 4.17 (dd, 1H, *J*=2.2, 4.9 Hz, H-2), 4.10 (m, 1H, H-5), 4.03 (dd, 1H, *J*=4.9, 9.0 Hz, H-3), 3.94 (brd, 1H, H-6), 3.83 (brd, 1H, H-6'), 2.03 (s, 3H, Ac), 1.74 (brs, 1H, OH), 1.57 [s, 3H, –(O)$_2$C*Me*$_2$], 1.34 [s, 3H, –(O)$_2$C*Me*$_2$], 1.30 (s, 3H, Thr–γH). MALDI TOF·MS *m/z* Calcd for $C_{33}H_{40}N_2O_{10}Na$ $(M+Na)^+$: 647.25, found: 647.71. Anal. Calcd for $C_{33}H_{40}N_2O_{10}\cdot 1/2H_2O$: C, 62.54; H, 6.52; N, 4.42%. Found: C, 62.86; H, 6.51; N, 4.89%.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-[2-acetamido-2-deoxy-3,4-*O*-isopropylidene-6-*O*-(α,α-dimethyl-4-nitrobenzyl)-dimethylsilyl-α-D-galactopyranosyl]-L-threonine allyl ester 7. Procedure A (from 6).** The compound **6** (30 mg, 0.05 mmol) was isopropylidenated with, 2,2-dimethoxypropane (0.1 ml, 0.82 mmol), and camphorsulfonic acid (5.0 mg, 0.02 mmol) in dry CH_3CN (1 ml) as described for **10**. The product was purified by preparative t.l.c. with toluene–EtOH (1:1) to give **7** (31 mg, 91%), R_f 0.29 (toluene–EtOH, 1:1), $[\alpha]_D$=+60.3° (*c* 1). ^{1}H NMR: δ 8.09 (d, 2H, *J*=8.8 Hz, Ar), 7.76 (d, 2H, *J*=7.3 Hz, Ar), 7.60 (m, 2H, Ar), 7.39–7.30 (m, 4H, Ar), 5.85 (m, 1H, –C*H*=CH_2), 5.74 (d, 1H, *J*=7.3 Hz, AcNH), 5.40 (d, 1H, *J*=9.5 Hz, Thr–NH), 5.31 (m, 2H, –CH=C*H*$_2$), 4.69 (d, 1H. *J*=3.4 Hz, H-1), 4.07 [brs, 1H, H-4), 4.02 (brs, 1H, H-5), 3.96 (dd, 1H, *J*=4.9, 9.0 Hz, H-3), 2.01 (s, 3H, Ac), 1.61 [s, 3H, –(O)$_2$C*Me*$_2$], 1.41 (s, 6H, –C*Me*$_2$Ar), 1.40 [m, 6H, –(O)$_2$C*Me*$_2$ and Thr–γH], 0.01 (s, 6H, –Si*Me*$_2$–). MALDI TOF·MS *m/z* Calcd for $C_{44}H_{55}N_3O_{12}SiNa$ $(M+Na)^+$: 868.34, found: 868.63. Anal. Calcd for $C_{44}H_{55}N_3O_{12}Si$: C, 62.47; H, 6.55; N, 4.97%. Found: C, 62.26; H, 6.58; N, 4.79%.

Procedure B (from 12). The compound **12** (187 mg, 0.30 mmol) was silylated with **5** (93 mg, 0.36 mmol) and NaI (134 mg, 0.90 mmol) in DMF (1.5 ml) at −20° C for 45 min. Work-up and purification by preparative t.l.c afforded **7** (235 mg, 97%).

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-[2-acetamido-2-deoxy-3,4-*O*-isopropylidene-6-*O*-(α,α-dimethyl-4-aminobenzyl)-dimethylsilyl-α-D-galactopyranosyl]-L-threonine allyl ester 13.** A mixture of **7** (54 mg, 0.06 mmol), AcOH (0.06 ml, 1.15 mmol), and powdered Zn (400 mg, 6.12 mmol) was stirred in THF (1 ml) at room temperature for 45 min. The mixture was diluted with EtOAc, filtered through Celite, washed with sat. $NaHCO_3$ and brine, dried

over Na_2SO_4, and concentrated in vacuo. The product was chromatographed on silica gel with toluene–EtOAc (1:9) to give **13** (49 mg, 94%), R_f 0.49 ($CHCl_3$–EtOH, 9:1), $[\alpha]_D$=+45.6° (*c* 1.4). ^{1}H NMR: δ 7.75 (d, 2H, *J*=7.3 Hz, Ar), 7.60 (d, 2H, *J*=7.6 Hz, Ar), 7.40–7.29 (m, 4H, Ar), 7.01 (brd, 2H, Ar), 6.56 (brd, 2H, Ar), 5.84 (m, 1H, –C*H*=CH_2), 5.76 (d, 1H, *J*=7.3 Hz, AcN*H*), 5.55 (d, 1H, *J*=9.5 Hz, Thr–N*H*), 5.29 (m, 2H, –CH=CH_2), 4.66 (d, 1H. *J*=3.4 Hz, H-1), 4.34 (dd, 1H, *J*=1.7, 9.5 Hz, Thr–αH), 4.06 [brs, 1H, H-4), 2.00 (s, 3H, Ac), 1.54 [s, 3H, -$(O)_2C$*Me*$_2$], 1.30–1.25 [m, 12H, –C*Me*$_2$Ar, –$(O)_2C$*Me*$_2$ and Thr–γH], 0.01 (s, 6H, –Si*Me*$_2$–). MALDI TOF·MS *m*/*z* Calcd for $C_{44}H_{58}N_3O_{10}Si$ $(M+H)^+$: 816.38, found: 816.64. Anal. Calcd for $C_{44}H_{57}N_3O_{10}Si$: C, 64.76; H,7.04; N, 5.15%. Found: C, 64.60; H, 7.03; N, 4.91%.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-[2-acetamido-2-deoxy-3,4-*O*-isopropylidene-6-*O*-(α,α-dimethyl-4-succin-mono-amidobenzyl)dimethylsilyl-α-D-galactopyranosyl]-L-threonine allyl ester 14.** A mixture of **13** (49 mg, 0.06 mmol), succinic anhydride (6.6 mg, 0.07 mmol), and 4-methylmorpholine (6.6 μl, 0.06 mmol) in CH_2Cl_2 (1 ml) was stirred under Ar at room temperature for 3 h. The mixture was concentrated in vacuo, before the residue was purified by gel permeation chromatography on Bio-beads S×3 with toluene–EtOAc (1:1). The obtained **14** (46 mg, 82%) was used without further purification, R_f 0.27 ($CHCl_3$–EtOH, 9:1). The NMR spectrum of **14** was not fully assigned due to the presence of more than three conformational isomers. ^{1}H NMR: δ 5.80 (m, 1H, –C*H*=CH_2), 5.21 (dd, 1H, *J*=1.5, 17.1 Hz, –CH=CH_2), 5.18 (dd, 1H, *J*=1.3, 10.5 Hz, –CH=CH_2), 4.44 (d, 1H, *J*=4.0 Hz, H-1), 4.59–4.40 (m, 4H, –CH_2CH=CH_2, Ar_2CHCH_2), 4.38–4.15 (m, 2H, Thr–αH, Ar_2CH–), 2.94–2.40 (m, 4H, –COCH_2CH_2CO–), 1.92 (s, 3H, Ac), 1.44, 1.39, and 1.29 (3brs, 12H, –C*Me*$_2$Ar and –$(O)_2C$*Me*$_2$), 0.18 (s, 6H, –Si*Me*$_2$–). MALDI TOF·MS *m*/*z* Calcd for $C_{48}H_{61}N_3O_{13}Si$·Na $(M+Na)^+$: 938.39, found: 938.80.

Attachment of 14 to resin (synthesis of 15). Commercial Fmoc–Gly–HMP-resin (73 mg, 56 μmol; 0.78 mmol/g) was stirred with 50% piperidine/NMP solution (1 ml) at room temperature for 1.5 h. The mixture was filtered on a sintered glass disk, washed successively with NMP, CH_2Cl_2 and ether. The resulting resin was dried in vacuo for a little while and used for further solid-phase synthesis. A mixture of **14** (129 mg, 0.14 mmol), 1 M HOBt/NMP (183 μl, 0.18 mmol), 1 M DCC/NMP (183 μl, 0.18 mmol) and 2 M ^{i}Pr$_2$NEt/NMP (71 μl, 0.14 mmol) in NMP (0.6 ml) was stirred at room temperature for 30 min. To the mixture was added the above *N*-deprotected resin and stirring was continued overnight. The mixture was filtered and the resin was washed with NMP, CH_2Cl_2 and ether. A small portion of resin (3 mg) was heated in a mixture of propionic acid (100 μl) and conc HCl (100 μl) at 150°C in an evacuated sealed tube. The mixture was concentrated in vacuo before dilution with 0.2N HCl. After filtration, the amino acids in the filtrate were analyzed. Since result of the analysis indicated that the loading efficiency of **14** was 36%, the resin was again exposed to the freshly prepared mixture of **14** (84 mg, 0.09 mmol), HOBt (0.12 mmol), DCC (0.12 mmol), and 2 M ^{i}Pr$_2$NEt/NMP (45 μl, 0.09 mmol) in NMP (0.7 ml). Amino acid analysis of the sample from the resultant resin exhibited a little increase in the total loading (41%). Then the unreacted amino group on the resin was capped with Ac_2O (0.1 ml) and pyridine (0.05 ml) in NMP (0.85 ml). The resin-attached **14** was also confirmed by the HPLC analysis and TOFMS of the acidic cleavage sample from resin **15**.

The unreacted glycosyl threonine unit was recovered from the first coupling mixture as follows. The combined filtrate and washings were concentrated in vacuo and the residue was chromatographed on Bio-beads S×3 with toluene–EtOAc (1:1). The fractions containing glycosyl threonine derivatives were collected and treated with 1 M Bu_4NF/THF (1.1 ml) and AcOH (0.14 ml) in THF (1 ml) for 10 h. The reaction was worked up as described for **12**. Chromatographic purification afforded a reasonable amount (65 mg, 50%) of recovered **12**.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(α,α-dimethyl-4-aminobenzyl)dimethylsilyl-L-threonine allyl ester 17.** Compound **16** (350 mg, 0.58 mmol) was reduced with powdered Zn (1.5 g) and AcOH (0.2 ml) in THF (5 ml) by the same procedure as described for **13**. The product was chromatographed on silica gel with hexane–EtOAc (1:1) to give **17** (331 mg, 96%), R_f 0.40 (hexane–EtOAc, 1:1), $[\alpha]_D$=−7.5° (*c* 0.9). ^{1}H NMR: δ 7.75 (brd, 2H, *J*=7.3 Hz, Ar), 7.64 (dd, 2H, *J*=7.8, 9.5 Hz, Ar), 7.41–7.29 (m, 4H, Ar), 7.00 (brd, 2H, *J*=8.5 Hz, Ar), 6.59 (brd, 2H, *J*=8.5 Hz, Ar), 5.88 (m, 1H, –C*H*=CH_2), 5.42 (d, 1H, *J*=9.5 Hz, Thr–N*H*), 5.31 (brd, 1H, *J*=17.3 Hz, –CH=CH_2), 5.23 (brd, 1H, *J*=10.5 Hz, –CH=CH_2), 4.64–4.54 (m, 2H), 4.48–4.35 (m, 3H), 4.31–4.24 (m, 2H), 3.45 (brs, 2H, NH_2), 1.28 and 1.27 (2s, 6H, –C*Me*$_2$Ar), 1.11 (d, 3H, *J*=6.3 Hz, Thr–γH), −0.04 and −0.08 (2s, 6H, –Si*Me*$_2$–). Anal. Calcd for $C_{33}H_{40}N_2O_5Si·H_2O$: C, 67.10; H,7.17; N, 4.74%. Found: C,67.40; H, 7.05; N, 4.46%.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(α,α-dimethyl-4-succin-mono-amidobenzyl)dimethylsilyl-L-threonine allyl ester 18.** Compound **17** (633 mg, 1.11 mmol) was converted to **18** with succinic anhydride (122 mg, 1.22 mmol) and 4-methylmorpholine (121 μl, 1.11 mmol) by the same procedure as described for **14**. Chromatography on Bio-beads S×3 with toluene–EtOAc (1:1) afforded **18** (732 mg, 98%), R_f 0.28 ($CHCl_3$–MeOH, 9:1), $[\alpha]_D$=−7.3° (*c* 1). ^{1}H NMR: δ 7.76 (brd, 2H, *J*=7.6 Hz, Ar), 7.61 (dd, 2H, *J*=7.7, 10.4 Hz, Ar), 7.41–7.20 (m, 6H, Ar), 7.15 (brd, 2H, *J*=8.8 Hz, Ar), 5.86 (m, 1H, –C*H*=CH_2), 5.37 (d, 1H, *J*=9.8 Hz, Thr–N*H*), 5.29 (dd, 1H, *J*=1.2, 17.2 Hz, –CH=CH_2), 5.22 (dd, 1H, *J*=1.2, 10.5 Hz, –CH=CH_2), 4.63–4.51 (m, 2H), 4.47–4.35 (m, 3H), 4.31–4.22 (m, 2H), 2.67 and 2.51 (2brt, 4H, –COCH_2CH_2CO–), 1.30 and 1.29 (2s, 6H, –C*Me*$_2$Ar), 1.09 (d, 3H, *J*=6.1 Hz, Thr–γH), −0.02 and −0.08 (2s, 6H, –Si*Me*$_2$–). Anal. Calcd for $C_{37}H_{44}N_2O_8Si·3/2H_2O$: C, 63.50; H,6.77; N, 4.00%. Found: C, 63.76; H, 6.45; N, 3.57%.

***O*-(*tert*-Butyl)-L-seryl-*O*-(*tert*-butyl)-L-seryl-*O*-(*tert*-butyl)-L-seryl-*O*-(*tert*-butyl)-L-threonyl-N$^{\epsilon}$-(*tert*-butoxycarbonyl)-L-lysyl-N$^{\epsilon}$-(*tert*-butoxycarbonyl)-L-lysyl-L-threonine allyl ester 21.** As described for **15**, the carboxylic acid **18** (305 mg, 0.45 mmol) was activated with 1 M DCC/NMP (589 μl, 0.59 mmol), 1 M HOBt/NMP (589 μl, 0.59

mmol), and 2 M $^{i}Pr_2NEt$/NMP (227 μl, 0.45 mmol) in NMP (0.6 ml), and then stirred with H–Gly–HMP-resin (293 mg, 0.23 mmol) overnight. A part of the obtained resin (3 mg) was hydrolyzed with HCl as described above. The hydrolysate was submitted to amino acid analysis, which showed 1:1.5 Thr/Gly ratio. The resin (385 mg) was treated with Ac_2O in pyridine and used for further solid-phase synthesis. The peptide assembly was performed with Fmoc–Lys(Boc)–OH, Fmoc–Thr(^{t}Bu)–OH, and Fmoc–Ser(^{t}Bu)–OH on an automated synthesizer according to FastMoc program, where HBTU/HOBt/$^{i}Pr_2NEt$ was the condensing agent. *N*-Deprotection in each cycle was effected with 20% piperidine/NMP. After six cycles of amino acid coupling and *N*-deprotection, the heptapeptide-bound resin was stirred with CsF (152 mg, 0.20 mmol) and AcOH (0.3 ml, 1.0 mmol) in DMF (5 ml) overnight. The mixture was filtered and the resin was washed with DMF, CH_2Cl_2, and ether. The combined filtrate and washings were concentrated in vacuo and the residue was extracted with $CHCl_3$. The extract was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by preparative HPLC on C18 silica gel (Mightysil, 10×250 mm^2) with a gradient elution of aq CH_3CN containing 0.1% TFA (45–75%/0–30 min, 2.5 ml/min) to give **21** (64 mg, 88%). ^{1}H NMR (CD_3OD): δ 5.85 (m, 1H, –C*H*=CH_2), 5.25 (dd, 1H, *J*=1.5, 15.6 Hz, –CH=C*H*$_2$), 5.13 (dd, 1H, *J*=1.5, 10.5 Hz, –CH=C*H*$_2$), 4.55 (brd, 2H, *J*=5.6 Hz, –C*H*$_2$CH=CH_2), 4.47 (brt, 1H, *J*=6.4 Hz, Ser–αH), 4.38 (d, 1H, *J*=3.2 Hz, Thr–αH), 4.36–4.28 (m, 3H, Ser–αH, 2 Lys–αH), 4.26 (d, 1H, *J*=6.9 Hz, Thr–αH), 4.22 (dd, 1H, *J*=3.0, 6.4 Hz, Thr–βH), 4.09 (dd, 1H, *J*=3.4, 6.3 Hz, Thr–βH), 3.99 (dd, 1H, *J*=3.0, 6.4 Hz, Ser–αH), 3.77 (dd, 1H, *J*=4.6, 10.0 Hz, Ser–βH), 3.65 (dd, 1H, *J*=4.4, 9.3 Hz, Ser–βH), 3.62–3.56 (m, 3H, 3 Ser–βH), 3.52 (dd, 1H, *J*=6.1, 12.7 Hz, Ser–βH), 2.92 (m, 4H, Lys–εH), 1.61 (m, 4H, Lys–βH), 1.34 (m, 4H, Lys–βH), 1.42–1.25 (m, 26H, 2 *t*-Bu, 4 Lys–γH, 4 Lys–δH), 1.16–1.13 (m, 36H, 4 *t*-Bu), 1.09 (d, 3H, *J*=6.4 Hz, Thr–γH), 1.02 (d, 3H, *J*=6.3 Hz, Thr–γH). MALDI TOF·MS *m/z* Calcd for $C_{58}H_{108}N_9O_{17}Na$ $(M+H)^+$: 1203.52, found: 1203.0.

Deallylation of 15 (synthesis of 22). The above glycosyl threonine-bound resin **15** (79 mg, 0.04 mmol) was stirred with $Pd(PPh_3)_4$ (9 mg, 8 μmol) and *N*-methylaniline (87 μl, 0.8 mmol) in THF (1 ml) under Ar overnight. The mixture was filtered and the resin was washed successively with THF, CH_2Cl_2 and ether. A small resin sample (2 mg) was stirred with TFA (30 μl) and the cleavage product was analyzed by HPLC on C18 silica gel and TOF mass spectra. The presence of the unreacted allyl ester derivative (*m/z* 584.24) in the hydrolysate indicated 39% conversion. The deallylation was completed by two more repetition of this procedure to give **22**. The deallylated product in the hydrolysate was confirmed as Fmoc–Thr(GalNAc)–OH, MALDI TOF·MS *m/z* 567.15 [Calcd for $C_{27}H_{32}N_2O_{10}Na$ $(M+Na)^+$, 567.19].

***N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-prolyl-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonyl-*O*-(*tert*-butyl)-L-seryl-*O*-(*tert*-butyl)-L-seryl-*O*-(*tert*-butyl)-L-seryl-*O*-(*tert*-butyl)-L-threonyl-N$^{\epsilon}$-(*tert*-butoxycarbonyl)-L-lysyl-N$^{\epsilon}$-(*tert*-butoxycarbonyl)-L-lysyl-L-threonine allyl ester 26.** A mixture of the deallylated resin **22** (111 mg, 0.06 mmol), heptapeptide **21** (92 mg, 0.08 mmol), 0.45 M HBTU/DMF (214 μl, 0.10 mmol), 1 M HOBt/NMP (30 μl, 0.03 mmol) and 2 M $^{i}Pr_2NEt$/NMP (48 μl, 0.10 mmol) in NMP (0.5 ml) was stirred overnight. The resin was collected by filtration and washed successively with NMP, CH_2Cl_2 and ether. Monitoring of the condensation was performed using a small resin sample (4 mg). HPLC and MS analyses of the hydrolyzed sample revealed the complete conversion of **22**, where generation of glycosyl octapeptide was detected as MALDI TOF·MS *m/z* 1304.55 [Calcd for $C_{59}H_{90}N_{11}O_{22}$ $(M+H)^+$, 1304.62]. The resin **23** thus obtained was stirred with 10% piperidine/NMP (1 ml) for 1.5 h. After filtration and washing with NMP, the resin was again treated with 10% piperidine/NMP (1 ml) for another 30 min. The resin was washed with NMP, CH_2Cl_2 and ether. A mixture of Fmoc–Pro–OH (24 mg, 0.07 mmol), 0.45 M HBTU/DMF (0.2 ml, 0.09 mmol), 1 M HOBt/NMP (90 μl, 0.09 mmol) and 2 M $^{i}Pr_2NEt$/NMP (72 μl, mmol) was stirred for 30 min. The *N*-deprotected resin was added to the activated proline mixture and stirring was continued overnight. The coupling was readily monitored by HPLC and MS of the hydrolyzed sample, MALDI TOF·MS *m/z* 1401.67 [Calcd for $C_{64}H_{97}N_{12}O_{23}$ $(M+H)^+$, 1401.67]. After washing, the resin (**24**) was *N*-deprotected as described above. In a similar manner, Boc–Ala–OH (14 mg, 0.07 mmol) was activated and reacted with the resin to give **25** (131 mg).

The resin **25** (65 mg) was stirred with CsF (15 mg, 0.10 mmol) and AcOH (30 μl, 0.10 mmol) in DMF (0.5 ml) for two days. The resin was filtered off and washed with DMF, CH_2Cl_2 and ether. The combined filtrate and washings were concentrated in vacuo and the residue was extracted with $CHCl_3$. The extract was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was chromatographed by preparative HPLC on C18 silica gel with a gradient elution of aq. CH_3CN containing 0.1% TFA (50–80%/0–30 min). The collected glycopeptide fractions were concentrated in vacuo to give **26** (13 mg, 86%). The isopropylidene group was readily removed during concentration of the acidic solution. $[\alpha]_D=-5.3°$ (*c* 0.2, MeOH). ^{1}H NMR: δ 7.65–7.12 (m, 6H, 3 Ser–N*H*, 3 Thr–N*H*), 5.91 (m, 1H, –C*H*=CH_2), 5.32 (d, 1H, *J*=17.3 Hz, –CH=C*H*$_2$), 5.22 (d, 1H, *J*=10.5 Hz, –CH=C*H*$_2$), 4.98 (d, 1H, *J*=3.7 Hz, H-1), 4.65 (brd, 2H, *J*=5.6 Hz, –C*H*$_2$CH=CH_2), 4.49–4.41 (m, 3H, 2 Ser–αH, Thr–αH), 4.37–4.30 (m, 3H, Ser–αH, 2 Thr–βH), 4.20 (brd, 1H, *J*=3.1 Hz, Thr–βH), 4.19–4.14 (m, 3H, H-2, 2 Thr–αH), 3.93 (brs, 1H, H-4), 3.91–3.81 (m, 3H, H-3, 2 Ser–βH), 3.73 (brd, 1H, *J*=4.9 Hz, Ser–βH), 3.12–3.02 (m, 4H, 2 Lys–εH), 1.22, 1.21, 1.95, and 1.18 (4s, 36H, 4 *t*-Bu), 1.17, 1.15, and 1.13 (3brs, 9H, 3 Thr–γH). HR FAB·MS *m/z* Calcd for $C_{83}H_{148}N_{13}O_{28}$ $(M+H)^+$: 1775.0557, found: 1775.0554.

L-Alanyl-L-prolyl-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonyl-L-seryl-L-seryl-L-seryl-L-threonyl-L-lysyl-L-lysyl-L-threonine 27. The resin **25** (64 mg, 8.35 μmol) was deallylated by the same procedure as described for the synthesis of **22** and the glycopeptide was detached from the resin by treatment with TFA (1 ml). The cleavage solution and aq. CH_3CN washings were concentrated in vacuo. The crude product was purified by reversed

phase chromatography as described above. By the gradient elution of aq. CH_3CN containing 0.1% TFA (5–25%/0–20 min), was isolated pure **27** (8 mg, 79%). $[\alpha]_D=-44.1°$ (*c* 0.3, H_2O), lit.[6] −33.4° (*c* 1, H_2O). ^1H NMR (D_2O, *t*-BuOH at δ 1.23): δ 4.94 (brs, 1H, H-1), 4.68 (brt, 1H, *J*=6.4 Hz, Pro–αH), 3.98 (brs, 1H, H-4), 3.75 and 3.64 (2m, 2H, Pro–δH), 2.99 (m, 4H, Lys–ϵH), 2.40 (m, 1H, Pro–βH), 2.13–1.93 (m, 3H, Pro–βH, 2 Pro–γH), 2.04 (s, 3H, Ac), 1.92–1.70 (m, 4H, Lys–γH), 1.66 (m, 4H, Lys–δH), 1.53 (d, 3H, *J*=6.6 Hz, Ala–βH), 1.46 (m, 4H, Lys–βH), 1.33 (d, 3H, *J*=6.3 Hz, Thr–γH), 1.21 (d, 3H, *J*=6.4 Hz, Thr–γH), 1.18 (d, 3H, *J*=6.1 Hz, Thr–γH). HR FAB·MS *m/z* Calcd for $C_{49}H_{88}N_{13}O_{22}$ $(M+H)^+$: 1210.6167, found: 1210.6176.

Acknowledgements

We thank Dr T. Chihara and his staff for elemental analyses, Mr M. Chijimatsu for amino acid analyses, and Ms A. Takahashi for technical assistance.

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