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Carbohydrate Research 329 (2000) 773–780

CARBOHYDRATE RESEARCH

Solid-phase synthesis of the glycopeptide of human glycophorin AM bearing the consecutive sialyl-T antigen

Sumie Ando [a,c], Yuko Nakahara [a,c], Yukishige Ito [a,c], Tomoya Ogawa [a,c], Yoshiaki Nakahara [a,b,c,*]

[a] *Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-0198, Japan*
[b] *Department of Industrial Chemistry, Tokai University, Kitakaname 1117, Hiratsuka-shi, Kanagawa 259-1292, Japan*
[c] *CREST, Japan Science and Technology Corporation (JST), Japan*

Received 19 June 2000; accepted 31 July 2000

Abstract

Fmoc solid-phase synthesis of the *N*-terminal glycopentapeptide of human glycophorin AM, bearing the consecutive sialyl-T antigen, was accomplished using glycosylated amino acid building blocks on a weakly acid-labile resin with high efficiency. The benzylated glycopeptide was treated with TMSOTf-thioanisole in TFA and then with aq $NaHCO_3$ to afford the deprotected glycopeptide in good yield.

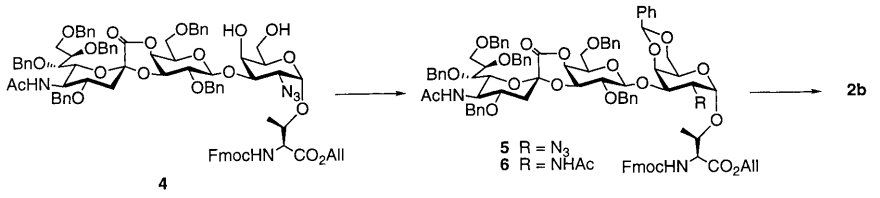
Keywords: Glycophorin A; Solid-phase synthesis; Sialic acid

1. Introduction

Solid-phase methodology has been utilized extensively to synthesize a variety of compounds because it allows easy separation of the product from the reaction media by simple filtration and accordingly brings about a considerable reduction in total time of synthesis. Development of automation techniques and the ready availability of functionalized resins have greatly facilitated solid-phase synthesis [1]. We have long been interested in the synthesis of glycopeptides that would be useful tools to study the biological roles of protein-bound oligosaccharides. In these studies we have employed the benzyl group as a persistent protecting group for carbohydrate moieties and have performed Fmoc peptide chemistry [2]. In order to encourage this strategy toward the larger glycopeptides or full-length glycoproteins, block coupling of segmentary glycopeptides, in which the suitably protected glycopeptide segments would be required as the N- and C-components, is indispensable. Recently, we have reported the solid-phase synthesis of a trimeric disaccharide-bound glycopeptide by combination of stepwise coupling and small-segment coupling methods using a newly designed silyl linker [3]. In the key steps of the synthesis, two equivalents of disaccharide-linked amino acids **3a** and **3b** were used as the building blocks through activation with HBTU [*O*-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate], HOBt (1-hydroxybenzotriazole), and DIEA (*N*,*N*-diisopropylethylamine). Eventually, the glycoheptapeptide in a

* Corresponding author. Tel./fax: +81-463-502075.
E-mail address: yonak@keyaki.cc.u-tokai.ac.jp (Y. Nakahara).

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00235-4



Scheme 1.

protected form was released from the resin by fluoridolysis in fairly good overall yield. On the other hand, the glycosylated amino acid units such as **3a** and **3b** are prepared by a lengthy process, and the unconsumed glycoamino acids recovered from coupling reactions cannot be reused out of concern for the possibility of racemization. Therefore, it is preferable to save the amount of valuable glycoamino acid used in each coupling reaction.

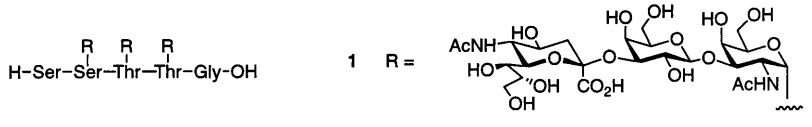
In this context, we have embarked on studies to screen coupling reagents and resins that are both compatible with the use of lesser amounts (1.5 equivalents) of glycoamino acid units.

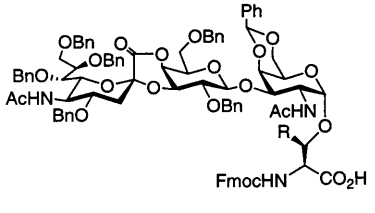
In this paper we report the solid-phase synthesis of an *N*-terminal fragment of human glycophorin AM, bearing the consecutive sialyl-T antigen [α-D-Neu5Ac-(2→3)-β-D-Gal-(1→3)-α-D-GalNAc] (**13**) performed under screened conditions. Its deprotection to compound **1** is also described.

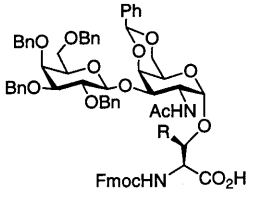
2. Results and discussion

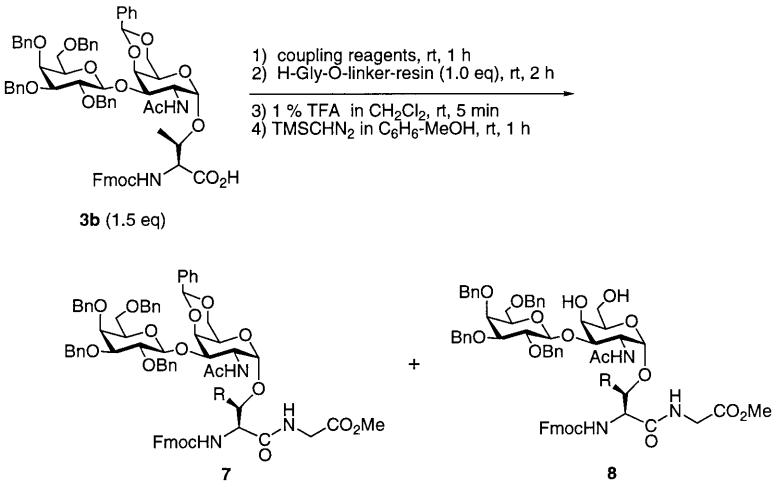
The necessary trisaccharide-linked amino acids were prepared as reported in the previous papers [2e,f]. The protected glycosyl serine **2a** was synthesized via stereocontrolled glycosylations with Gal*p*N_3, Gal*p*, Ser and Neu*p*5Ac synthons, while the threonine congener **2b** was obtained from diol **4** [4] via **5** and **6** in a similar manner (Scheme 1). A synthesis of the acylated trisaccharide linked-threonine building block has been reported recently [5].

Prior to synthesis of the target sialoglycopeptide **13**, the efficiency of the solid-phase coupling was studied. Disaccharide-linked threonine **3b** was reacted with glycine-loaded 2-chlorotrityl resin or glycine-loaded HMPB–BHA[4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid–benzhydrylamine] resin using several activating reagents. Both of these resins are weakly acid labile, and the protected glycopeptides can be released with 1% TFA in dichloromethane. The glycothreonine **3b** was









Scheme 2.

Table 1
Coupling reaction of glycosylated threonine **3b** and glycine-preloaded resin

Entry	Linker	Coupling reagents (eq. to CO_2H)	Solvent	HPLC yield,%		
				7	**8**	Total
1	2-chlorotrityl	DCC–HOBt (1.5:1.5)	DMF	24	27	51
2	2-chlorotrityl	HBTU–HOBt–DIEA (1.5:1.5:3.0)	DMF	31	13	44
3	2-chlorotrityl	HATU–HOAt–DIEA (1.5:1.5:3.0)	DMF	38	14	52
4	HMPB–BHA	HBTU–HOBt–DIEA (1.5:1.5:3.0)	DMF	47	30	77
5	HMPB–BHA	HATU–HOAt–DIEA (1.5:1.5:3.0)	DMF	65	25	90
6	HMPB–BHA	HATU–HOAt–DIEA (1.0:1.0:2.0)	DMF	37	17	54
7	HMPB–BHA	TFFH–HOAt–DIEA (1.5:1.5:3.0)	DMF	51	21	72
8	HMPB–BHA	PyBroP–DIEA (1.5:1.5)	DMF	22	18	40
9	HMPB–BHA	HATU–HOAt–DIEA (1.5:1.5:3.0)	NMP	69	20	89

activated with coupling reagents at room temperature for 1 h before mixing with the resin. The coupling reaction was performed under stirring by a vortex tube mixer at room temperature for 2 h. The yields were determined from the HPLC of the methyl esters derived from the acidolysis products **7** and **8**. A part of the product was debenzylidenated during the cleavage step. The yields were calculated by comparison with standard solutions of **7** and **8** (Scheme 2), and results of the coupling reactions are summarized in Table 1.

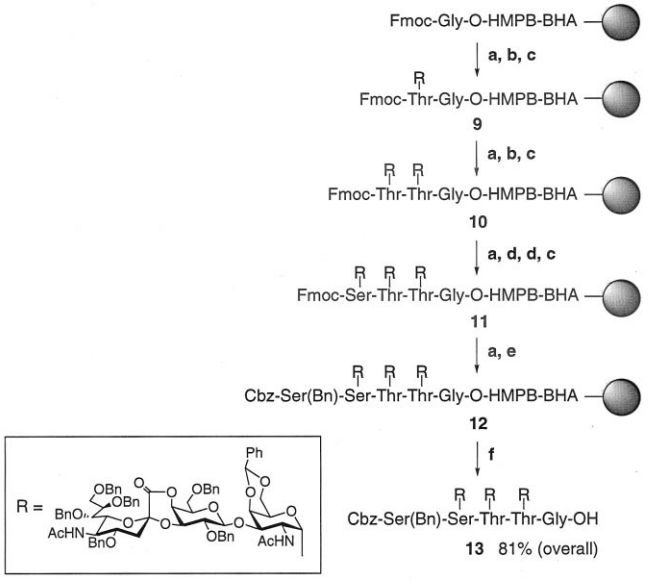
Entries 1 and 2 show the reactions on the 2-chlorotrityl resin with the most commonly used DCC–HOBt and HBTU–HOBt–DIEA in DMF. The coupling yields were only moderate in both cases. HATU [*O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]–HOAt (1-hydroxy-7-azabenzotriazole)–DIEA [6] also gave a similar result (entry 3). On the other hand, pronounced yield enhancement was observed when HMPB–BHA resin was employed (entries 4 and 5). Use of a smaller equivalent of the coupling reagents diminished the yield as anticipated (entry 6). Two other coupling reagents, TFFH (tetramethylfluoroformamidium hexafluorophosphate) [7] and PyBroP (bromotris(pyrrolidino)phosphonium hexafluorophosphate) [8] were also examined, but no significant improvement was observed (entries 8 and 9). The use of NMP (*N*-methylpyrrolidone) as the solvent in place of DMF gave a comparable result.

Based on these results, the solid-phase synthesis of *N*-terminal glycopentapeptide of glycophorin AM **13** was executed on a HMPB–BHA resin using 1.5 equivalents of HATU–HOAt–DIEA in NMP.

The synthetic cycles involving *N*-deprotection with 20% piperidine in NMP, peptide coupling, and capping with Ac_2O were carried out with **2b**, **2a**, and *O*-benzyl-*N*-benzyloxycarbonyl-L-serine as acyl donors. The coupling efficiency was monitored by a ninhydrin test, and the estimated yields of intermediary peptides **9**, **10**, **11** (after the first coupling), and **12** were 96.1, 99.2, 92.0, and 99.8%, respectively. When the coupling yield was less than 95%, a double coupling procedure was applied. Accordingly, the double coupling with **2a** for **11** led to an increase in yield up to 99.7% (Scheme 3).

The glycopeptide thus synthesized was cleaved from the solid support using a solution of 1% TFA–1% benzaldehyde in dichloromethane at room temperature for 5 min. The added benzaldehyde was used to suppress debenzylidenation in the acidic mixture. Purification by gel-permeation chromatography gave the glycopentapeptide **13** in 81% overall yield. The glycopeptide **13** was identified by ^1H NMR spectroscopy and MALDI-TOF mass spectrometry. A small amount of monodebenzylidenated compound was observed in the mass spectrum.

Full deprotection of glycopeptide **13** was accomplished by treatment with 1 M trimethylsilyl trifluoromethanesulfonate–thioanisole in TFA [9], followed by lactone hydrolysis with 0.1 M sodium hydrogen carbonate in deuterium oxide [2f,10] to exclusively afford **1** (Scheme 4).



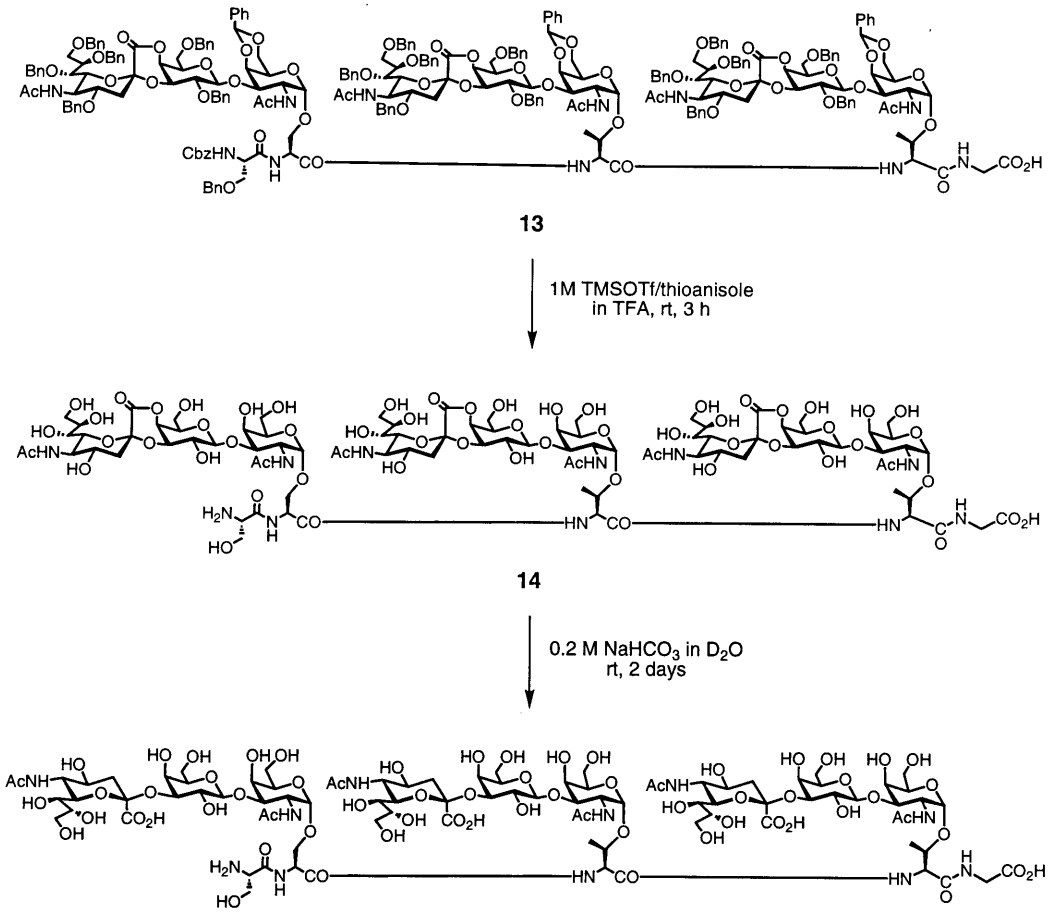
Scheme 3. Solid-phase glycopeptide synthesis: (a) 20% piperidine in NMP, rt, 0.5 h × 2; (b) **2b** (1.5 equiv), HATU–HOAt–DIEA, rt, 2 h; (c) Ac_2O–DIEA–HOBt, rt, 0.5 h; (d) **2a** (1.5 equiv), HATU–HOAt–DIEA, rt, 2 h; (e) Cbz–Ser(Bn)–OH (4.0 equiv), HATU–HOAt–DIEA, rt, 2 h; (f) 1% TFA + 1% PhCHO in CH_2Cl_2, rt, 5 min.

In conclusion, the synthesis of a glycopentapeptide bearing the bulky consecutive sialyl T-antigen was accomplished saving the amount of valuable acyl donors **2a** and **2b** by means of Fmoc methodology with HATU–HOAt–DIEA on an acid-labile resin. Studies on the synthesis of larger glycopeptides using the protected segment **13** are in progress. The deprotected glycopeptide **1**, representing the epitope of human blood group type M, will be a useful tool for related biological studies.

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 F_{254} (E. Merck 230–400 mesh). TLC was performed on Silica Gel 60 F_{254} (E. Merck). ^1H and ^{13}C NMR spectra were recorded with either a JEOL ECP 500, α 400 or EX 400 spectrometer. Chemical shifts were expressed in ppm downfield from the signal for internal Me_4Si for solutions in $CDCl_3$ and *t*-BuOH for solutions in D_2O. MALDI-TOF mass spectra were obtained with a Shimadzu MALDI-4 [Norharmane or 2,5-dihydroxybenzoic acid (DHBA) were used as matrix]. H-Gly-preloaded 2-chlorotrityl and Fmoc Gly-preloaded HMPB–BHA resins were purchased from Calbiochem–Novabiochem Japan Ltd. All the solid-phase reactions were performed at rt in capped polypropylene test tubes or in Assist mini columns with stirring on a vortex tube-mixer. HPLC analyses for **7** and **8** were performed on a column [Kanto Chemical, LiChroCART 250-4, LiChrospher Si 60 (5 μm, 4 mm i.d. × 250 mm), MeOH–$CHCl_3$ (gradient mode, 0–10% for 30 min, 1 mL/min]. The analysis of **1** was performed on a reversed-phase column [Kanto Chemical, MightysilRP-18 (5 μm, 3 mm i.d. × 250 mm)].

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)*-



Scheme 4.

lactone]-L-*threonine allyl ester* (**5**).—A mixture of **4** [4] (1.30 g, 0.69 mmol), α,α-dimethoxytoluene (0.91 mL, 6.0 mmol), and *p*-TsOH (10 mg, 0.05 mmol) in CH_3CN (40 mL) was stirred at rt for 15 min, and the reaction was quenched with a few drops of pyridine before being concentrated in vacuo. The residue was chromatographed on a silica gel column (4:1 toluene–EtOAc to 80:19:1 toluene–EtOAc–MeOH) to give **5** (976 mg, 86%). R_f 0.67 (1:1 toluene–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$, Me_4Si): 7.75 (d, *J* 7.5 Hz, Ar), 7.62 (d, *J* 7.3 Hz, Ar), 7.54 (d, *J* 6.8 Hz, Ar), 7.12–7.47 (m, Ar) (total 43 H), 5.93 (m, 1 H, All), 5.78 (d, 1 H, *J* 9.5 Hz, NH), 5.47 [s, 1 H, $PhCH(O)_2$], 5.26–5.38 (m, 2 H, All), 5.18 (d, 1 H, *J* 3.5 Hz, H-4b), 5.10 (d, 1 H, *J* 3.1 Hz, H-1a), 2.16 (dd, 1 H, *J* 13.4, 4.3 Hz, H-3c eq), 1.70 (s, 3 H, Ac), 1.32 (d, 3 H, *J* 6.2 Hz, Thr-CH_3); Anal. Calcd for $C_{94}H_{97}N_5O_{21}$: C, 69.15; H, 5.99; N, 4.29. Found: C, 69.35; H, 6.00; N, 4.08.

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-*galacto-pyranosyl)-(1→3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy-α-*D-*galactopyranosyl-(1c→4b)-lactone]*-L-*threonine allyl ester* (**6**).—To a solution of **5** (907 mg, 0.56 mmol) in THF (15 mL), was added portionwise AcOH (1.5 mL, 26 mmol) and Zn powder (3.66 g, 56 mmol). The mixture was stirred at rt for 30 min, and Ac_2O (3.0 mL, 32 mmol) was added. The resulting mixture was stirred at rt for 30 min and then filtered through Celite. The Celite was washed with EtOAc. The filtrate and washings were combined and washed with water, satd $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was chromatographed on a silica gel column with 2:1 toluene–EtOAc to afford **6** (802 mg, 88%). R_f 0.19 (1:1 toluene– EtOAc); $[\alpha]_D$ +81.3° (*c* 1.0); ^{1}H NMR (400 MHz, $CDCl_3$, Me_4Si): 7.76 (d, 2 H, *J* 7.6 Hz, Ar), 7.62 (d, 2 H, *J* 7.3 Hz, Ar), 7.56 (dd, 2 H, *J* 7.9, 1.5 Hz, Ar), 7.14–7.51 (m, 37 H, Ar), 5.85 (m, 1 H, All), 5.61 (d, 1 H, *J*

9.0 Hz, NH), 5.53 (d, 1 H, *J* 9.5 Hz, NH), 5.46 [s, 1 H, $PhCH(O)_2$], 5.27–5.35 (m, 2 H, All), 5.16 (d, 1 H, *J* 3.5 Hz, H-4b), 4.98 (brs, 1 H, H-1a), 2.11 (dd, 1 H, *J* 13.3, 4.5 Hz, H-3c eq), 1.85 (s, 3 H, Ac), 1.71 (s, 3 H, Ac), 1.27 (d, 3 H, *J* 6.1 Hz, Thr-CH_3); Anal. Calcd for $C_{96}H_{101}N_3O_{22}\cdot 0.5H_2O$: C, 69.55; H, 6.20; N, 2.53. Found: C, 69.32; H, 6.13; N, 2.45.

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-*galacto-pyranosyl)-(1→3)-2-acetamido-4,6*-O-*benzylidene-2-deoxy-α-*D-*galactopyranosyl-(1c→4b)-lactone]*-L-*threonine* (**2b**).—A mixture of **6** (1.33 g, 0.8 mmol), $Pd(PPh_3)_4$ (90 mg, 78 μmol), and dimedone (1.13 g, 8 mmol) in dry THF (80 mL) was stirred under Ar at rt for 3 h and then concentrated in vacuo. The residue was chromatographed on a silica gel column over a gradient of 20:1, 10:1 $CHCl_3$–EtOH, 10:1:0.5 $CHCl_3$–EtOH–AcOH, and then on a C_{18} reversed-phase column with 95% aq CH_3CN containing 0.1% AcOH to afford **2b** (1.10 g, 85%). R_f 0.20 (93.5:6:0.5 $CHCl_3$–MeOH–AcOH); $[\alpha]_D$ +87.5° (*c* 1.0); ^{1}H NMR (500 MHz, $CDCl_3$, Me_4Si): 7.74 (d, 2 H, *J* 7.4 Hz, Ar), 7.61 (d, 2 H, *J* 7.4 Hz, Ar), 7.53 (d, 4 H, *J* 7.2 Hz, Ar), 7.11–7.38 (m, 35 H, Ar), 6.09 (brs, 1 H, NH), 5.66 (brs, 1 H, NH), 5.41 [s, 1 H, $PhCH(O)_2$], 5.13 (s, 1 H, H-4b), 5.08 (d, 1 H, *J* 3.1 Hz, H-1a), 2.09 (brd, 1 H, *J* 13.0 Hz, H-3c eq), 1.91 (s, 3 H, Ac), 1.72 (s, 3 H, Ac), 1.64 (t, 1 H, *J* 13.0 Hz, H-3c ax), 1.17 (d, 3 H, *J* 5.7 Hz, Thr-CH_3); Anal. Calcd for $C_{93}H_{97}N_3O_{22}$: C, 69.43; H, 6.08; N, 2.61. Found: C, 69.14; H, 6.07; N, 2.59.

Gly-preloaded HMPB–BHA resin.—Commercial Fmoc-Gly-preloaded HMPB–BHA resin (0.69 mmol/g, 362 mg, 0.25 mmol) was treated with 20% piperidine–NMP using the standard synthesizer program of deprotection. A cycle of the program involved a deprotection for 21 min, and three cycles of the deprotection step were repeated to give the Fmoc-deprotected Gly-preloaded HMPB–BHA resin (307 mg, quant). Efficiency of the deprotection was monitored by the conductivity of the dibenzofulvene-piperidine adduct.

Solid-phase coupling of **3b** *and Gly-preloaded resin*

DCC–HOBt coupling (entry 1). To a solution of **3b** (16.9 mg, 14.6 μmol) in DMF (100 μL) were added 1.0 M DCC in NMP (22 μL, 22 μmol), 1.0 M HOBt in NMP (22 μL, 22 μmol) at rt. The mixture was stirred at rt for 1 h. The resultant mixture was added to the Gly-preloaded 2-chlorotriryl resin (0.54 mmol/g, 18.0 mg, 9.7 μmol) that was pre-swelled in DMF (100 μL). The mixture was mixed using a vortexing test tube mixer for 2 h at rt. The resin was collected by filtration, washed with DMF and dry CH_2Cl_2, and dried in vacuo. The resin (21.4 mg) was suspended in 1% TFA in CH_2Cl_2 (1 mL) and mixed with a vortex mixer at rt for 5 min. The reaction mixture was filtered and the resin was twice washed with CH_2Cl_2. The filtrate and washings were combined and concentrated with toluene. The residue was dissolved to 7:3 C_6H_6–MeOH (1 mL) and treated with 2 M $TMSCHN_2$ in hexane (50 μL, 100 μmol) to afford methyl esters **7** and **8**. The yields were calcd on the basis of the integrated values of HPLC peaks by comparison with the standard solution. The results are summarized in Table 1.

HBTU–HOBt–DIEA coupling (entries 2 and 4). To a solution of **3b** (17.5 mg, 15 μmol) in DMF (100 μL), were added 0.5 M HBTU–DMF (44 μL, 22 μmol), 0.5 M HOBt–DMF (44 μL, 22 μmol) and 2.0 M DIEA–DMF (22 μL, 44 μmol) at rt. The mixture was stirred at rt for 1 h. The resultant mixture was added to the Gly-preloaded 2-chlorotriryl resin (0.54 mmol/g, 19.0 mg, 10 μmol) [or Gly-preloaded HMPB–BHA resin (0.81 mmol/g, 12.3 mg, 10 μmol)] that was pre-swelled in DMF (100 μL). The mixture was mixed using a vortexing test tube mixer for 2 h at rt. The resin was collected by filtration, washed with DMF and dry CH_2Cl_2, and dried in vacuo. Dipeptides **7** and **8** were obtained by the acidic cleavage from the resin and esterification in a similar manner described in Section 3.6.1 and the yields were calcd by HPLC.

Other couplings (entries 3, and 5–9). Disaccharide threonine **3b** (17.5 mg, 15 μmol) was activated with 0.5 M HATU–0.5 M HOAt–2.0 M DIEA (or 0.5 M TFFH–0.5 M HOAt–2.0 M DIEA or 0.5 M PyBroP–2.0 M DIEA) and reacted with glycine preloaded resin in DMF (or NMP) in a manner similar to that described above for Sections 3.6.1 and 3.6.2.

N-*(Benzyloxycarbonyl)*-O-*benzyl*-L-*seryl*-O-*[(5-acetamido-4,7,8,9-tetra*-O-*benzyl-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosyl-*

onic 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-*seryl*-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-*threonyl*-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-*threonyl*-L-*glycine* (**13**). —To a solution of **2b** (48 mg, 30 μmol) in NMP (200 μL), were added 0.5 M HATU–NMP (88 μL, 44 μmol), 0.5 M HOAt–NMP (88 μL, 44 μmol) and 2.0 M DIEA–NMP (44 μL, 88 μmol) at rt. The mixture was stirred at rt for 1 h. The resultant mixture was added to the Gly-preloaded HMPB–BHA resin (25 mg, 20 μmol) that had previously been swelled in NMP (200 μL). The mixture was agitated using a vortexing test tube mixer for 2 h at rt. The resin was collected by filtration, washed with NMP and dry CH_2Cl_2, and dried in vacuo to afford **9** (67 mg). A portion of **9** (4 mg) was used for the ninhydrin test to monitor the coupling efficiency (96.1% coupling yield). The rest of the resin **9** was suspended in NMP (200 μL) and treated with Ac_2O capping solution (0.5 M Ac_2O–0.125 M DIEA–0.015 M HOBt in NMP) (1 mL) at rt for 1 h using vortexing test tube mixer to cap the unreacted amino group. The resin was rinsed three times with NMP and then twice treated with 20% piperidine in NMP (1 mL) at rt for 0.5 h. After filtration and washing with NMP and dry CH_2Cl_2, the resin was swelled in NMP (200 μmL) again and reacted with activated ester of **2b**, prepared from a solution of **2b** (48 mg, 30 μmol) in NMP (200 μL) and 0.5 M HATU–NMP (88 μL, 44 μmol), 0.5 M HOAt–NMP (88 μL, 44 μmol) and 2.0 M DIEA–NMP (44 μL, 88 μmol) at rt for 1 h as described above. The mixture was vortex mixed for 2 h at rt, filtered, washed with NMP and dry CH_2Cl_2, and dried in vacuo to give tripeptide resin **10** (84 mg, coupling yield by ninhydrin test 99.2%). The resin **10** (80 mg) was then capped with Ac_2O capping solution and N-deprotected with 20% piperidine to react with **2a**. The glycosylated serine **2a** (48 mg, 30 μmol) was activated with HATU–HOAt–DIEA in NMP at rt for 1 h and added to the N-deprotected tripeptide resin. The coupling procedure of **2a** was the same as **2b**, but the coupling yield was 92.0%. So the crude tetrapeptide **11** (86 mg) was treated with **2a** and HATU–HOAt–DIEA again to complete the coupling. After the second coupling, the yield by ninhydrin test was improved to 99.7%. The tetrapeptide **11** (91 mg) thus obtained was then coupled with *O*-benzyl-*N*-benzyloxycarbonyl-L-serine (28 mg, 85 μmol) using 0.5 M HATU–NMP (160 μL, 80 μmol), 0.5 M HOAt–NMP (160 μL, 80 μmol) and 2.0 M DIEA–NMP (80 μL, 160 μmol) to furnish the pentapeptide resin **12** (88 mg, 99.8% coupling yield).

A portion of the resin **12** (21 mg) was suspended in 1% TFA and 1% benzaldehyde in CH_2Cl_2 (1 mL) and mixed with vortexing tube mixer at rt for 5 min. The reaction mixture was filtered and the resin was washed with CH_2Cl_2 twice. The filtrate and washings were combined and concentrated with toluene. The residue was purified by a gel-permeation column (Biobeads S-X1, 15 mm i.d. × 500 mm, 1:1 toluene–EtOAc) to give glycosylpentapeptide **13** (18 mg, 81% from Fmoc-Gly-preloaded resin); R_f 0.52 (18:2:1 $CHCl_3$–MeOH–AcOH); $[\alpha]_D$ + 83.3° (*c* 0.5); ^{1}H NMR (500 MHz, 20% MeOH–$CDCl_3$, Me_4Si): δ 5.39 [s, 3 H, $PhCH(O)_2$], 5.16 (d, 1 H, *J* 3.6 Hz, H-4b), 5.04 (d, 1 H, *J* 3.0 Hz, H-1a), 1.93–2.08 (m, 3 H, H-3c eq), 1.55–1.66 (m, 3 H, H-3c ax), 1.22 (bs, 6 H, Thr-CH_3), MALDI-TOF MS (norharmane): *m*/*z* Calcd for $C_{253}H_{275}N_{11}O_{63}$ 4477.9. Found 4505.3 $[M+Na]^+$.

L-*Seryl*-O-*[(5-acetamido-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(β*-D-*galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α*-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*seryl*-O-*[(5-acetamido-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(β*-D-*galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α*-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*threonyl*-O-*[(5-acetamido-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(β*-D-*galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α*-D-*galactopyranosyl-(1c→4b)-lactone]*-L-*threonyl*-L-*glycine* (**14**) *and* L-*seryl*-O-*[(5-acetamido-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-(β*-D-

*galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-*D*-galactopyranosy]-*L*-seryl-O-[(5-acetamido-3,5-dideoxy-*D*-glycero-α-*D*-galacto-2-nonulopyranosylonic acid)-(2→3)-(β-*D*-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-*D*-galactopyranosyl]-*L*-threonyl-O-[(5-acetamido-3,5-dideoxy-*D*-glycero-α-*D*-galacto-2-nonulopyranosylonic acid)-(2→3)-(β-*D*-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-*D*-galactopyranosyl]-*L*-threonyl-*L*-glycine* (**1**). —The protected glycopeptide **13** (8.9 mg, 2.0 μmol) was stirred with 1 M TMSOTf–thioanisole in TFA (200 μL, 200 μmol) [9] at rt for 3 h. The mixture was added dropwise to dry cold ether (6 mL) to precipitate glycopeptide, and the ethereal layer was pipetted out after centrifugation. The precipitate was chromatographed on a gel-permeation column (Sephadex LH-20) with 10% aq CH_3CN to give two major fractions. The first fraction (1.3 mg) consisted of the glycopeptide which kept lactonic structure, **14** and carboxylic acid **1** (**14**:**1** = 3:1). The second (2.9 mg) was lactone **14** (total 89%). **14**: R_f 0.49 (2:2:2:1 *n*-BuOH–MeOH–water–AcOH); $[\alpha]_D$ +43.5° (*c* 0.17); ^{1}H NMR [400 MHz, D_2O, *t*-BuOH (1.24)]: δ 5.28 (d, 3 H, *J* 3.9 Hz, H-4: Gal), 5.02 (d, 1 H, *J* 2.0 Hz, H-1: GalNAc), 4.92 (d, 1 H, *J* 3.4 Hz, H-1: GalNAc), 2.58 (dd, 3 H, *J* 13.0, 5.0 Hz, H-3 eq: NeuAc), 2.02, 2.00, 1.98, 1.96 (4 s, 18 H, NAc), 1.79 (t, 3 H, *J* 13.0 Hz, H-3 ax: NeuAc), 1.31 (d, 3 H, *J* 6.3 Hz, Thr-CH_3), 1.26 (d, 3 H, *J* 6.3 Hz, Thr-CH_3). MALDI-TOF MS (DHBA): *m*/*z* Calcd for $C_{91}H_{143}N_{11}O_{61}$ 2367.2. Found 2390.5 $[M+Na]^+$.

The mixture of **14** and **1** (1.3 mg) was dissolved in 0.2 M $NaHCO_3$–D_2O (0.75 mL), and the solution (pH ~ 8.5) was left at rt for 2 days, the progress of lactone hydrolysis being monitored by ^{1}H NMR spectroscopy. The mixture was chromatographed on a gel-permeation column (Sephadex LH-20) with 10% aq CH_3CN to give **1** (1.4 mg, quant). HPLC analysis on a C_{18} column with a gradient elution of aq CH_3CN [A: 0.1% aq TFA, B: 80% aq CH_3CN–0.1% TFA, A/B = 100/0 (0–3 min)–0/100 (3–40 min)] showed the product **1** of more than 94% purity. ^{1}H NMR [500 MHz, D_2O, *t*-BuOH (1.24)]: δ 5.01 (d, 1 H, *J* 3.6 Hz, H-1: GalNAc), 4.98 (d, 1 H, *J* 3.6 Hz, H-1: GalNAc), 4.89 (d, 1 H, *J* 3.6 Hz, H-1: GalNAc), 4.51–4.54 (m, 3 H, H-1: Gal), 2.75 (dd, 3 H, *J* 12.3, 4.5 Hz, H-3 eq: NeuAc), 2.02, 2.01, 2.00, 1.97 (4 s, 18 H, NAc), 1.79 (t, 3 H, *J* 12.3 Hz, H-3 ax: NeuAc), 1.36 (d, 3 H, *J* 5.3 Hz, Thr-CH_3), 1.25 (d, 3 H, *J* 5.9 Hz, Thr-CH_3). MALDI-TOF MS (DHBA): *m*/*z* Calcd for $C_{91}H_{149}N_{11}O_{64}$ 2421.2. Found 2446.0 $[M+Na]^+$, 2466.7 $[M-H+2Na]^+$, 2486.9 $[M-H+Na+K]^+$, 2510.6 $[M-2H+2Na+K]^+$, 2532.5 $[M-3H+3Na+K]^+$, 2549.2 $[M-3H+2Na+2K]^+$.

Acknowledgements

This work was financially supported by the CREST program of the Japan Science and Technology Corporation. We are grateful to Drs J. Uzawa, H. Koshino and Ms T. Chijimatsu for NMR measurements and Mr M. Chijimatsu for amino acid analysis. We thank Dr T. Chihara and his staff for elemental analyses, and Ms A. Takahashi for technical assistance.

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