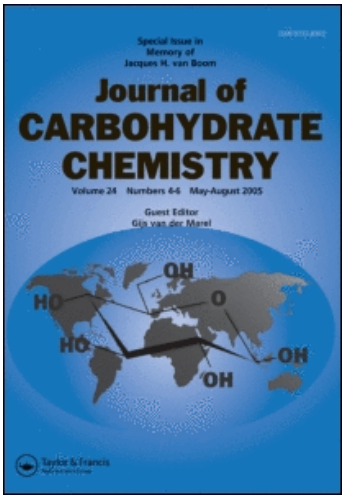

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SYNTHESIS AND PROPERTIES OF NEOGLYCOCONJUGATES CARRYING A DIMERIZATION MOTIF OF GLYCOPHORIN A TRANSMEMBRANE DOMAIN[1]

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ABSTRACT

A new class of glycopeptides **8** and **10**, were synthesized from 3-carboxypropyl lactoside **4** and tricosapeptide **6** on a solid support. Their amino acid sequences include a dimerization motif of glycophorin A transmembrane domain. The dimerizable properties of the synthesized glycopeptides and non-glycosylated peptides were investigated by Tris-Tricine-SDS-PAGE.

INTRODUCTION

Neoglycoconjugates have been designed and synthesized as excellent tools to study carbohydrate-mediated biological events and to develop useful molecules such as artificial vaccines.[5] Several examples have been reported in terms of the method used for the conjugation between carbohydrates and natural or unnatural polymer carriers. Most of these studies have been directed to efficiently accumulate carbohydrates on the surface of the carriers, but little attention seems to have been paid in designing the molecular architecture of carrier molecules to implement the specific functions.

In 1992, Lemmon et al.[6] reported production and characterization of a chimera protein which carried a dimerization motif derived from glycophorin A transmembrane domain (GpA-TM) . From their biochemical studies, they showed a pattern of seven amino acids (LIxxGVxxGVxxT) in GpA-TM (Figure 1), that is sufficient to drive the specific

GpA transmembrane domain

H-Ile-Thr-Leu-Ile-Ile-Phe-Gly-Val-Met-Ala-Gly-Val-Ile-Gly-Thr-Ile-Leu-Leu-Ile-Ser-Tyr-Gly-Ile-OH

Synthetic peptide containing LIxxGVxxGVxxT (**1a**)

H-Leu-Leu-Leu-Ile-Leu-Leu-Gly-Val-Leu-Leu-Gly-Val-Leu-Leu-Thr-Leu-Leu-Leu-Leu-Leu-Leu-Leu-Leu-OH

Single mutant of **1a** (**1b**)

H-Leu-Leu-Leu-Ile-Leu-Leu-Gly-Val-Leu-Leu-Ser-Val-Leu-Leu-Thr-Leu-Leu-Leu-Leu-Leu-Leu-Leu-Leu-OH

Figure 1. Sequence of GpA transmembrane domain and synthetic peptides. Seven key amino acids contribute to the dimerization of GpA-TM are enclosed in boxes. A Ser is enclosed in a circle.

dimerization of transmembrane α-helices in staphylococcal nuclease/GpA chimera and displacement of the second glycine (G) residue by serine made the protein lose the dimerization property.[7] It has been also proposed that the hydrophobic transmembrane domains play their roles not only in anchoring molecules into lipid bilayer but also in assembling molecules and then promoting intermolecular recognition.[8] These interesting features prompted us to design new glycoconjugates which contain an artificial intermolecular recognition sequence.

In this paper, we describe a novel design and synthesis of neoglycopeptide carrying dimerization motif.

RESULTS AND DISCUSSION

Synthesis of Neoglycopeptide

Whether the specific amino acid sequence as described by Lemmon et al., when incorporated in a short peptide, can also bring about dimerization is not yet established. In order to ascertain this we first synthesized compound **1a** (Figure 1) involving the above sequence necessary for dimerization and examined the property by electrophoresis. The peptide **1b**, which was not expected to dimerize owing to the substitution of Gly11 by Ser, was also prepared. Subsequently, we designed neoglycopeptide **8** and **10** with β-lactoside linked to the polypeptide via a 3-carboxypropyl linker as a first example of this series.

Synthesis was carried out as follows. The β-lactosyl derivative **4** was synthesized from α-D-hepta-*O*-acetyllactosyl bromide[9] (**2**) in two steps, i.e., the bromide **2** was glycosylated with excess amount of 1,4-butanediol in the presence of silver oxide, followed by the oxidation with pyridinium dichromate (PDC) (Scheme 1).

The synthetic peptides **1a** and **1b** (Figure 1 and Scheme 2) were prepared by solid-phase synthesis techniques. The coupling yield of the peptides was determined by the ninhydrin test at each step. The total coupling yields of peptide-resins **5a** and **5b** were 84% and 77%, respectively. Fmoc cleavage of **5** was performed with 20% piperidine in *N*-methylpyrrolidone (NMP) to give peptide-resin **6**. Cleavage of the peptide from the resin was performed by treatment with 95% aqueous TFA at room temperature for 1.5 h, under which conditions the protecting groups on amino acids were also removed concomitantly.

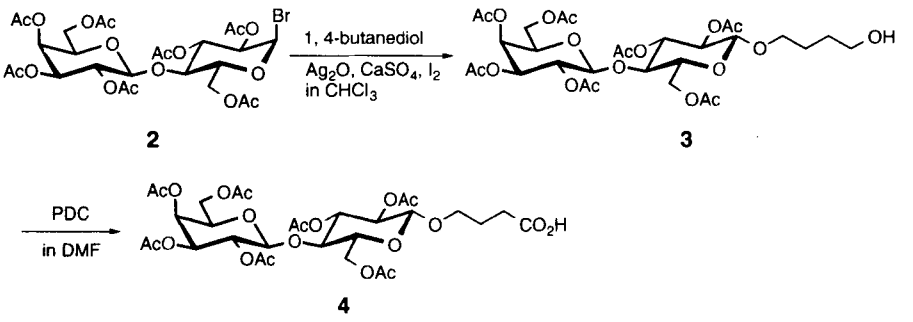
After the cleavage, the peptide **1** was purified by reversed phase HPLC in aqueous 2-propanol with 0.1% trifluoroacetic acid (TFA). Synthetic peptide easily aggregated and was only slightly soluble in 2-propanol with 0.1% TFA, especially in the case of **1a**. So the separation by HPLC was inefficient. The aggregated peptide was dissociated by dissolving in a mixture of dichloromethane and trifluoroethanol (3 : 1),[10] concentrated and then subjected repeatedly to purification by HPLC.

Coupling of the lactosyl derivative **4** with the peptide-resin **6** was carried out manually, i.e., **4** (4 equiv) was activated by dicyclohexylcarbodiimide (DCC) (10 equiv) and 1-hydroxybenzotriazole (HOBt) (10 equiv), then mixed with **6** (1 equiv) on a vortex mixer overnight at room temperature. The coupling yield of the glycopeptide-resin **7** calculated by ninhydrin test was about 95%. After the cleavage by aqueous TFA, the crude glycopeptide **8** (**8a** in 97% and **8b** in 93% yield by weight) was purified by reversed phase HPLC in aqueous 2-propanol with 0.1% TFA.

To obtain deprotected lactosyl peptide, **7a** was deacetylated with sodium methoxide in methanol (pH 8.5 - 9.0). The deacetylated glycopeptide-resin **9a** was treated with 95% aqueous TFA to give **10a**. No significant degradation of lactoside occurred, but contamination with a trace amount of the mono-acetylated derivative was observed in the mass spectrum.

Electrophoresis of Peptides and Gylcopeptides

The dimerization properties of synthetic peptide **1** and glycopeptide **8** and **10** in SDS solution were analyzed by Tris-Tricine [*N*-tris(hydroxymethyl)methylglycine]-SDS-polyacrylamide gel electrophoresis (PAGE).[11] The SDS-PAGE of **1a** and **1b** was shown in left panel of Figure 2. In the case of **1a**, main band was observed at 5 kDa, a dimer of **1a** (lane 2). On the other hand, the band of **1b** was at 2.5 kDa, a monomer size (lane 4).



Scheme 1

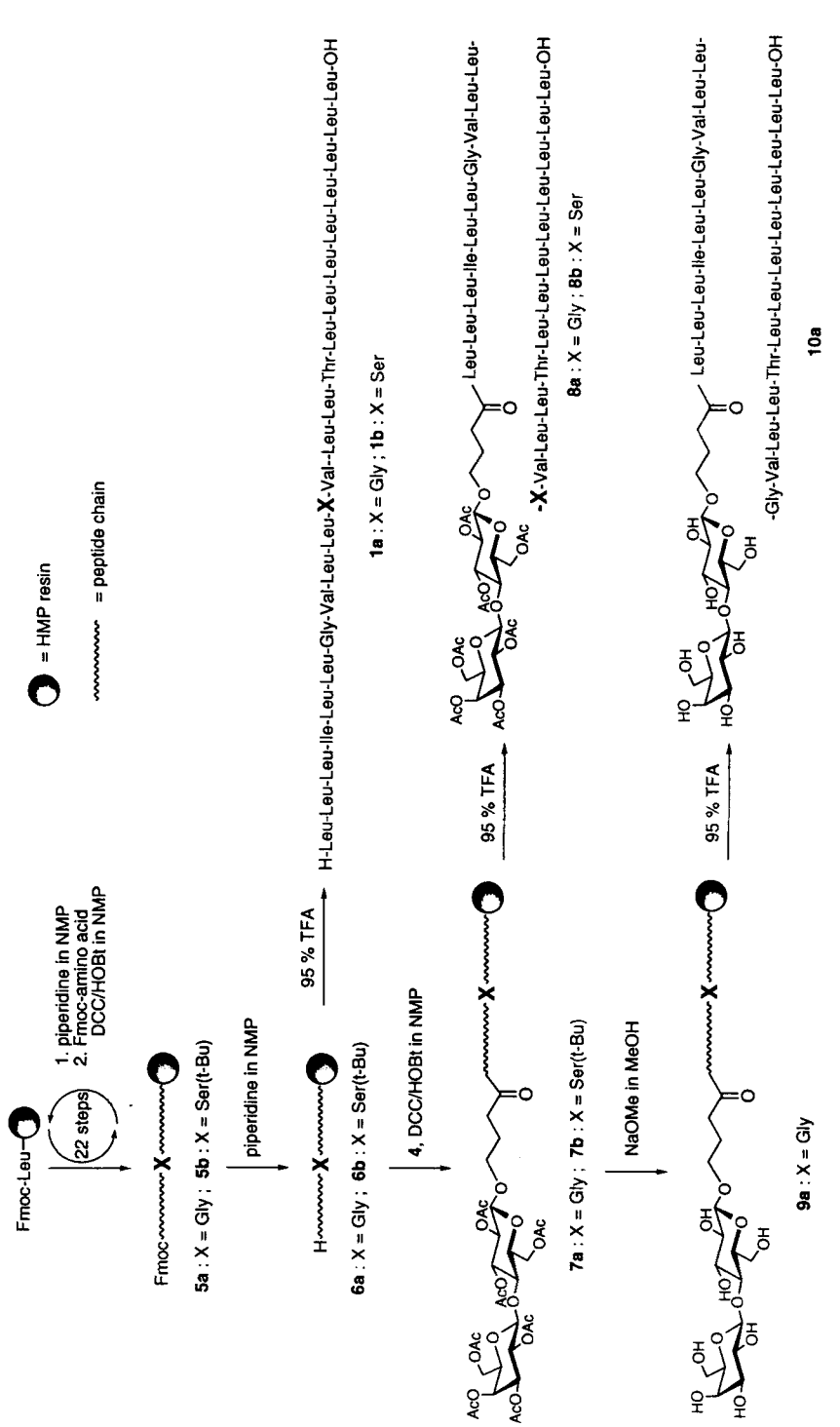
Apparently the displacement of glycine by serine resulted in the disruption of dimerization. Furthmayr et al.[12] reported the equilibrium between dimer and monomer was dependent on the temperature of incubation. Therefore we incubated the samples for 5 min at 37 ℃ and 100 ℃, respectively. Analysis of the samples revealed that properties of these peptides were temperature-independent (lane 2 vs. 3 and lane 4 vs. 5).

A similar experiment was carried out with the glycopeptides and the result is shown in the right panel of Figure 2. The two different incubation conditions were applied to the glycopeptides **8a** and **8b**. One is at 37 ℃, another is at 100 ℃, but no temperature-effect was again observed (lane 8 vs. 9 and lane 10 vs. 11). Regardless of the incubation condition, the SDS-PAGE of **8a** and **10a** shows a distinct band, which has an apparent molecular mass of a dimer, with a smear below the dimer band (lanes 8, 9 and 12). In contrast, **8b** clearly ran as a monomer (lanes 10 and 11). The results of the electrophoretic analysis led us to conclude that the LIxxGVxxGVxxT motif promotes dimerization between such small peptides as a 23mer and the ability is retained even after the introduction of the sugar derivative.

CONCLUSION

In this paper, we described the synthesis of a new class of glycopeptides and preliminary results on their electrophoretic analyses, where the dimerization of synthetic peptide **1a** and glycopeptide **8a** and **10a** was demonstrated.

In order to examine a possible acceleration of the intermolecular reaction between two glycan parts by taking advantage of this interesting interhelical interaction, further investigation will be necessary with respect to improving the solubility of peptide in solvents, controlling the reversibility between monomer and dimer, and so on. The study is ongoing.



Scheme 2

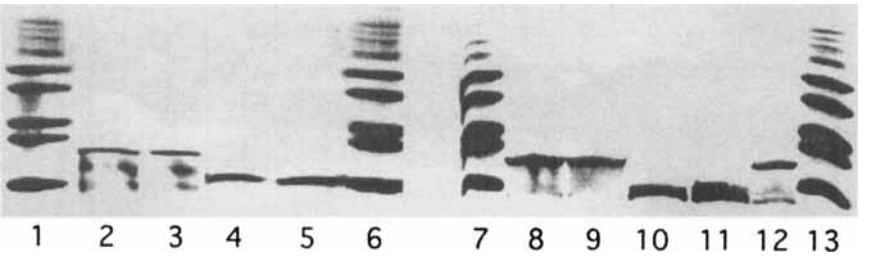


Figure 2. Tricine-SDS-PAGE of peptides and glycopeptides.
Lanes 1, 6, 7, 13 show low molecular mass markers (Pharmacia), of 2.5, 6.2, 8.2, 10.7, 14.4 kDa from the bottom. Lanes 2 and 3 show 4 μg of **1a**, incubated in SDS for 5 min at 37 °C and 100 °C, respectively, before loading. Lanes 4 and 5 show 4 μg of **1b**, incubated in SDS for 5 min at 37 °C and 100 °C, respectively, before loading. Lanes 8 and 9 show 20 μg of **8a**, incubated at 37 °C and 100 °C, respectively, before loading. Lane 10 and 11 show 20 μg of **8b**, incubated in SDS for 5 min at 37 °C and 100 °C before loading, respectively. Lane 12 shows 4 μg of **10a**, incubated for 5 min at 100 °C.

EXPERIMENTAL

General.

Column chromatography was performed on silica gel-60 (Merck 230-400 mesh). Optical rotations were determined with a Jasco DIP-370 polarimeter for solutions in $CHCl_3$. ^1H and ^{13}C NMR were recorded with either JEOL EX 270 [^1H (270 MHz), ^{13}C (67.8 MHz)] or JEOL α600 (600 MHz) spectrometers. Chemical shifts are expressed in ppm downfield from the signal for internal Me_4Si for solutions in $CDCl_3$. MALDI-TOF mass-spectra were obtained with a Bruker REFLEX (α-cyano-4-hydroxycinnamic acid or 2-mercaptobenzothiazole were used as a matrix). FAB mass-spectra were obtained with ThermoQuest TSQ 700 (*m*-nitrobenzyl alcohol was used as a matrix). Peptide synthesis was performed with an Applied Biosystems Model 431A peptide synthesizer. Fmoc Leu-preloaded HMP resin, Fmoc amino acids in cartridges, and the reagents for the peptide synthesis were purchased from Applied Biosystems Inc. HPLC was performed using C_8 reverse phase column [Cica-MERCK, Hiber LiChroCART LiChrospher 100 RP-8 (e) (5 μm), ϕ 4 x 250 mm (flow rate: 1 mL/min) for analysis, and Cica-MERCK, Hiber LiChroCART LiChrospher 100 RP-8 (e) (5 μm), ϕ 10 x 250 mm (flow rate: 4 mL/min) or Nakarai COSMOSIL $5C_8$, ϕ 20 x 250 mm (flow rate: 8 mL/min) for preparative HPLC] with a gradient elution of aqueous 2-propanol containing 0.1% TFA (concentration of 2-propanol: 0 - 20 min; 70 - 100%, 20 - 30 min; 100 -100%). Tris-Tricine-SDS-PAGE was performed using Bio-Rad Mini-Protein II with ATTO Cross Power 150.

4-Hydroxybutyl *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetylglucopyranoside (3). According to the manner of Reynolds and Evans,[13] 1,4-butanediol (5.41 g, 60 mmol), silver oxide (835 mg, 3.6 mmol), Drierite (545 mg, 4 mmol) preheated at 180 ℃ under reduced pressure for 2 h, and dry $CHCl_3$ (2 mL) were placed in a brown flask and stirred for 1 h at room temperature. To the mixture, iodine (30 mg, 0.12 mmol) and a solution of *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetylglucopyranosyl bromide (α-D-hepta-*O*-acetyllactosyl bromide, **2**)[9] (4.20 g, 6 mmol) in dry $CHCl_3$ (15 mL) were added. The reaction mixture was stirred for 24 h and filtered through a layer of Celite. The solid was washed with $CHCl_3$. The filtrate and washing were combined and washed with water and brine, dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column (hexane / ethyl acetate) to give **3** (3.00 g, 70%): Rf 0.20 (hexane / ethyl acetate = 1 : 4); $[\alpha]_D$ -16.7°(*c* 1.0); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.57-1.73 (m, 4 H, 2- and 3-CH_2 of butyl), 1.97 (s, 3 H, Ac), 2.05 (s, 3 H, Ac), 2.07 (s, 3 H, Ac), 2.13 (s, 9 H, Ac x 3), 2.16 (s, 3 H, Ac), 3.49-3.67 (m, 4 H, H-5, 1-CH_2 of butyl, 4-CH_2 of butyl x 2), 3.80 (dd, 1 H, *J* = 9.9, 9.2 Hz, H-4), 3.83-3.92 (m, 2 H, H'-5, 1-CH_2 of butyl), 4.04-4.17 (m, 3 H, H'-6, H-6 x 2), 4.47 (d, 1 H, *J* = 7.9 Hz, H-1), 4.49 (d, 1 H, *J* = 7.9 Hz, H'-1), 4.49-4.52 (m, H'-6), 4.89 (dd, 1 H, *J* = 9.6, 7.9 Hz, H-2), 4.95 (dd, 1 H, *J* = 10.5, 3.3 Hz, H'-3), 5.11 (dd, 1 H, *J* = 10.5, 7.9 Hz, H'-2), 5.20 (t, 1 H, *J* = 9.2 Hz, H-3), 5.35 (d, 1 H, *J* = 3.3 Hz, H'-4); ^{13}C NMR ($CDCl_3$) δ 20.4 (Ac), 20.56 (Ac), 20.63 (Ac), 20.7 (Ac), 20.8 (Ac), 25.8 (2- or 3- CH_2 of butyl), 29.3 (3- or 2- CH_2 of butyl), 60.8 (C-6), 61.9 (C'-6), 62.2 (4-CH_2 of butyl), 66.6 (C'-4), 69.1 (C'-2), 69.9 (1-CH_2 of butyl), 70.6 (C'-5), 70.9 (C'-3), 71.7 (C-2), 72.6 (C-5), 72.8 (C-3), 76.2 (C-4), 100.5 (C-1), 101.0 (C'-1), 169.0 (CO), 169.6 (CO), 169.7 (CO), 170.0 (CO), 170.1 (CO), 170.3 (CO), 170.4 (CO).

Anal. Calcd for $C_{30}H_{44}O_{19}$ (708.67): C, 50.85; H, 6.26. Found: C, 50.39; H, 6.22.

3-Carboxypropyl *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetylglucopyranoside (4).[14] A mixture of **3** (2.13 g, 3 mmol) and pyridinium dichromate (3.66 g, 10.5 mmol) in dry DMF (7 mL) was stirred for 20 h at room temperature. The reaction mixture was poured into H_2O (70 mL). The aqueous solution was extracted with diethyl ether (100 mL x 4). The extract was washed with H_2O (200 mL) and brine (200 mL), dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column (ethyl acetate / methanol) to give **4** (921 mg, 43%). The spectral data of **4** were identified with the data in ref. 14.

L-Leucyl-L-leucyl-L-leucyl-L-isoleucyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-threonyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucine (1a). An Fmoc-protected tricosapeptide-linked resin **5a** (580 mg, 71% by weight) was synthesized after twenty-two cycles of the standard synthesizer program of condensation with the DCC-HOBt activated Fmoc amino acids (1 mmol each) starting from Fmoc Leu-preloaded NMP resin (357 mg, 0.30 mmol). Efficiency of the condensation at each step was monitored by utilizing the ninhydrin test, and the overall coupling yield of the tricosapeptide was estimated as 84%.

A part of **5a** (101 mg, 28 μmol) was treated with 20% piperidine in NMP (2 mL) to remove the Fmoc group. The mixture was shaken for 1 h at room temperature and filtered. The resulting resin was washed with NMP and CH_2Cl_2, dried under reduced pressure. To this resin (**6a**, 95 mg), 95% aqueous TFA (2 mL) was added at 0 ℃ and the mixture was shaken for 1.5 h at room temperature. After the filtration, the resin was washed with TFA and CH_2Cl_2. The combined filtrate and washings were concentrated. The crude **1a** (64 mg, 91% by weight, 70% purity by HPLC) was suspended in 2-propanol, shaken for several minutes and centrifuged. The supernatant was separated by decantation and the precipitate was resuspended in 2-propanol, shaken for several minutes and centrifuged. This solubilization procedure was repeated twice. The supernatants were combined and concentrated to give the residue (15 mg), which was then purified by preparative HPLC to give tricosapeptide **1a** (2.9 mg). The precipitate (46 mg) separated by centrifugation was disaggregated by dissolving into a mixture of CH_2Cl_2 / trifluoro-ethanol (3 : 1) and proved to be the aggregated form of **1a** by HPLC and ^{1}H NMR. ^{1}H NMR (600 MHz, d_6-acetone) δ 0.88 - 1.03 (m, 114 H), 1.13 - 1.17 (m, 7 H) (CH_3 of Leu, Ile and Val, γ-CH_2 of Ile), 1.19 (d, 3 H, J = 6.4 Hz, γ-CH_3 of Thr), 1.60 - 2.05 (m, 53 H, β-CH and γ-CH of Leu, γ-CH_2 and β-CH of Ile), 2.32 - 2.40 (m, 1 H, β-CH of Val), 2.43 - 2.50 (m, 1 H, β-CH of Val), 3.69 (dd, 1H, J = 10.3 and 4.9 Hz, α-CH_2 of Gly), 3.72 (dd, 1H, J = 10.3 and 4.9 Hz, α-CH_2 of Gly), 3.78 - 3.95 (m, 6 H), 4.07 - 4.24 (m, 16 H), 4.39 - 4.46 (m, 2 H) (β-CH of Thr and α-CH of amino acids), 5.09 (bd, 1 H, J = 5.9 Hz, OH of Thr), 7.59 (d, 1 H, J = 6.8 Hz), 7.65 (d, 1 H, J = 4.9 Hz), 7.67 (d, 1 H, J = 6.8 Hz), 7.71 (d, 1 H, J = 5.4 Hz), 7.97 (d, 1 H, J =4.4 Hz), 7.99 (d, 1 H, J = 4.4 Hz), 8.11 (d, 1 H, J = 3.9 Hz), 8.16 (d, 1 H, J = 3.9 Hz), 8.19 (d, 1 H, J = 4.4 Hz), 8.24 - 8.35 (m, 9 H), 8.42 (d, 1 H, J = 4.9 Hz), 8.46 (d, 1 H, J = 3.9 Hz), 8.60 (dd, 1 H, J = 6.6 and 4.6 Hz), 8.66 (d, 1 H, J = 4.9 Hz), 8.81 (d, 1 H, J = 4.9 Hz), 8.83 (d, 1 H, J = 3.9 Hz) (NH of amino acids). MALDI-TOF MS ($C_{126}H_{231}N_{23}O_{25}$): *m/z* (positive ion mode) 2491.4 $[M+Na]^+$, 2507.4 $[M+K]^+$; (negative ion mode) 2466.9 [M-1].

L-Leucyl-L-leucyl-L-leucyl-L-isoleucyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-seryl-L-valyl-L-leucyl-L-leucyl-L-threonyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucine (1b). An Fmoc-protected tricosapeptide-linked resin **5b** (459 mg, 70% by weight) was synthesized in a similar manner as described for **5a**, from Fmoc Leu-preloaded NMP resin (294 mg, 0.25 mmol). Efficiency of the condensation at each step was monitored by utilizing the ninhydrin test and the overall coupling yield of the tricosapeptide was estimated as 70%.

In a similar manner as described for **1a**, a part of **5b** (50 mg, 14 mmol) was treated with piperidine then TFA to give **1b** (34 mg, 100% by weight, 43% purity by HPLC). After suspending in 2-propanol and centrifugation, the supernant was purified by preparative HPLC to give tricosapeptide **1b** (3.7 mg). ^{1}H NMR (600 MHz, d_6-acetone) δ 0.88 - 1.01 (m, 114 H), 1.14 - 1.16 (m, 7 H) (CH_3 of Leu, Ile and Val, γ-CH_2 of Ile), 1.23 (d, 1 H, J = 6.4 Hz, γ-CH_3 of Thr), 1.60 - 2.10 (m, 53 H, β-CH and γ-CH of Leu, γ-CH_2 and β-CH of Ile), 2.34 - 2.50 (m, 2 H, β-CH of Val), 2.83 (br s, 2 H, OH of Thr and Ser), 3.68 - 3.73 (m, 2 H), 3.79 (dd, 1H, J = 9.3 and 4.9 Hz, α-CH_2 of Gly), 3.82 - 3.85 (m, 1H) , 3.89 - 3.97 (m, 2 H), 4.04 - 4.27 (m, 19 H), 4.37 - 4.45 (m, 2 H) (β-CH of Thr and Ser and α-CH of amino acids), 7.58 (d, 1 H, J = 7.3 Hz), 7.66 (d, 1 H, J = 5.4 Hz), 7.69 (d, 1 H, J = 5.4 Hz), 7.93 - 7.96 (m, 3 H), 8.10 (t, 1 H, J = 3.7 Hz,), 8.16 (d, 1 H, J =4.4 Hz), 8.18 (d, 1 H, J = 3.9 Hz), 8.20 - 8.42 (m, 9 H), 8.26 (d, 1 H, J = 3.9 Hz), 8.31 (d, 1 H, J = 3.9 Hz), 8.45 (d, 1 H, J = 4.9 Hz), 8.62 (d, 1 H, J = 4.9 Hz), 8.75 (d, 1 H, J = 3.9 H), 8.83 (d, 1 H, J = 2.9 Hz) (NH of amino acids). FAB MS ($C_{127}H_{233}N_{23}O_{26}$): *m/z* (positive ion mode) 2520.0 $[M+Na]^+$, 2535.3 $[M+K]^+$; (negative ion mode) 2497.4 [M-1].

4-[*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetylglucopyranosyloxy]butyryl-L-leucyl-L-leucyl-L-leucyl-L-isoleucyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-threonyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucine (8a). A mixture of **4** (20 mg, 27.7 μmol) in NMP (0.5 mL), 1 M DCC in NMP (0.1 mL, 100 μmol) and 1 M HOBt in NMP (0.1 mL, 100 μmol) was stirred at room temperature for 1 h. To this activated ester, was added **6a** (30.5 mg, 8.5 μmol). The suspension was shaken on a vortex mixer (Iwaki TM-252) for 24 h at room temperature. The reaction mixture was filtered and the resin was washed with NMP and CH_2Cl_2. The resulting resin **7a** was dried under reduced pressure (31.5 mg), treated with 95% aqueous TFA (1 mL) at room temperature for 1.5 h. The resin was filtered off and washed with TFA and CH_2Cl_2. The filtrate and washings were combined and

concentrated (26.3 mg, 97% by weight, 60% purity by HPLC). A portion was dissolved into 2-propanol and purified by HPLC. ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ = 4 : 1) δ 0.86 - 1.01 (m, 114 H), 1.11 - 1.13 (m, 7 H) (CH_3 of Leu, Ile and Val, γ-CH_2 of Ile), 1.27 (d, 3 H, J = 6.4 Hz, γ-CH_3 of Thr), 1.55 - 2.00 (m, 55 H, β-CH and γ-CH of Leu, γ-CH_2 and β-CH of Ile, 2-CH_2 of propyl), 1.98 (s, 3 H, Ac), 2.06 (s, 3 H, Ac), 2.07 (s, 6 H, Ac x 2), 2.08 (s, 3 H, Ac), 2.14 (s, 3 H, Ac), 2.17 (s, 3 H, Ac), 2.26 - 2.34 (m, 1 H, β-CH of Val), 2.36 - 2.43 (m, 3 H, β-CH of Val, 3-CH_2 of propyl), 3.57 - 3.66 (m, 2 H, H-5 and 1-CH_2 of propyl), 3.69 - 3.76 (m, 2 H, α-CH_2 of Gly), 3.80 - 3.91 (m, 9 H, α-CH of amino acids, H-4, H'-5 and 1-CH_2 of propyl), 3.93 - 4.18 [m, (20 H, overlapped with a solvent peak), β-CH of Thr, α-CH of amino acids, H-6 x 2 and H'-6 x 2], 4.37 - 4.43 (m, 2 H, α-CH of amino acids), 4.51 (d, 1 H, J = 8.3 Hz, H-1), 4.55 (d, 1 H, J = 7.8 Hz, H'-1), 4.86 (ap t, 1 H, J = 9.3 Hz, H-2), 4.99 (dd, 1 H, J = 10.4 and 3.2 Hz, H'-3), 5.10 (dd, 1 H, J = 10.4 and 7.8 Hz, H'-2), 5.20 (t, 1 H, J = 9.3 Hz, H-3), 5.36 (d, 1 H, J = 3.4 Hz, H'-4), 7.18 (d, 1 H, J = 6.4 Hz), 7.57 (bs, 1 H), 7.72 (dd, 1 H, J =10.8 and 7.8 Hz), 7.88 (bs, 1 H), 8.05 - 8.27 (m, 17 H), 8.59 - 8.62 (m, 1 H), 8.70 (bs, 1 H) (NH of amino acids). FAB MS ($C_{156}H_{271}N_{23}O_{44}$): *m/z* (positive ion mode) 3173.3 $[M+H]^+$, 3195.3 $[M+Na]^+$, 3210.6 $[M+K]^+$, 3217.2 $[M+2Na-H]^+$.

4-[*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetylglucopyranosyloxy]butyryl-L-leucyl-L-leucyl-L-leucyl-L-isoleucyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-seryl-L-valyl-L-leucyl-L-leucyl-L-threonyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucine (8b). In a similar manner as described for **8a**, **4** (25 mg, 34.6 μmol) in NMP (0.5 mL) activated with 1 M DCC-NMP in NMP (0.1 mL, 100 μmol) and 1 M HOBt (0.1 mL, 100 μmol) was reacted with **6b** (31.5 mg, 8.5 μmol). After the cleavage with TFA, crude **8b** (25.4 mg, 94% by weight, 43% purity by HPLC) was obtained and purified with HPLC. ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ = 4 : 1) δ 0.85 - 1.00 (m, 114 H), 1.10 - 1.11 (m, 7 H) (CH_3 of Leu, Ile and Val, γ-CH_2 of Ile), 1.26 - 1.30 (m, 3 H, γ-CH_3 of Thr), 1.57 - 2.00 (m, 55 H, β-CH and γ-CH of Leu, γ-CH_2 and β-CH of Ile, 2-CH_2 of propyl), 1.98 (s, 3 H, Ac), 2.06 (s, 3 H, Ac), 2.07 (s, 6 H, Ac x 2), 2.08 (s, 3 H, Ac), 2.15 (s, 3 H, Ac), 2.17 (s, 3 H, Ac), 2.30 - 2.42 (m, 4 H, β-CH of Val, 3-CH_2 of propyl), 3.61 - 3.65 (m, 4 H, α-CH of amino acids, H-5 and 1-CH_2 of propyl), 3.74 (dd, 1 H, J = 11.7 and 4.9 Hz, α-CH_2 of Gly), 3.81 - 3.91 (m, 6 H, α-CH of amino acids, H-4, H'-5 and 1-CH_2 of propyl), 3.93 - 4.25 [m, (23 H, overlapped with a solvent peak), β-CH of Thr and Ser, α-CH of amino acids, H-6 x 2 and H'-6 x 2], 4.37 - 4.44 (m, 2 H, α-CH of amino acids), 4.51 (d, 1 H, J = 7.8 Hz, H-1), 4.55 (d, 1 H, J = 8.3 Hz, H'-1), 4.86 (ap t, 1 H, J = 8.8 Hz, H-2), 4.99 (dd, 1 H, J = 10.4 and 3.4 Hz, H'-3), 5.10 (dd, 1 H, J = 10.4 and 8.3 Hz, H'-2), 5.20 (t, 1 H, J = 9.3 Hz, H-3), 5.36 (d, 1 H, J =

3.4 Hz, H'-4), 7.16 (bs, 1 H), 7.57 (bs, 1 H), 7.74 (bs, 1 H), 7.96 - 8.30 (m, 17 H), 8.48 (bs, 1 H), 8.61 (bs, 1 H), 8.71 (bs, 1 H) (NH of amino acids). MALDI-TOF MS ($C_{157}H_{273}N_{23}O_{45}$): *m/z* (positive ion mode) 3223.6 $[M+Na]^+$, 3239.6 $[M+K]^+$; (negative ion mode) 3199.8 [M-1]. FAB MS: *m/z* (positive ion mode) 3202.8 $[M+H]^+$, 3224.5 $[M+Na]^+$.

4-[*O*-(β-D-Galactopyranosyl)-(1→4)-glucopyranosyloxy]butyryl-L-leucyl-L-leucyl-L-leucyl-L-isoleucyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-glycyl-L-valyl-L-leucyl-L-leucyl-L-threonyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucyl-L-leucine (10a). A suspension of glycopeptide resin **7a** (29.1 mg) in methanol (1 mL) synthesized from **6a** (29 mg, 8.7 μmol) and **4** (25 mg, 34.6 μmol), was treated with 0.2 M sodium methoxide in methanol at pH 8.5 - 9.0 (using moist pH paper) with shaking by vortex mixer for 4 h at room temperature. The resulting resin **9a** was washed with methanol and dichloromethane followed by treatment with 95% aqueous TFA to give crude **10a** (20.7 mg, 83% by weight, 53% purity by HPLC). A portion of crude **10a** was purified with HPLC. ^1H NMR (600 MHz, CD_3OD) δ 0.89 - 1.03 (m, 114 H), 1.10 - 1.15 (m, 7 H) (CH_3 of Leu, Ile and Val, γ-CH_2 of Ile), 1.23 (d, 3 H, J = 6.4 Hz, γ-CH_3 of Thr), 1.57 - 2.02 (m, 55 H, β-CH and γ-CH of Leu, γ-CH_2 and β-CH of Ile, 2-CH_2 of propyl), 2.24 - 2.43 (m, 3 H, β-CH of Val, 3-CH_2 of propyl), 2.49 - 2.62 (m, 1 H, β-CH of Val), 3.47 - 4.52 (m, 40 H, β-CH of Thr, α-CH of amino acids, H-2, 3, 4, 5, 6, H'-2, 3, 4, 5, 6 and 1-CH_2 of propyl), 4.32 (d, 1 H, J = 7.8 Hz, H-1), 4.37 (d, 1 H, J = 7.8 Hz, H'-1), 7.30 (d, 1 H, J = 7.8 Hz), 7.73 (bs, 1 H), 7.83 (bs, 1 H), 7.97 (dd, 1 H, J = 9.3 and 4.9 Hz), 8.00 (bs, 1 H), 8.03 (bs, 1 H), 8.10 (bs, 2 H), 8.17 (bs, 1 H), 8.23 (bs, 2 H), 8.26 (bs, 2 H), 8.30 (bs, 2 H), 8.34 (d, 1 H, J = 4.9 Hz), 8.38 (bs, 1 H), 8.42 (d, 1 H, J = 4.9 Hz), 8.47 (d, 1 H, J = 4.9 Hz), 8.58 (d, 1 H, J = 4.4 Hz), 8.61 (bs, 1 H), 8.64 (d, 1 H, J = 3.4 Hz), 8.70 (bs, 1 H) (NH of amino acids). MALDI-TOF MS ($C_{142}H_{257}N_{23}O_{37}$): *m/z* (positive ion mode) 2902.4 $[M+Na]^+$, 2918.3 $[M+K]^+$, 2941.9 $[M-H+Ac+Na]^+$, 2959.3 $[M-H+Ac+K]^+$; (negative ion mode) 2878.6 [M-1]. FAB MS : m/z 2900.7 $[M+Na]^+$, 2917.8 $[M+K]^+$.

Gel Electrophoresis (Tris-Tricine-SDS-PAGE[11])

A separating gel containing 16.2% T [T denotes the total percentage concentration of both monomer (acrylamide and bis(acrylamide))] and 6% C (C denotes the percentage concentration of the cross-linker relative to the total concentration) was prepared, overlaid by a 10% T, 3% C spacer gel and a 4% T, 3% C stacking gel according to the method of Schägger and von Jagow.[11] The samples were incubated for 5 min at 37 °C or 100 °C in 4% SDS, 12% glycerol (w/v), 50 mM Tris, 2% mercaptoethanol (v/v), 0.01% bromo

phenol blue, adjusted with HCl to pH 6.8 prior to loading. The 0.2 M Tris solution, adjusted with HCl to pH 8.9 was used for the anode buffer and the 0.1% SDS solution containing 0.1 M Tris and 0.1M Tricine was used as the cathode buffer. The electrophoresis was performed at room temperature, cooled only by the ambient air. All electrophoresis runs started at 80 V. Gels were stained with a silver staining method (Wako Silver Stain Kit).

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REFERENCES AND NOTES

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