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Solid-phase Synthesis of CD52 Glycopeptide and an Efficient Route to Asn–Core Pentasaccharide Conjugate

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Abstract—The intact peptide sequence (**18**) as well as its glycoform carrying an *N*-linked core pentasaccharide (**1**) of CD52 antigen were prepared by means of solid-phase synthesis employing Fmoc-amino acids and benzyl-protected oligosaccharide–asparagine conjugate (**3**) as building blocks. It was concluded that the pentasaccharide structure had little influence on further peptide elongation in solid-phase synthesis and the benzylated pentasaccharide moiety was sufficiently stable to the 95% TFA acidic conditions used to release glycopeptide from the supporting resin. The paper also describes an efficient route leading to asparagine–core pentasaccharide conjugate (**3**) which was prepared in seven steps for an overall yield of 23% from monosaccharide units **5**, **6**, **7** and **8**. © 1997 Elsevier Science Ltd.

Introduction

With the increased understanding of the biological functions of glycoproteins as the recognition signals for various phenomena,[1] such as cell-growth regulation, cell-recognition, invasion, metastasis, host immune surveillance and differentiation, the synthesis of glycopeptides and glycoproteins has become the focus of numerous research efforts and many breakthroughs have been made.[2] However, glycopeptide synthesis is still recognized as a challenging problem in current organic chemistry, because in order to pursue synthetic studies toward glycopeptides, a sophisticated and interactive combination of carbohydrate chemistry and peptide chemistry is required.[3] Although recent studies have resulted in dramatic progress in both fields, they are still considered quite separately and the most optimized strategies established for each of them may not be compatible with each other. Therefore, for the successful synthesis of a complex glycopeptide, proper choices of the synthetic and protective strategies as well as the coupling and deprotection conditions are fundamentally important. In addition, synthesis of a large quantity of complex oligosaccharides is still a time-consuming and sometimes difficult task. Therefore, all the present synthetic practices towards glycopeptides used relatively simple oligosaccharide moieties, but naturally occurring glycoproteins may contain rather complicated oligosaccharide structures. Recently, we have disclosed the synthesis of an *N*-linked glycopeptide carrying core pentasaccharide in which the glycoasparagine building block was designed on the basis of the Fmoc method and used in solution to construct a tripeptide sequence of human chorionic gonadotropin (hCG).[4] However, if one wants to synthesize an *N*-linked glycopeptide carrying oligosaccharide structures so complex as a core pentasaccharide by solid-phase synthesis, one must consider several possible problems in advance, even though the solid-phase strategy has been successfully employed in the synthesis of glycopeptides containing relatively simple oligosaccharide structures:[5] (1) the influences of large sugar moieties on the coupling reaction of asparagine to the peptide backbones, as well as the influences of sugar moieties on the coupling reactions of further peptide elongation; (2) the stability of the sugar moiety, especially the α-mannopyranosyl 1→6 and the β-mannopyranosyl 1→4 linkages which are rather labile to acidic conditions, against the 95% TFA treatment used in the standard Fmoc method for releasing the peptide chain from the resin in the solid-phase synthesis of glycopeptides. In order to address all these problems, we have chosen the glycopeptide structure of a CD52 antigen containing an *N*-linked core pentasaccharide structure (**1**) as the target for the practice of solid-phase synthesis.

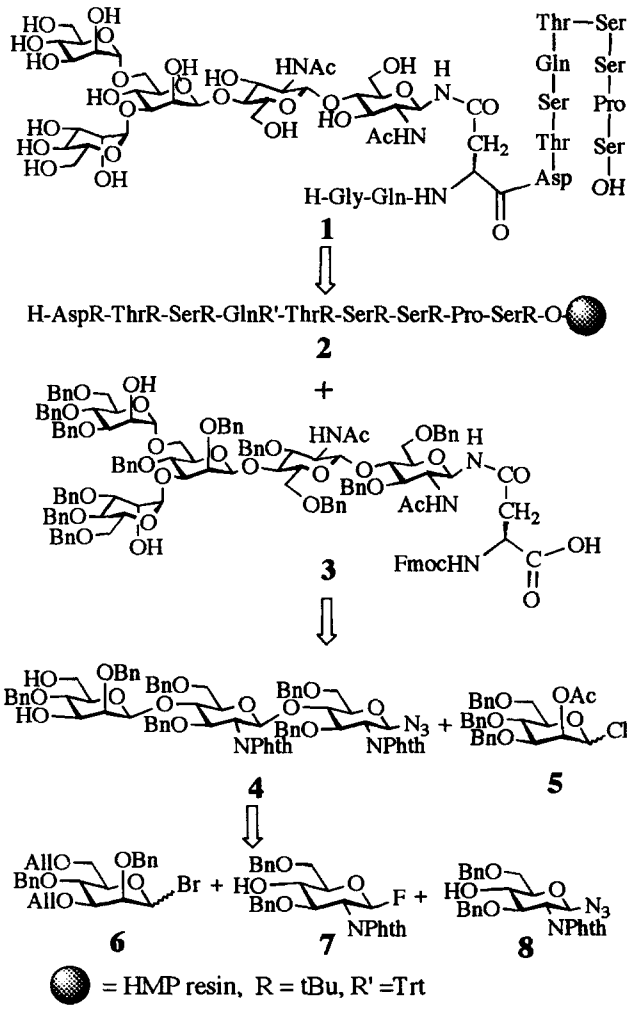
CD52 antigen is a GPI-anchored glycoprotein expressed on virtually all human lymphocytes. Monoclonal antibodies against this antigen are potent effectors of complement-mediated lysis and have been widely used in vivo and in vitro for the control of graft versus host disease and for the prevention of bone marrow transplant rejection.[6] Structural studies on CD52 revealed that it is a remarkable glycoprotein in that it is overwhelmingly glycosylated: although it has an apparent molecular weight of 21–28 kDa, its peptide chain is made up of only 12 amino acid residues. It is actually the shortest cell-surface glycoprotein ever found.[6b] Within the structure of CD52, there is only one *N*-glycosylation site carrying complex-type, tetra-antennary oligosaccharide chains and the GPI anchor is linked to the C-terminal of peptide chain.[6c] The simplicity of the CD52 peptide sequence makes CD52

glycopeptide an ideal synthetic target as well as a good model for pursuing strategic studies on glycopeptide synthesis and for studying the influences of *N*-linked oligosaccharides on the conformation and the biological properties of a glycopeptide.

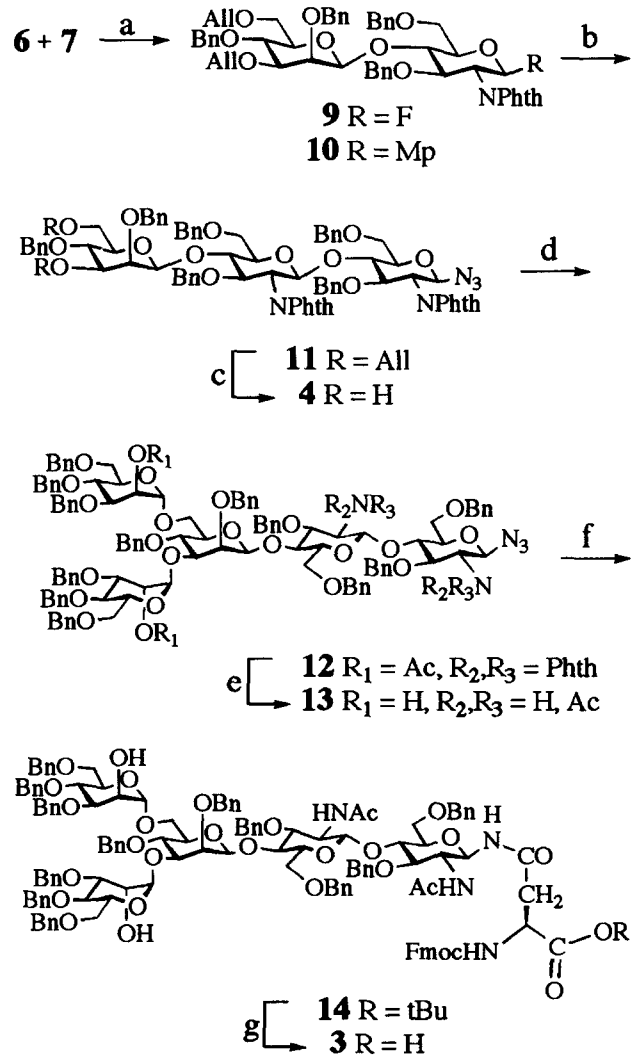
Results and Discussion

For the synthesis of target compound **1**, we employed a strategy (shown in Scheme 1) that was based on solid-phase synthesis involving Fmoc-amino acids and benzyl-protected oligosaccharides (**3**).[5] Using benzyl as protective groups of oligosaccharides has several advantages. First, benzylated sugars have high reactivity during glycosylation reactions, which is of obvious significance for the synthesis of complex oligosaccharides. Second, the stability of benzyl groups towards basic conditions eliminates the possible side reactions during the elongation of peptide chain, such as potential acyl exchange between acylated sugar moieties and peptide *N*-terminals. Third, benzyl groups can be removed easily under mild conditions, e.g. catalytic hydrogenolysis. In addition, the strategy of using benzyl as a protective group can be advantageous when the synthetic target contains sialic acid.[7]

Therefore, our present study began with the preparation of the key building block **3** (Scheme 1). For the preparation of a key intermediate trisaccharide **4**, based on the idea of orthogonal glycosylation strategy[8] we designed monosaccharide units **6**, **7** and **8** for direct glycosylations. Compounds **6**,[9] **7**[8] and **8**[4] were prepared from D-mannose and D-glucosamine, respectively. Then, as shown in Scheme 2, reaction of **6** and **7**, promoted by silver alumina-silicate,[10] afforded a 91% yield of mixture of **9** and its α-isomer in a ratio of 1.4:1.0, with the anomeric fluoride untouched. Compound **9** was easily isolated from the reaction mixture by column chromatography and was then employed



Scheme 1. Retro-synthesis of CD52 glycopeptide.



Scheme 2. Synthesis of core pentasaccharide-Asn conjugate (**3**). (a) Silver alumina-silicate, MS 4A, CH_2Cl_2, 0 °C to rt, 3 h, 91% (α:β = 1.0:1.4). (b) MS 4A, Cp_2HfCl_2, $AgClO_4$, **8**, CH_2Cl_2, −20 °C to rt, overnight, 76%. (c) $[Ir(COD)(PMePh_2)_2]PF_6$, THF, rt, 1 h; then HgO/$HgCl_2$, acetone, rt 1 h, 86%. (d) AgOTf, MS 4A, **5**, CH_2Cl_2, −40 °C to rt, overnight, 97%. (e) (i) $NH_2CH_2CH_2NH_2$, *n*-BuOH, 90 °C, 44 h; (ii) Ac_2O in MeOH (1:2), 0 °C, 2 h, 92%. (f) (i) Lindlar catalyst, H_2, MeOH, rt, overnight, 75%. (g) TFA–CH_2Cl_2 (1:2), rt, 2 h, 98%.

directly as the glycosyl donor for the next glycosylation. Compared to the process we recently reported,[4] the new route saved two steps: (1) the oxidative cleavage of the protective group in **10**, in which step purification of the resulting hemiacetals from the brown contaminants was proved to be time-consuming and the yield relatively low (70%), and (2) fluorination of the resulting hemiacetals. Reaction of **9** with **8** in the presence of hafnocene dichloride–silver perchlorate (1:2)[11] resulted in trisaccharide **11** (76%) stereoselectively. Deallylation of **11** was achieved by an iridium-catalyzed process[12] to afford **4** (86%) which was then reacted with **5**, using silver trifluoromethanesulfonate as a promoter, to give pentasaccharide **12** (97%) stereoselectively. In the structure of **12**, the 2-hydroxyl groups of two α-mannosyl residues, protected as acetates, were easily discriminated from all other hydroxyl groups, and it is at these positions that further elongation occurs in natural *N*-linked oligosaccharide chains. Therefore, the present synthetic strategy is also compatible with the synthesis of more extended structures. Dephthaloylation of **12** with ethylenediamine in *n*-butanol[13] followed by subsequent acetylation of the resulting product in acetic anhydride and methanol were carried out in a one-pot–two-step manner without purification to produce **13** in 92% yield. During all these transformations, anomeric azide was unaffected. Then, reduction of the azide group in **13** with a Lindlar catalyst under a hydrogen atmosphere followed by selective acylation of the resulting oligosaccharyl amine by activated asparagine, using standard 1-hydroxybenzotriazole (HOBt)/*N*,*N*′-dicyclohexylcarbodiimide (DCC) activation method,[14] gave the desired product **14** (75%). Finally, the carboxylic group of **14** was deprotected by treatment with 34% TFA in dichloromethane to give **3** (98%), which was ready to be applied to the coupling with peptide backbone by the Fmoc method. Therefore, by the present route, core pentasaccharide-asparagine conjugate **3** was prepared in only seven steps from monosaccharide units **5**, **6**, **7** and **8** in an overall yield of 23% and the syntheses of related pentasaccharides were carried out on multi-gram scales.

For the synthesis of target compound **1** (Scheme 3), a peptide fragment $Asp^4 \sim Ser^{12}$ linked to HMP-resin (**15**) was first constructed, starting from serine-preloaded HMP-resin, with an automatic synthesizer using the ready-made program and standard Fmoc method on a 0.25-mmol scale with an efficiency of 99.4% (monitored by ninhydrin reaction). The coupling reactions were pursued in *N*-methylpyrrolidone (NMP) using DCC-HOBt as the activating agent and *t*-butyl and trityl as the protective groups of the side chains of Asp/Thr/Ser and Gln, respectively. After deprotection of *N*-terminal in **15** with 20% piperidine in NMP at room temperature, the resulting peptide **2** (quantitative yield) having free *N*-terminal was coupled with Asn-pentasaccharide conjugate **3** manually, i.e. the mixture of **3** (2.4 equiv) activated by HOBt-DCC (2.8 equiv) and **2** (1.0 equiv) suspended in NMP was shaken on a vortex mixer overnight. Thus, after filtration, washing and drying overnight in vacuo, glycosylated peptide fragment linked to resin (**16**) was obtained in 98% yield and the excess amount of **2** was recovered from the solution as a mixture of **3**, its activated ester and some other side products. Compound **16** was applied to automatic synthesizer again and the peptide chain elongation was carried out on a 0.1-mmol scale according to the ready-made program to give **17** (94% by weight). The result has clearly shown that coupling of the Asn–pentasaccharide conjugate **3** with a resin-peptide could be easily and efficiently carried out under common reaction conditions and the benzylated oligosaccharide chain did not show any steric problem for further elongation of the peptide chain. Releasing glycopeptide from the resin was realized by treating the loaded resin **17** with a 95% TFA aqueous solution containing 2.5% 1,2-dimercaptoethane, under which conditions the protective groups on amino acid residues were also removed concomitantly. Figure 1 is the high performance gel permeation chromatogram of the product released from resin. Peak 1 was the expected benzylated glycopeptides, containing both fully benzylated (MS: M+Na = 3201) and substantial amounts of debenzylated derivatives, including mono-, di- and tri-debenzylated (MS: M+Na = 3113, 3024 and 2932, respectively). The well-separated peaks 2–5 contained the glycopeptides carrying either a disaccharide (MS: M+1 = 1975,

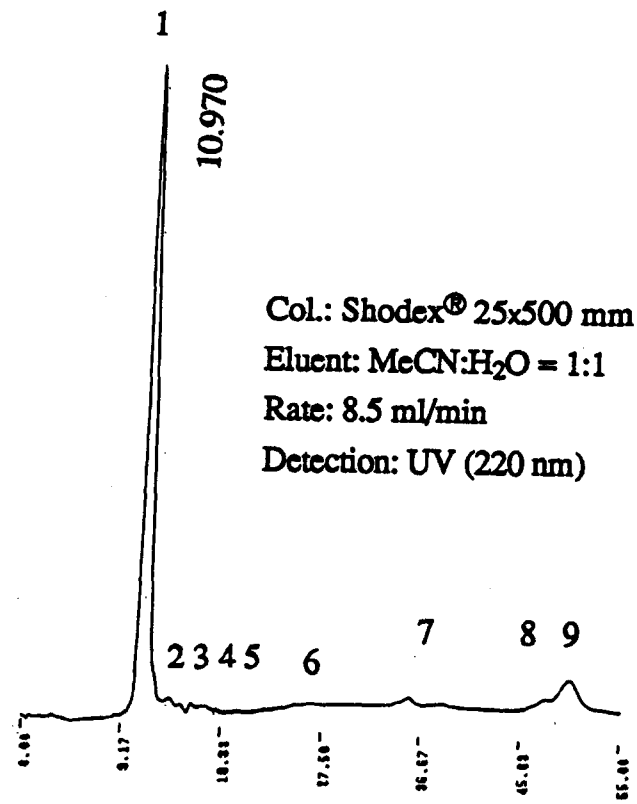
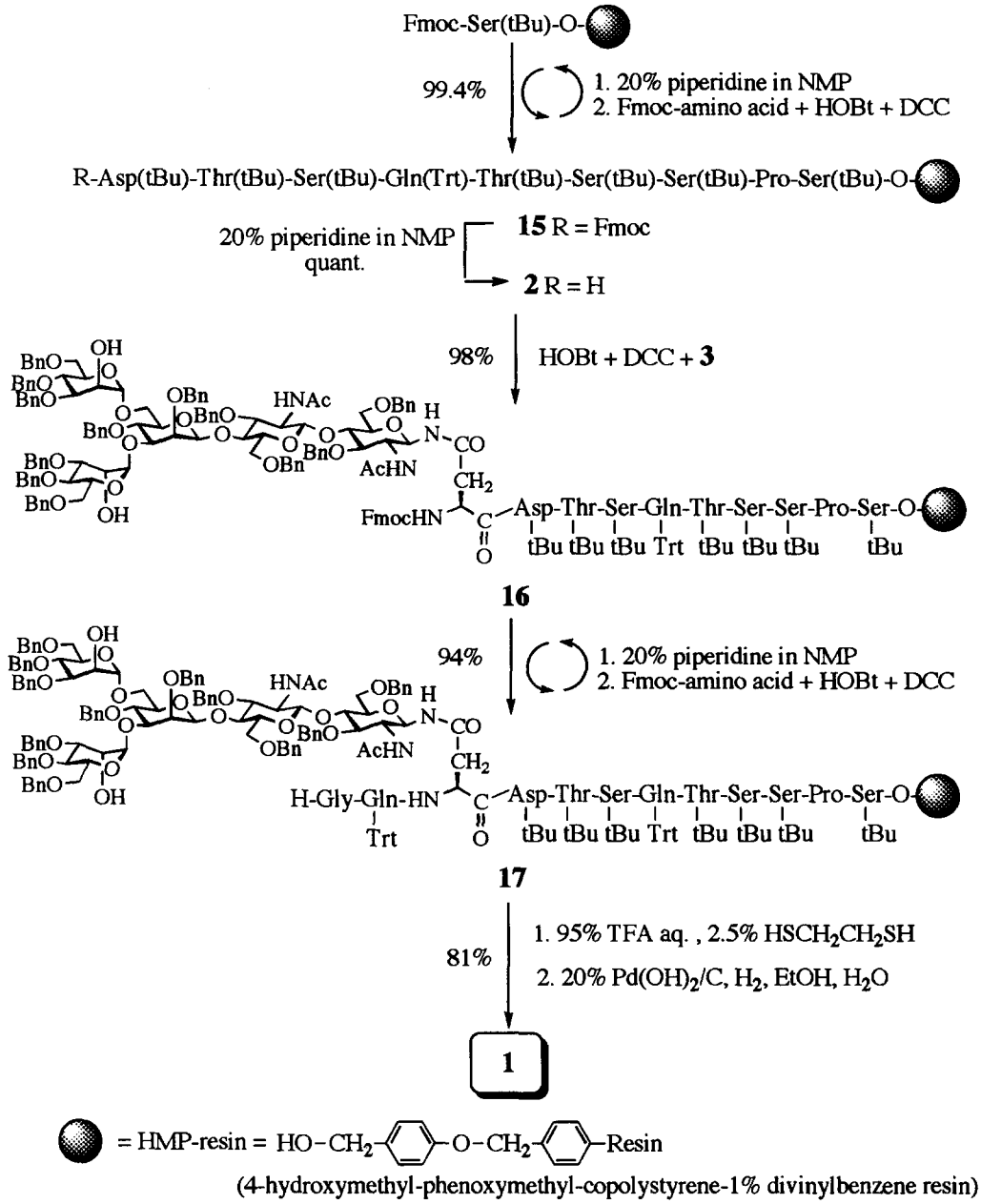


Figure 1. High performance gel permeation chromatogram of benzylated CD52 glycopeptide released from resin support. Peak 1: CD52 glycopeptide containing pentasaccharide structures. Peaks 2–5: CD52 glycopeptide containing di- or monosaccharide structures and nonglycosylated peptide deleting Asn. Peaks 6–9: nonpeptide nonsugar products.



Scheme 3. Solid-phase synthesis of CD52 glycopeptide.

M+Na = 2001) or a monosaccharide (MS: M+Na = 1614, M+K =1630) unit as well as the asparagine-deleting peptide (MS: M+Na = 1118). Peaks 6–9 were non-peptide, non-sugar products. As shown in Figure 1, among the product mixture, only a minor quantity (<2%) of glycopeptides containing degraded sugar moieties was formed during the cleavage of peptide chain from the resin. Therefore, although substantial amounts of glycopeptide was debenzylated, the backbone of the oligosaccharide chain was sufficiently stable to the 95% TFA condition used. Then, the fractions (Peak 1) containing the fully benzylated glycopeptide as well as its mono-, di- and tri-debenzylated derivatives were completely debenzylated by hydrogenolysis using 20% $Pd(OH)_2$ on charcoal as the catalyst (room temperature, 4 days). Filtration followed by in vacuo concentration of the reaction mixture afforded a solid residue which was purified first by high performance gel filtration to afford the expected product **1** (81% from **17**, purity ; 95% by NMR) as a white solid and then by reversed phase HPLC. The structure of **1** was established both by ^1H NMR (Fig. 2) and MS (M+1 = 2102.7, M+Na = 2125.7, M+K = 2141.0, M+2Na−1= 2147.1).

In order to pursue the investigations of the influences of *N*-linked glycan both on the solution conformation and on the biological activities of CD52 glycopeptide, we

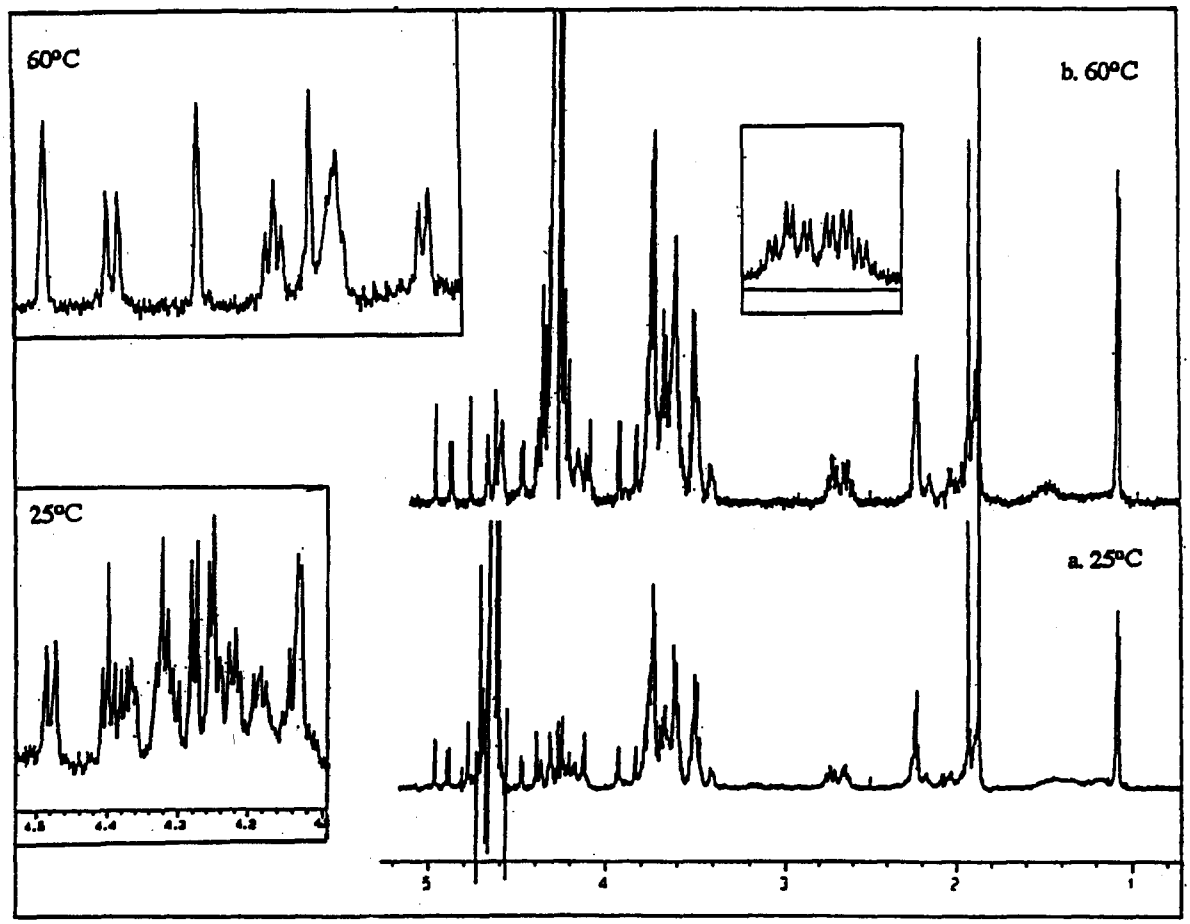
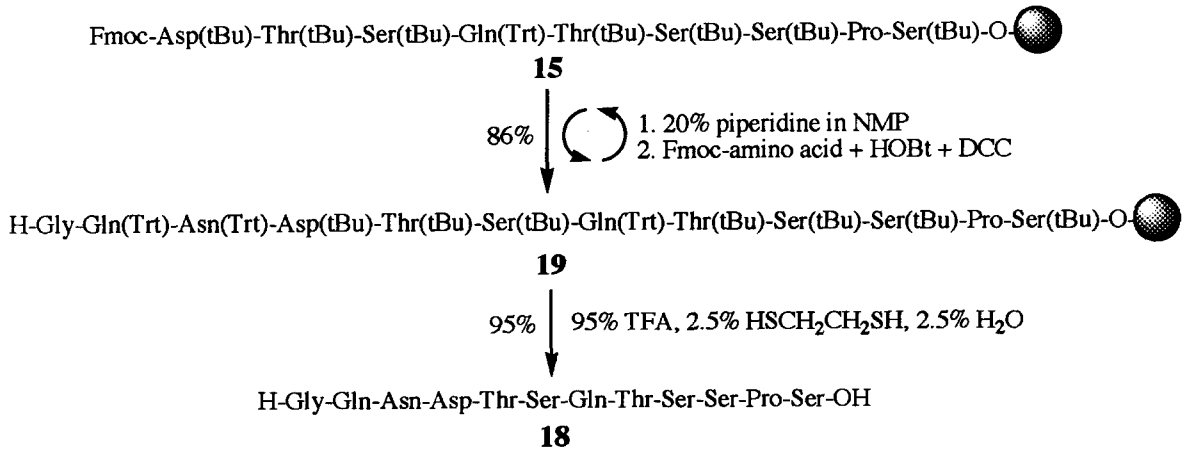


Figure 2. ^{1}H NMR spectra of CD52 glycopeptide (**1**) recorded at (a) 25 °C and (b) 60 °C.

have also prepared the non-glycosylated CD52 peptide **18** by solid-phase synthesis. As shown in Scheme 4, peptide fragment $Asp^4 \sim Ser^{12}$ on resin support (**15**) was applied to the automatic synthesizer for the elongation of the peptide chain on a 0.1 mmol scale. The resulting resin-peptide **19** (86% yield based on weight) was then treated with a 95% TFA aqueous solution containing 2.5% 1,2-dimercaptoethane to give the free peptide **18** (95% yield, 98% purity by NMR and HPLC) which was further purified by RP-HPLC.

In summary, *N*-linked CD52 glycopeptide was prepared by means of solid-phase synthesis, which represents a first example for synthesis of an intact natural glycopeptide sequence carrying an *N*-linked core pentasaccharide. The results revealed that the benzylated pentasaccharide moiety did not affect the coupling efficiencies during further elongation of the peptide chain and that the benzylated pentasaccharide moiety was sufficiently stable to the acidic conditions used to release glycopeptide chain from the supporting resin,



Scheme 4. Solid-phase synthesis of CD52 peptide.

and thus paved a way to the synthesis of more complex glycopeptides containing complicated sugar structures. The non-glycosylated CD52 peptide was also prepared by solid-phase synthesis, and the influences of the oligosaccharide chain on the conformation as well as the bio-activities of the peptide are now under further investigation. In addition, we have also presented herein an alternative and efficient route leading to Asn-linked core pentasaccharide. Thus, Asn–core, the pentasaccharide conjugate **3**, was prepared in seven steps for an overall yield of 23% from monosaccharide units **5**, **6**, **7** and **8**.

Experimental

General methods

Optical rotations were measured at 23 ± 2 °C with a Jasco DIP 370 polarimeter. ^1H and ^{13}C NMR spectra were recorded with either a Jeol EX 270 or a Jeol α600 spectrometer, for solutions in $CDCl_3$ with TMS as internal standard otherwise indicated. MALDI-TOF mass-spectra were obtained with a Bruker REFLEX (2,6-dihydroxybenzyoic acid was used as a matrix). Analytical TLCs and preparative TLCs were performed on precoated silica gel 60 F254 glass plates (Merck). Silica gel column chromatography (CC) was performed with silica gel 60 (Merck). Molecular sieves were purchased from Nakarai Chemical and were activated at 180 °C under vacuum immediately prior to use. All reactions except hydrogenation were performed in anhydrous solvent under dry N_2 atmosphere. Peptide synthesis was performed with an Applied Biosystem Model 431A Peptide Synthesizer. Fmoc-Ser-preloaded HMP-resin, Fmoc-amino acids in cartridges and the reagents for peptide synthesis were purchased from Applied Biosystems.

3,6-Di-*O*-allyl-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl fluoride (9). To a stirred mixture of compound **7** (0.70 g, 1.4 mmol), silver alumina-silicate (3.00 g, 9.0 mmol) and molecular sieves 4A (5.0 g) in CH_2Cl_2 (20 mL) was added a solution of compound **6** (1.80 g, 3.6 mmol) in CH_2Cl_2 (8 mL) at −20 °C. After stirring at 0 °C for 1 h and at room temperature for 2 h, the mixture was filtered through Celite and the filtrate was evaporated to dryness in vacuo. The residue was separated by silica gel CC (toluene:EtOAc, 8:1) to afford compound **9** (0.68 g, 53%)[4] and its α-isomer **9α** (0.49 g, 38%) as glassy solids. **9**: $[\alpha]_D$ +29.1° (c 3.1 in $CHCl_3$); R_f 0.56 (toluene:EtOAc, 6:1); ^1H NMR (270 MHz): δ 8.19–6.75 (m, 24 H, H-aromatic), 5.96–5.71 (m, 2 H, 2 H-All$^{\beta}$), 5.85 (dd, 1 H, $J_{1,2}$ 7.3 Hz, $J_{1,F}$ 54.1 Hz, H-1^1), 5.33–5.01 (m, 4 H, 2 H-All$^{\gamma,\gamma'}$), 4.96, 4.91, 4.87, 4.84, 4.68, 4.58, 4.49, 4.47 (4 AB, 8 H, 4 H-Bn), 4.51 (s, 1 H, H-1^2), 4.30–4.20 (ABX, 2 H, H-2^1,3^1), 4.11 (dd, 1 H, $J_{3,4} = J_{4,5}$ 8.5 Hz, H-4^2), 3.99–3.92 (m, 4 H, 2 H-All$^{\alpha,\alpha'}$), 3.82 (dd, 1 H, $J_{3,4} = J_{4,5}$ 9.6 Hz, H-4^1), 3.74–3.54 (m, 6 H, H-5^1, 6^1, $6'^1$, 2^2, 6^2, $6'^2$), 3.31 (m, 1 H, H-5^2), 3.25 (dd, 1 H, $J_{2,3}$ 3.0 Hz, H-3^2). **9α**: $[\alpha]_D$ +58.4° (c 1.8 in $CHCl_3$); R_f 0.66 (toluene:EtOAc, 6:1); ^1H NMR (270 MHz): δ 7.70–6.76 (m, 24 H, H-aromatic), 5.96–5.83 (m, 2 H, 2 H-All$^{\beta}$), 5.82 (dd, 1 H, $J_{1,2}$ 7.2 Hz, $J_{1,F}$ 54.1 Hz, H-1^1), 5.32–5.12 (m, 4 H, 2 H-All$^{\gamma,\gamma'}$), 5.27 (s, 1 H, H-1^2), 4.90, 4.66, 4.61, 4.57, 4.56, 4.51, 4.50, 4.19 (4 AB, 8 H, 4 H-Bn), 4.35 (dd, 1 H, $J_{3,4}$ 8.2 Hz, $J_{2,3}$ 10.9 Hz, H-3^1), 4.31 (dd, 1 H, H-2^1), 3.93–3.88 (m, 4 H, H-4^1, 6^1, $6'^1$, 4^2), 4.09–3.92 (m, 4 H, 2 H-All$^{\alpha,\alpha'}$), 3.80 (m, 1 H, H-5^2), 3.78–3.54 (m, 3 H, H-5^1, 2^2, 3^2), 3.63 (dd, 1 H, $J_{5,6}$ 4.8 Hz, $J_{6,6'}$ 10.8 Hz, H-6^2), 3.56 (dd, 1 H, $J_{5,6'}$ 2.2 Hz, H-$6'^2$).

3,6-Di-*O*-allyl-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl azide (11). After the mixture of silver perchlorate (2.40 g, 11.2 mmol), hafnocene dichloride (1.60 g, 5.6 mmol) and MS 4A (12.0 g) in CH_2Cl_2 (40 mL) was stirred at room temperature for 0.5 h, a solution of compound **8** (2.10 g, 4.2 mmol) in CH_2Cl_2 (40 mL) was introduced. The mixture was stirred at −20 °C for 0.5 h and was then added a solution of compound **9** (3.2 g, 3.5 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred at room temperature overnight and filtered through Celite. The filtrate was washed with aq. $NaHCO_3$ and then brine. The organic layer was dried over anhydrous Na_2SO_4, concentrated, and then purified by silica gel CC (toluene:EtOAc, 5:1) to afford **11** (3.74 g, 76%)[4] as a foamy solid.

2,4-Di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl azide (4). A solution of $[Ir(COD)(PMePh_2)_2]PF_6$ (0.15 g, 0.18 mmol) in THF (100 mL) was stirred under H_2 until the red solution became colorless. Then, the H_2 atmosphere was replaced with N_2 and a solution of **11** (4.50 g, 3.2 mmol) in THF (100 mL) was added into. The mixture was stirred at room temperature for 1 h and concentrated in vacuo. The residue was dissolved in 90% aq. acetone (200 mL) and treated with HgO (0.06 g, 0.25 mmol) and $HgCl_2$ (4.40 g, 16.3 mmol) for 1 h. It was filtered through Celite and the filtrate was concentrated in vacuo. CC of the residue gave compound **4** (3.60 g, 86%)[4] as a white solid.

2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)-[2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl azide (12). To a stirred mixture of **4** (3.44 g, 2.6 mmol), AgOTf (3.99 g, 15.5 mmol) and MS 4A (15.0 g) in CH_2Cl_2 (40 mL) was added a solution of compound **8** (4.74 g, 9.3 mmol) in CH_2Cl_2 (30 mL) at −40 °C. The mixture was gradually warmed up to room temperature and stirred overnight. It was filtered through Celite, and the filtrate was concentrated and purified by silica gel

CC (toluene:EtOAc, 8:1) to give **12** (5.70 g, 97%)[4] as a white solid.

3,4,6-Tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)-[3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl azide (13). A solution of **12** (0.25 g, 0.11 mmol) and ethylenediamine (0.2 mL) in *n*-butanol (7 mL) was stirred at 90 °C for 2 days. After concentration in vacuo, the residue was dissolved in MeOH (10 mL), and then, to the solution was added acetic anhydride (5 mL) at 0 °C. Two hours later, the mixture was concentrated in vacuo and the residue was purified by preparative TLC ($CHCl_3$:MeOH, 20:1) to afford **13** (0.21 g, 92%)[4] as a white solid.

***N*²-(9-Fluorenylmethoxycarbonyl)-*N*⁴-{3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)-[3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl}-L-asparagine tert-butyl ester (14)**. To a solution of Fmoc–Asn–Bu^t (0.20 g, 0.49 mmol) in CH_2Cl_2 (7 mL) was subsequently added 1 M HOBt (in NMP) (0.5 mL) and 1 M DCC (in NMP) (0.5 mL) and the mixture was stirred at room temperature for 1 h (activation of asparagine).

A mixture of compound **13** (0.20 g, 0.1 mmol), Lindlar catalyst (50 mg) and MeOH (40 mL) was stirred at room temperature for 5.5 h. After filtration through Celite and concentration in vacuo, to the resulting residue was added CH_2Cl_2 (2 mL) and then, freshly prepared solution of activated asparagine (Fmoc–Bu^t–AsnOBt). The mixture was stirred at room temperature overnight and then concentrated in vacuo. The residue was separated by a LH-20 column followed by HPLC purification (Intersil Prep-Sil column, 10 × 250 mm; det. 254 nm; eluent: 1.3% EtOH in $CHCl_3$, 5 mL/min; retention time, 6.36 min) to afford **14** (0.18 g, 75%)[4] as a white solid.

***N*²-(9-Fluorenylmethoxycarbonyl)-*N*⁴-{3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)-[3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-(1→3)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl}-L-asparagine (3)**. The solution of compound **14** (70 mg, 0.029 mmol) in TFA and CH_2Cl_2 (1:2, 6 mL) was stirred at rt for 1.5 h. After the reaction mixture was concentrated in vacuo and coevaporated with ethanol, the residues was purified by passing through a LH-20 column (2.5 × 65 cm, eluent: $CHCl_3$:MeOH = 1:1) to afford **3** as a white solid (66.8 mg, 98%).[4]

All compounds **9**, **11**, **4**, **12**, **13**, **14** to **3** were identical to authentic samples both for their physical and for their spectroscopic properties.[4]

L-Glycyl-L-glutaminyl-{*N*⁴-α-D-mannopyranosyl-(1→6)-[α-D-mannopyranosyl-(1→3)]-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl}-L-asparaginyl-L-glutaryl-L-threonyl-L-seryl-L-glutaminyl-L-threonyl-L-seryl-L-seryl-L-prolyl-L-serine (1). An Fmoc-protected nonapeptide linked to resin (**15**, 674 mg) was synthesized after eight cycles of the standard synthesizer program of condensation with the DCC-HOBt-activated Fmoc amino acids (1 mmol each) starting from Fmoc Ser-preloaded HMP resin (357 mg, 0.25 mmol). Efficiency of the condensation at each step was monitored by untilizing ninhydrin test and the overall yield of **14** was 99%.

A part of the resin **15** (34 mg, 11 mmol) was treated with 20% piperidine in NMP at room temperature for 2 h to remove Fmoc group, while **3** (64 mg, 28 mmol, 2.4 equiv) was activated by stirring with M DCC-NMP (34 mL, 2.9 equiv) and M HOBt-NMP (34 mL, 2.9 equiv) at room temperature for 1 h. The resulting resin **3**, after washed with NMP, CH_2Cl_2 and drying under high vacuum, was added to the activated **3**. The mixture was shaken on a vortex mixer (Iwaki TM-252) for 16 h at room temperature, and then filtered off and washed subsequently with NMP and CH_2Cl_2 and then dried under vacuum overnight. Condensation of the filtrate and washings gave recovered **3** and its activated ester (38 mg). The resulting resin **16** (56.1 mg, 98%) was applied to the automatic synthesizer again, and Glu and Gly were subsequently introduced. After deprotection of Fmoc with piperidine, washing and drying, glycopeptide-linked resin **17** (53.0 mg, 91%) was obtained.A part of **17** (31 mg) was treated with a mixture of TFA (1.9 mL), $HSCH_2CH_2SH$ (0.05 mL) and water (0.05 mL) at room temperature for 2 h. The mixture was filtered off and the filtrate was concentrated. The residue was suspended in water (5 mL) and extracted with ether (3 × 10 mL). The water solution was concentrated and the residue was dissolved in 50% MeCN aq. solution (1.0 mL). It was separated by gel filtration HPLC (Col. Shodex® 25 × 500 mm; eluent. H_2O:MeCN = 1:1, 8.5 mL/min, Fig. 1). The fractions containing the mixture of glycopeptides carrying fully benzylated as well as mono-, di- and tri-debenzylated pentasaccharide structures (Peak 1, R_t = 10.97 min) were collected and concentrated (15 mg). A part of it (5.8 mg) was dissolved in water (5.5 mL) and aldehyde-free EtOH (4.5 mL) and was stirred with 20% $Pd(OH)_2$/C (3 mg) under hydrogen atmosphere at room temperature for 4 days. After filtration and concentration in vacuo, the residue was purified by gel filtration HPLC (Col., Shodex® 25 × 500 mm; eluent, H_2O:MeCN = 1:1, 8.5 mL/min) to give the expected glycoprotein **1** (R_t = 13.87 min; 3.7 mg, 81% yield based on **17**; 95% purity by NMR) and it was further purified by RP-HPLC (Col., LichroCART®, 10 × 250 mm; eluent, 2.6% *i*-PrOH in H_2O, 3 mL/min; R_t = 9.82 min). **1**: $[\alpha]^{23}_D$ −38.0 (*c* = 0.1 in H_2O); ^1H NMR (600 MHz, in D_2O, acetone δ = 2.23 as standard, 60 °C): δ 5.10 (d, 1 H, $J_{1,2}$ = 1.0 Hz, H-1^4), 5.02 (d, 1 H, $J_{1,2}$ = 8.9 Hz, H-1^2), 4.90 (d, 1 H, $J_{1,2}$ = 1.7 Hz, H-1$^{4'}$), 4.80 (dd, 1H, H-α^{Asp}), 4.76 (s, 1 H, H-1^3),

4.70–4.74 (m, 2H, H-$\alpha^{Asn,\ Ser}$), 4.61 (bd, $J_{1,2}$ = 7.6 Hz, 1 H, H-1[1]), 4.47–4.32 (m, 10 H, H-$\alpha^{3\times Ser,\ 2\times Thr,\ Pro,\ 2\times Glu}$, H-$\beta^{2\times Thr}$), 4.00–3.50 (m, 42 H, H-α,α'^{Gly}, H-β, $\beta'^{4\times Ser}$, H-δ, δ'^{Pro}, H-2, 3, 4, 5, 6 and 6′ of five sugar rings), 2.72–2.90 (m, 4 H, H-β, $\beta'^{Asp,\ Asn}$), 2.25–2.31 (m, 5 H, H-γ, $\gamma'^{2\times Gln}$, β^{Pro}), 2.38–2.00 (m, 7 H, H-β, $\beta'^{2\times Gln}$, β', γ, γ'^{Pro}), 2.06 (s, 3 H, Ac), 2.00 (s, 3 H, Ac), 1.19 (d, 6 H, J = 6.3 Hz, H-$\gamma^{2\times Thr}$). MALTI-TOP-MS ($C_{79}H_{129}N_{17}O_{49}$, average MW 2101.0): *m/z* 2102.7 (M+1), 2125.7 (M+Na), 2141.0 (M+K), 2147.1 (M+2 × Na−1), 2163.3 (M+Na+K−1), 2169.2 (M+3 × Na−2), 2185.9 (M+2 × Na+K−2).

L-Glycyl-L-glutaminyl-L-asparaginyl-L-glutaryl-L-threonyl-L-seryl-L-glutaminyl-L-threonyl-L-seryl-L-seryl-L-prolyl-L-serine (18). A part of the resin **15** (292 mg, 0.1 mmol) was applied to automatic synthesizer, and Asn, Glu and Gly were subsequently introduced. After deprotection of Fmoc with piperidine, washing and drying, peptide-linked resin **19** (320 mg, 86%) was obtained. A part of **19** (75 mg) was treated with a mixture of TFA (1.9 mL), $HSCH_2CH_2SH$ (0.05 mL) and water (0.05 mL) at room temperature for 2 h. The mixture was filtered off and the filtrate was concentrated. The residues was suspended in water (5 mL) and extracted with ether (3 × 10 mL). The water solution, after passing through a C_{18} cartridge, was concentrated to get the crude product (29.5 mg; 98% purity by HPLC). A part of the crude product was dissolved in 50% MeCN aq. solution and further purified by RP-HPLC (Col., LichroCART®, 10 × 250 mm; eluent, 4% *i*-PrOH in H_2O, 3 mL/min; R_t = 7.38 min) to give pure **18**: $[\alpha]^{23}_D$ −88.3 (*c* = 1.0 in H_2O); ^1H NMR (600 MHz, in D_2O, acetone δ = 2.23 as standard, 24 °C): δ 4.80 (dd, 1 H, $J_{\alpha,\beta}$ = 6.3 Hz, $J_{\alpha,\beta'}$ = 6.8 Hz, H-α^{Asp}), 4.77 (dd, 1 H, $J_{\alpha,\beta}$ = 5.9 Hz, $J_{\alpha,\beta'}$ = 6.9 Hz, H-α^{Ser}), 4.69 (dd, 1H, $J_{\alpha,\beta}$ = 6.3 Hz, $J_{\alpha,\beta'}$ = 7.3 Hz, H-α^{Asn}), 4.49 (t, 2 H, $J_{\alpha,\beta}$ = $J_{\alpha,\beta'}$ =4.7 Hz, H-$\alpha^{2\times Ser}$), 4.48 (dd, 1 H, $J_{\alpha,\beta}$ = 4.4 Hz, $J_{\alpha,\beta'}$ = 7.8 Hz, H-α^{Pro}), 4.45 (t, 1 H, $J_{\alpha,\beta}$ = 5.7 Hz, H-α^{Ser}), 4.41 (dd, 1 H, $J_{\alpha,\beta}$ = 5.4 Hz, $J_{\alpha,\beta'}$ = 9.4 Hz, H-α^{Gln}), 4.37 (d, 1 H, $J_{\alpha,\beta}$ = 4.4 Hz, H-α^{Thr}), 4.36 (d, 1 H, $J_{\alpha,\beta}$ = 4.4 Hz, H-α^{Thr}), 4.34 (dd, 1 H, $J_{\alpha,\beta}$ = 5.9 Hz, $J_{\alpha,\beta'}$ = 8.3 Hz, H-α^{Gln}), 4.27 (dq, 1 H, $J_{\beta,\gamma}$ = 6.3 Hz, H-β^{Thr}), 4.23 (dq, 1 H, $J_{\beta,\gamma}$ = 6.3 Hz, H-β^{Thr}), 3.95 (dd, 1 H, $J_{\beta,\beta}$ = 11.7 Hz, H-β^{Ser}), 3.87–3.81 (m, 8 H, H-$\beta^{6\times Ser}$, $\alpha^{2\times Gly}$), 3.79 (ddd, 1 H, H-δ^{Pro}), 3.77 (dd, 1 H, $J_{\beta,\beta}$ = 11.7 Hz, H-β^{Ser}), 3.70 (ddd, 1 H, $J_{\gamma,\delta}$ = 2.7 Hz, $J_{\gamma,\delta'}$ = 7.1 Hz, $J_{\delta,\delta'}$ = 12.4 Hz, H-δ^{Pro}), 2.96 (dd, 1 H, $J_{\alpha,\beta}$ = 5.9 Hz, $J_{\beta,\beta'}$ = 17.1 Hz, H-β^{Asp}), 2.87 (dd, 1 H, $J_{\alpha,\beta}$ = 7.3 Hz, H-β'^{Asp}), 2.84 (dd, 1 H, $J_{\alpha,\beta}$ = 6.3 Hz, $J_{\beta,\beta'}$ = 15.6 Hz, H-β^{Asn}), 2.75 (dd, 1 H, $J_{\alpha,\beta'}$ = 7.8 Hz, H-β'^{Asn}), 2.39–2.32 (m, 4 H, H-$\gamma^{4\times Gln}$), 2.29 (m, 1 H, $J_{\alpha,\beta}$ = 8.0 Hz, $J_{\beta,\beta'}$ = 13.2 Hz, H-β^{Pro}), 2.13 (m, $J_{\alpha,\beta}$ = 5.4 Hz, $J_{\beta,\beta'}$ = 14.1 Hz, $J_{\beta,\gamma}$ = 7.4 Hz, $J_{\beta,\gamma'}$ = 8.3 Hz, H-β^{Gln}), 2.07 (m, $J_{\alpha,\beta}$ = 5.9 Hz, $J_{\beta,\beta'}$ = 14.6 Hz, $J_{\beta,\gamma}$ = 7.3 Hz, $J_{\beta,\gamma'}$ = 7.8 Hz, H-β^{Gln}), 2.03–1.93 (m, 5 H, H-$\beta^{2\times Gln}$, $\gamma^{2\times Pro}$, β^{Pro}), 1.18 (d, 3H, $J_{\beta,\gamma}$ = 6.8 Hz, H-γ^{Thr}), 1.17 (d, 3H, $J_{\beta,\gamma}$ = 6.4 Hz, H-γ^{Thr}). MALTI-TOP-MS ($C_{45}H_{73}N_{15}O_{24}$, everage MW 1208.5): *m/z* 1209.7 (M+1), 1231.6 (M+Na), 1247.9 (M+K), 1253.4 (M+2 × Na−1).

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