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Synthesis of an *N*-glucoasparagine analog as a building block for a V3-loop glycopeptide from GP120 of HIV-I

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Abstract

The preparative synthesis of a new N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine mimetic **1**, starting from 2-amino-1,5-anhydro-2-deoxy-glucitol hydrochloride and Z-Asp-(OH)-OBn is described. This glycosyl-amino acid unit **1** is expected to show higher stabilities towards in vivo conditions. Further, the use of **1** as building block for the synthesis of modified glycopeptides using solid phase support is reported. The glycopeptide Ac-SXNTRKSIHIGPGRAF-NH_2 having sugar-modified Asn_2 mimics parts of the V3-loop structure containing the principle neutralizing determinant (PND) of HIV-1 and the naturally conserved glycosylation site within the V3 loop.

Keywords: Glycopeptides; Mimetics; Carbohydrates; Solid phase synthesis; HIV

1. Introduction

Binding of the HIV-1 external envelope glycoprotein gp120 to the CD4 receptor on human cells is the first step in HIV:cell interaction and virus entry [1]. After attachment to CD4, gp 120 interacts with one of the chemokine receptors CCR5 or CXCR4, recently identified as the major coreceptors for HIV-1 [2]. It was shown that the interaction between gp120 and the two coreceptors is mediated by the third variable region (V3 loop) of gp120 [3]. Mutational studies with the V3 loop suggest that coreceptor usage and HIV:cell fusion are linked to V3 loop amino acid sequences [4]. Thus, the V3 loop of the HIV-1 gp120 envelope glycoprotein is an important domain involved in virus entry by making contact to target cell receptors. In a second step, the V3 loop may interact with the chemokine receptor to permit HIV:cell fusion. This second step of HIV:cell interaction can be inhibited by synthetic V3 loop peptides [5] containing the highly conserved GPGRAF-motif

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of the subtype B strains [6]. Thus peptides corresponding to the V3 loop sequence GPGRAF were able to block HIV-1 infection in vitro. One main therapeutic aim is the extracellular blockade of virus attachment and the fusion process which follows. A possible strategy in blocking viral infection is the use of synthetic peptides mimicking the gp120 V3 loop domain. Enhanced antiviral activity was observed using multibranched peptide constructs [7] indicating that small V3 peptides can interfere with gp120:coreceptor binding. Since gp120 and especially the V3 loop is highly glycosylated [8] and V3 loop glycosylation is important for viral function and infectivity [9], we are interested in the synthesis of glycosylated V3 loop peptides and the influence of glycosylation on the three dimensional structure of the V3 loop peptide. Therefore, we report a method for the synthesis of V3 glycopeptides containing the GPGRAF-motif and the flanking glycosylation site.

Because the moderate metabolic stability of glycopeptides limits their potential as therapeutic agents, modified analogs such as glycopeptides containing C-glycosylated amino acids have been reported [10]. Herein we describe the synthesis of the glycosyl-amino acid unit N^{γ}-(3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxyglucitol-2)-L-asparagine **1** as a mimetic of N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine **2** and the incorporation of **1** as a new building block [11] in solid phase synthesis of the potential anti-HIV active peptide **3** (Fig. 1). This glycopeptide contains a monosaccharide at the amino acid position which is the naturally conserved 306 N-linked glycosylation site of the gp120 V3 loop [9].

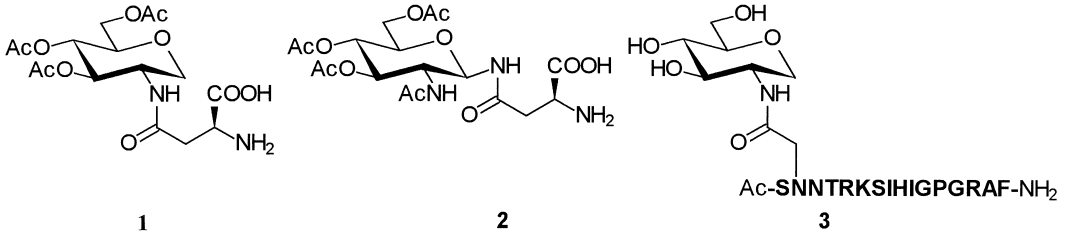
Building block **1** is characterized by a new amide linkage between the 2-amino function of the carbohydrate moiety and the side chain of the amino acid which ensures an improved stability towards enzymes, acids and bases, in contrast to the labile *N*-glycosidic linkage of **2**. In addition, the anomeric center has been reduced transforming the cyclic acetal structure to a stable cyclic ether. This compound represents a new approach toward the synthesis of glycosylamino acid mimetics which is distinguished by the need for only a few reaction steps starting from easily available starting materials and the compatibility with most of the generally applied protective groups.

2. Results and discussion

The modified *N*-glucoasparagine unit **1** was introduced into the peptide sequence as N^{α}-Fmoc protected derivative **9** (Scheme 1). Starting from the known 2-acetamido-2-deoxy-glucopyranosyl chloride **4** [12] the appropriate glucitol **5** [13] was obtained by reduction using AIBN/tributyltin hydride [14] in refluxing toluene in 84% yield. Deacetylation of **5** was performed by acid hydrolysis with hydrochloric acid (2.5 N) to give the 2-amino-1,5-anhydroglucitol hydrochloride **6** in 68% yield.

Deacetylation procedures under alkaline reaction conditions using $Ba(OH)_2$ or NaOH [15] led to difficulties in work up resulting in low yields of **6**. For the coupling step between **6** and Z-Asp(OH)-OBn **7** [16], the amino acid was activated as mixed anhydride using isobutylchloroformate [17]. This method offered the advantage of using the unblocked sugar moiety directly in aqueous solution which made the development of a protective-group pattern obsolete. After coupling the remaining hydroxy groups were acetylated to lead to **8** (75% overall). Catalytical hydrogenolysis of **8** gave **1** in quantitative yield, which was subsequently N^{α}-Fmoc protected (82%) leading to compound **9**. The employment of **1** as *N*-glucoasparagine analog was tested by synthesizing the small peptide **10** and subsequently the acetylated V3-loop peptide sequence **12** using **9** (Scheme 2).

The assembly of the *O*-acetylated glycopeptides **10** and **12** was done according to the peptide sequence starting at the C-terminal end using a fully automatic peptide synthesizer. PEGA-resin

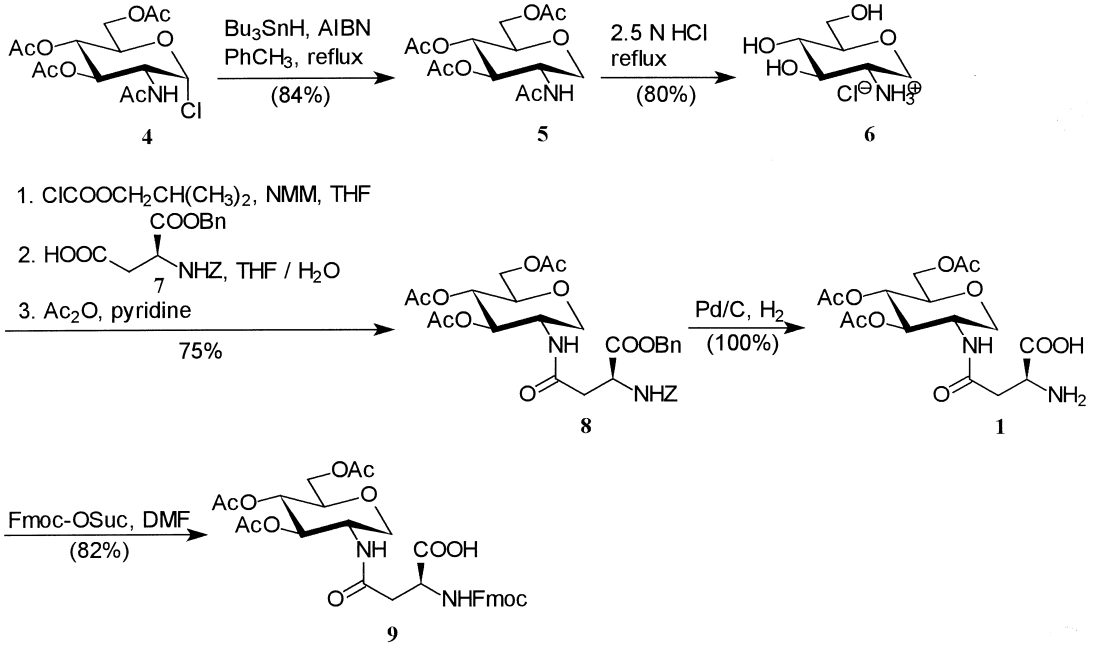


1 2 3

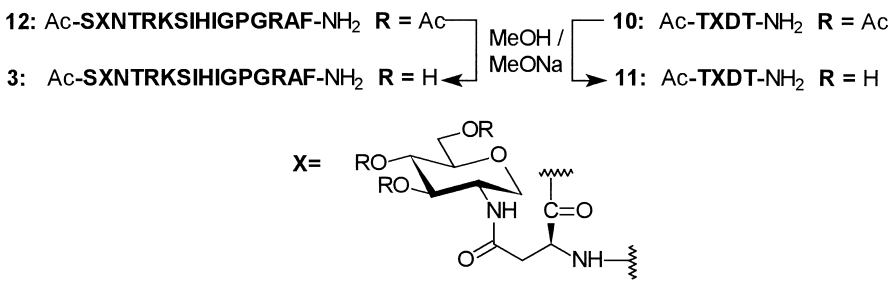
Fig. 1. Original **2** and modified **1** sugar-Asn building block and modified glycopeptide structure **3**.

[18] derivatised with Rink-linker [19] was chosen as polymer support in order to get high amounts of peptide–amides [20]. After cleavage of the resin and HPLC purification the O-acetylated glycopeptides were obtained in 21% (**10**) respectively 23% (**12**) yield. Deacetylation was performed in aqueous MeOH (50%) using catalytic amounts of NaOMe (1% in MeOH). The yields after HPLC purification were 60% (**11**) respectively 54% (**3**). NMR characterisation of the peptides **10**, **11** and **12** was performed by ^{1}H–^{1}H-COSY NMR experiments. For compound **3** a complete NMR assignment of all protons was performed by ^{1}H–^{1}H-TOCSY and ^{1}H–^{1}H-NOESY spectroscopy [21]. Moreover the assignment of several NH($i+1$)→CH$_\alpha$(i) NOEs led to complete characterisation of **3**. The NOE data indicate that the majority of the molecule is disordered, that means no single conformation is present in solution. However the N–N(i, $i+2$) NOE between the amide protons of Gly-13 and Ala-15 indicates a β-turn conformation. This interaction is surprising, because of the small size of the peptide. In comparison with HIV gp120 sequences as RP342, RP142 or RP70, peptide **3** seems to generate a similar shape. Chandrasekhar et al. [22] have shown for a similar sequence (RP) that the evaluation of a β-turn conformation was detectable for peptides with the GPGRAF-motif centered, but not for peptides with this motif near the C-terminus. However there is no evidence for an induced peptide conformation by the glucitol side-chain. Evidently, NOE interaction of the Asn-δ-NH with H-3 of the carbohydrate moiety on one hand and with the Asn-(β_2-H on the other hand proved the linkage between the aminoglucitol and the peptide backbone (Fig. 2, Table 1).

This is the first report on the synthesis of glycosylated V3 loop peptides as a new antiviral candidate. In contrast to other V3 loop peptide constructs [23] our glycosylated V3 peptide had no



Scheme 1. Synthesis of the N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine analog **1** and building block **9**.



Scheme 2. Synthesis of the deacetylated peptides **3** and **11**.

inhibitory effects on activated T cells or PBMC (data will be published in another context). Since we did not observe inhibition of T cell proliferation after incubation of T cells with the V3 loop glycopeptide its antiviral activity can be determined precisely and it might be an interesting future candidate for safe in vivo applications. Besides the direct antiviral effects observed by testing V3 loop

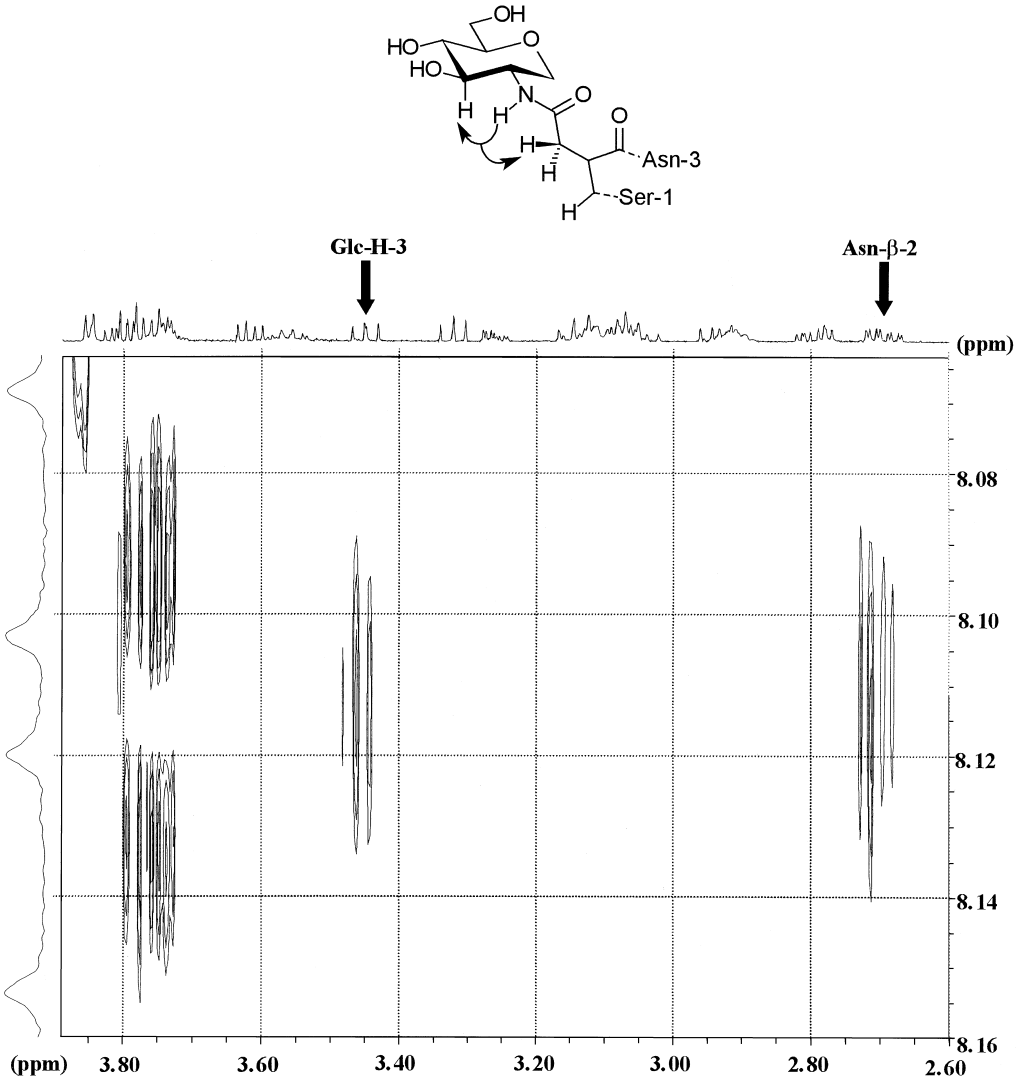
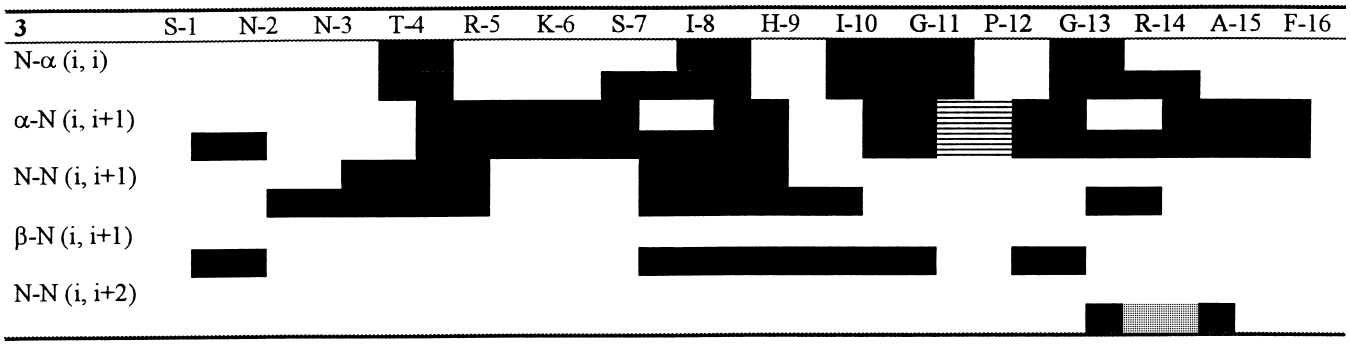


Fig. 2. Part of the NOESY-spectrum of compound **3**. The crosspeaks (as indicated above) are shown between the 2-NH proton and the Asn-β_2-resp. Glc-H-3 which indicate the linkage between the carbohydrate moiety and the peptide unit.

Table 1
Schematic diagram showing the magnitude of various NOE connectivities observed in NOESY spectra of peptide **3**



peptides [5,7] it is also conceivable, that glycosylated V3 loop peptides compared to normal peptides are more effective in the induction of antibodies able to neutralize HIV-1. This might be relevant for the development of a mixed HIV-1 vaccine based on recombinant gp120 and V3 loop peptides.

3. Experimental

Melting points were determined using a Mettler FP 82 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 400 or a Bruker DRX 500 spectrometer at 300 K. In 2D experiments 512 FIDs with 4096 complex data points and 32 scans each were acquired. The NOESY spectra were acquired with a 300 ms mixing time. The water resonance was suppressed by low-power presaturation during the relaxation delay (1.5 s). Data were processed on a PC using 2D WIN–NMR (950901.6) software (Bruker Franzen Analytik GmbH). The data were zero-filled twice in the t_1 dimension, and multiplied with a squared sinebell function (SSB 2) in both dimensions. Chemical shifts are given in ppm relative to internal acetone (δ 2.225 ppm) for solutions in D_2O/H_2O and TMS (δ 0.000 ppm) for other solutions. FAB mass spectra were measured on a double-focused VG-Analytical 70–250 S mass spectrometer with 3-nitrobenzyl alcohol as matrix. Optical rotations were recorded on a Perkin–Elmer Polarimeter 241, [*a*]-values are given in units of 10^{-1} deg cm^2 g^{-1}. MPLC was performed on silica gel Silitech 12–26, 6 μm (ICN) at 300–500 kPa. HPLC was performed on a Merck/Hitachi HPLC system with LiChrospher RP-18 columns (250×25 mm; 7 μm; flow rate 10 cm^3 min^{-1}) for preparative separation and LiChrospher RP-18 column (250×4 mm; 7 μm; flow rate 10 cm^3 min^{-1}) for analytical use with buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile). Peak detection was performed with a photodiode array detector at 215 nm. Peptide **9** and **11** were synthesized on a Pioneer Perseptive Applied Biosystems continues flow peptide synthesizer. DMF used for peptide synthesis was analysed for free amines by addition of Dhbt-OH prior to use. Reagents for peptide synthesis were purchased as follows: Dhbt-OH and TBTU from Fluka; HATU and Fmoc amino acids from Perseptive Applied Biosystems.

2-Amino-1,5-anhydro-2-deoxyglucitol hydrochloride (**6**).—A suspension of 2-acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy glucitol **5** [16] (2.50 g, 7.55 mmol) in 2.5 N hydrochloric acid (20 mL) was heated under reflux for 2 h. The resulting clear solution was concentrated under reduced pressure and the oily residue was dissolved in a minimum amount of ethanol. Diethyl ether was added dropwise under stirring until the product precipitated. The crude product was filtered off, recrystallized from propanol/ethanol (1:1) and dried to afford 1.02 g (68%) of **6** as a colourless solid. mp 199 °C decomp; $[\alpha]^{25}_D$ +17.8 (*c* 1; H_2O); ^{1}H NMR (D_2O) δ 3.15 (ddd, 1 H, $J_{1a,1e}$ 11.2, $J_{1a,2}$ 11.2, $J_{1e,2}$ 5.1 Hz, H-2), 3.23–3.32 (m, 2 H, H-4, H-5), 3.41 (dd, 1 H, $J_{1a,1e}$ 11.2, $J_{1a,2}$ 11.2 Hz; H-1a), 3.50 (dd, 1 H, $J_{2,3}$ 10.2, $J_{3,4}$ 8.1 Hz; H-3), 3.58 (dd, 1 H, $J_{5,6}$ 5.6, $J_{6,6*}$ 12.2 Hz; H-6), 3.75 (dd, 1 H, $J_{5,6*}$ 2.0, $J_{6,6*}$ 12.2 Hz; H-6*), 4.05 (dd, 1 H, $J_{1a,1e}$ 11.2, $J_{1e,2}$ 5.1 Hz; H-1e); ^{13}C NMR (D_2O) δ 51.87 (C-5), 61.10 (C-6), 66.44 (C-1), 70.23 (C-4), 74.56 (C-3), 80.98 (C-2). Anal. Calcd. for $C_6H_{14}NO_4Cl$: C, 36.17; H, 7.09; N, 7.03. Found: C, 36.33; H, 7.07; N 7.02.

N^{α}-*Benzyloxycarbonyl*-N^{γ}-*[3,4,6-tri*-O-*acetyl-1,5-anhydro-2-deoxyglucitol-2]*-L-*asparagine benzyl ester* (**8**).—*N*-Methylmorpholine (570 μL, 5.14 mmol) was added to a stirred and cooled solution (−10 °C) of N^{α}-benzyloxycarbonyl-L-asparagine benzyl ester **7** (1.93 g, 5.41 mmol) in dry THF (30 mL). Isobutylchloroformate (740 μL, 5.68 mmol) was added slowly and stirring was continued for 30 min at −10 °C. To a stirred suspension of 2-amino-1,5-anhydro-2-deoxy-glucitol hydrochloride **6** (972 mg, 4.87 mmol) in THF (15 mL) and triethylamine (0.68 mL, 4.87 mmol) water (2 mL) was added dropwise.

The resulting solution was added slowly to the cooled (−10 °C) solution of **6** under vigorous stirring. The mixture was stirred for 10 min at −10 °C and then allowed to warm to room temperature over 2 h. After evaporation the oily residue was dissolved in dry pyridine (25 mL) and treated with acetic anhydride (8 mL, 84.6 mmol). The mixture was stirred at room temperature for 16 h and then the solvent was removed under reduced pressure (codestillation with toluene). Purification of the crude product by MPLC chromatography on silica gel (EtOAc/light petroleum 1:2) gave **8** (2.3 g, 75%) as a colourless solid. M.p. 157 °C; $[\alpha]^{25}_D$ +38.2 (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.91 (s, 3 H; Ac–H_3) 1.96 (s, 3 H; Ac–H_3), 2.01 (s, 3 H; Ac–H_3), 2.57 (dd,1 H, $J_{\alpha,\beta}$ 4.6, $J_{\beta,\beta*}$ 15.8 Hz; Asn-β-H),

2.76 (dd, 1 H, J_{α,β^*} 5.1, J_{β,β^*} 15.8 Hz; Asn-β*-H), 2.95 (dd, 1 H, $J_{1a,1e}$ 11.2 Hz, $J_{1a,2}$ 11.2 Hz; H-1a), 3.42 (ddd, 1 H, $J_{4,5}$ 9.7, $J_{5,6}$ 2.5, $J_{5,6^*}$ 5.1 Hz; H-5), 3.95 (dd, 1 H, $J_{1a,1e}$ 11.2, $J_{1e,2}$ 5.1 Hz; H-1e), 4.01 (m, 1 H, H-2), 4.02–4.18 (m, 2H, $J_{5,6}$ 2.5, $J_{5,6^*}$ 5.1 Hz; H-6, H-6*), 4.52 (ddd, 1 H, $J_{\alpha,\beta}$ 4.6, J_{α,β^*} 5.1, $J_{a,\text{Asn–NH}}$ 8.1 Hz; Asn-α-H), 4.82 (dd, 1 H, $J_{2,3}$ 10.7, $J_{3,4}$ 10.2 Hz; H-3), 4.95 (dd, 1 H, $J_{3,4}$ 10.7, $J_{4,5}$ 9.7 Hz; H-4), 5.03 (s, 2 H; CH_2–Bn), 5.10 (s, 2 H; CH_2–Bn), 5.75 (d, 1 H, $J_{\text{NH-2,2}}$ 7.6 Hz; NH-2), 5.88 (d, 1 H, $J_{\alpha,\text{Asn-NH}}$ 8.1 Hz; Asn–NH), 7.23–7.31 (m, 10 H, Ar); ^{13}C NMR ($CDCl_3$) δ 19.58, 19.65, 19.72 (3×Ac–CH_3), 36.88 (Asn-β-C), 49.44 (C-2), 49.83 (Asn-α-C), 61.32 (C-6), 66.08 (Bn–CH_2), 66.47 (Bn–CH_2), 66.90 (C-1), 67.10 (C-4), 73.02 (C-3), 75.51 (C-5), 127.06 , 127.19, 127.42, 127.52, 127.60, 134.31, 135.13 (2×Ar), 168.27 (Z–CO), 168.84 (Ac–CO), 169.0 (NH–CO), 169.5 (CO) 169.8, 171.2 (2×Ac–CO); MS (FAB): 629.2 $(M+H)^+$. Anal. Calcd. for $C_{31}H_{36}N_2O_{12}$: C, 59.21; H, 5.78; N, 4.46. Found: C, 59.52; H, 5.73; N, 4.34.

N^{γ}-*[3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-glucitol-2]*-L-*asparagine* (**1**).—To a solution of **8** (500 mg, 0.8 mmol) in dry methanol (25 mL) Pd/C (50 mg) was added and the mixture was stirred under hydrogen atmosphere (1 bar) for 8 h. The catalyst was filtered off and the solution was concentrated under reduced pressure. Yield: 323.5 mg (100%), colourless foam. M.p. 115 °C; $[\alpha]^{25}_D$ +14.3 (*c* 1, H_2O); ^{1}H NMR (D_2O) δ 2.10 (s, 3 H; Ac–H_3), 2.12 (s, 3 H; Ac–H_3), 2.14 (s, 3 H; Ac–H_3), 2.94 (d, 2 H, $J_{\alpha,\beta}$ 5.6 Hz; Asn-β-H), 3.53 (dd, 1 H, $J_{1a,1e}$ 11.7, $J_{1a,2}$ 11.2 Hz; H-1a), 3.92 (ddd, 1 H, $J_{4,5}$ 10.2, $J_{5,6}$ 4.1, $J_{5,6^*}$ 2.0 Hz; H-5), 4.04 (dd, 1 H, $J_{1a,1e}$ 11.7, $J_{1e,2}$ 5.6 Hz; H-1e), 4.21 (dd, 1 H, $J_{5,6}$ 4.1, $J_{6,6^*}$ 12.7 Hz; H-6), 4.28 (ddd, 1 H, $J_{1a,2}$ 11.2, $J_{1e,2}$ 5.6, $J_{2,3}$ 9.2 Hz; H-2), 4.30 (t, 1 H, $J_{\alpha,\beta}$ 5.6 Hz; Asn-α-H), 4.37 (dd, 1 H, $J_{5,6^*}$ 2.0, $J_{6,6^*}$ 12.7 Hz; H-6*), 5.05 (dd, 1 H, $J_{2,3}$ 9.2, $J_{3,4}$ 9.7 Hz; H-3), 5.22 (dd, 1 H, $J_{3,4}$ 9.7, $J_{4,5}$ 10.2 Hz; H-4); ^{13}C NMR (D_2O) δ 20.45, 20.47, 20.52 (3×Ac–CH_3), 35.36 (Asn–β-C), 49.36 (C-2), 51.56 (Asn–α-C), 62.64 (C-6), 67.29 (C-1), 69.22 (C-4), 74.57 (C-3), 75.89 (C-5), 172.25 (Ac–CO), 173.29 (CO), 173.83, 174.08 (2×Ac–CO); MS (FAB): 405.2 $(M+H)^+$. Anal. Calcd. for $C_{16}H_{24}N_2O_{10}$: C, 47.52; H, 5.98; N, 6.93. Found: C, 47.48; H, 5.95; N, 6.85.

N^{α}-*(9′-Fluorenylmethyloxycarbonyl)*-N^{γ}-*[3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-glucitol-2]*-L-*asparagine* (**9**).—A solution of 9-fluorenylmethyl-succinimidyl carbonate (327 mg, 0.97 mmol) in DMF (4 mL) was added to a vigorous stirred solution of **1** (380 mg, 0.97 mmol) in 5 mL sat. $NaHCO_3$-solution at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 30 min, water (25 mL) was added and the solution was washed with diethyl ether (25 mL) and EtOAc (2×25 mL). Then the aqueous phase was cooled to 0 °C and acidified with hydrochloric acid (5 N) to pH 2. The precipitated product was extracted with EtOAc (4×25 mL) and the combined organic layers were washed with water (3×50 mL) and saturated NaCl solution (3×50 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give 520 mg of **9** (86%) as a colourless solid.

M.p. 218 °C; $[\alpha]^{25}_D$ +8.6 (*c* 1, DMSO); ^{1}H NMR ([D6]DMSO) δ 1.90 (s, 3 H; Ac–H_3), 1.96 (s, 3 H; Ac–H_3), 1.99 (s, 3 H; Ac–H_3), 2.39 (dd, 1H, $J_{\alpha,\beta}$ 8.1, J_{β,β^*} 15.2 Hz; Asn-β-H), 2.55 (dd, 1 H, J_{α,β^*} 5.6, J_{β,β^*} 15.2 Hz; Asn-β*-H), 3.23 (dd, 1H, $J_{1a,1e}$ 11.2, $J_{1a,2}$ 11.2 Hz; H-1a), 3.67 (ddd, 1 H, $J_{4,5}$ 10.5, $J_{5,6}$ 2.2, $J_{5,6^*}$ 5.1 Hz; H-5), 3.73 (dd, 1 H, $J_{1a,1e}$ 11.2, $J_{1e,2}$ 5.6 Hz; H-1e), 3.97–4.00 (m, 1 H, $J_{a,\beta}$ 8.1, J_{α,b^*} 5.6, Asn-α-H), 4.00 (dd, 1 H, $J_{5,6}$ 2.2, $J_{6,6^*}$ 12.2 Hz; H-6), 4.09 (dd, 1 H, $J_{5,6^*}$ 5.1, $J_{6,6^*}$ 12.2 Hz; H-6*), 4.18–4.31 (m, 4 H, $J_{1a,2}$ 11.2, $J_{1e,2}$ 5.6, $J_{2,3}$ 9.66 Hz; H-2, Fmoc–CH_2, Fmoc–CH), 4.78 (dd, 1 H, $J_{2,3}$ 9.7 $J_{3,4}$ 9.7 Hz; H-3), 4.97 (dd, 1 H, $J_{3,4}$ 9.7, $J_{4,5}$ 10.5 Hz; H-4), 7.29–7.93 (m, 8 H; Fmoc–Ar); ^{13}C NMR ([D6]DMSO) δ 21.22, 21.33 (3×Ac–CH_3), 36.71 (Asn-β-C), 47.40 (Fmoc–H), 49.46 (C-2), 51.25 (Asn-α-C), 63.01 (C-6), 66.58 (Fmoc–CH_2), 67.84 (C-1), 69.67 (C-4), 74.35 (C-3), 76.16 (C-5), 120.96, 126.41, 127.99, 128.57, 141.53, 144.52, 144.59 (Fmoc–Ar), 156.64 (Fmoc–CO), 163.42 (NH–CO), 170.37, 170.92, 171.12 (3×Ac–CO), 173.54 (CO). MS (FAB): 627.2 $(M+H)^+$. Anal. Calcd. for $C_{31}H_{34}N_2O_{12}$: C, 59.42; H, 5.47; N, 4.47. Found: C, 59.17; H, 5.51; N, 4.44.

General procedure for solid-phase synthesis of peptides (**3**) *and* (**10**–**12**).—The standard synthesis protocol from Perseptive Applied Biosystems synthesizer was performed using 4 equivalents of N-Fmoc-protected amino acid derivatives and piperidine (20% in DMF) as Fmoc removal reagent. Fmoc-Rink-PEGA resin (0.180 mmol/g substitution) was used in a 278 mg scale (50 μmol) for each peptide. DMF was used as solvent for all reactions. The building block **9** was coupled manually outside the synthesizer in equimolar value. The peptide synthesis was finished by acetylation with acetic anhydride (50% in DMF) and cleavage of the glycopeptides from the polymer with concurrent

removal of the Boc, *t*Bu, Pbf and trityl side-chain protecting groups by treating the resin with aq 95% CF_3CO_2H (2 mL) two times for each 1 h. The resin was washed with aq 95% CF_3CO_2H (5×2 mL, 2 min each). The combined filtrates were evaporated and the residue was coevaporated several times with toluene and 3:1 toluene–MeOH. Deacetylated compounds **11** and **3** were available by dissolving glycopeptides **10** resp. **12** in aq 50% MeOH (2 mL) and addition of a 1% solution of NaOMe in MeOH ($\approx 50\,\mu$L) until pH 9.0. The reaction was stopped by the addition of HOAc (10 μL).The deprotection was followed by analytical HPLC.

N^{α}-*Acetyl*-L-*threonyl*-N^{γ}-*[3,4,6-tri*-O-*acetyl-1,5-anhydro-2-deoxy-glucitol-2]*-L-*asparagyl*-L-*aspartyl*-L-*threonylamide* (**10**).—Acylation times for all amino acids were 30 min according to the standard protocol, but 24 h in case of building block **9**. The cleavage of **10** from the polymer was followed by purification using HPLC [buffer A-buffer B, 100/0→50/50 (10 min)]. Yield: 8 mg (21%) (calculated on the substitution of the resin). ^{1}H NMR (D_2O, pD 3.5) δ 1.14 (d, 3 H, $J_{\beta,\gamma}$ 6.4 Hz, Thr-γ-H_3), 1.18 (d, 3 H, $J_{\beta,\gamma}$ 6.4 Hz, Thr-γ-H_3), 2.01 (s, 3 H, Ac–H_3), 2.04 (s, 3 H, Ac–H_3), 2.04 (s, 3 H, Ac–H_3), 2.06 (s, 3 H, Ac–H_3), 2.63 (dd, 1 H, $J_{a,\beta}$ 7.1 Hz, J_{β,β^*} 15.4 Hz, Asn-β-H), 2.77 (dd, 1 H, J_{α,β^*} 6.2 Hz, J_{β,β^*} 15.4 Hz, Asn-β*-H), 2.83 (dd, 1 H, $J_{a,\beta}$ 7.3 Hz, J_{β,β^*} 17.1 Hz, Asn-β*-H), 2.92 (dd, 1 H, J_{α,β^*} 5.6 Hz, J_{β,β^*} 17.1 Hz, Asn-β*-H), 3.41 (dd, 1 H, $J_{1a,1e}$ 11.5 Hz, $J_{1a,2}$ 11.4 Hz, H-1a), 3.82 (ddd, 1 H, $J_{4,5}$ 9.9 Hz, $J_{5,6}$ 2.0 Hz, $J_{5,6^*}$ 4.6 Hz, H-5), 3.92 (dd, 1 H, $J_{1a,1e}$ 11.5 Hz, $J_{1e,2}$ 5.5 Hz, H-1e), 4.02 (ddd, 1 H, $J_{1a,2}$ 11.4 Hz, $J_{1e,2}$ 5.5 Hz, $J_{2,3}$ 10.4 Hz, H-2), 4.02 (dd, 1 H, $J_{5,6}$ 2.0 Hz, $J_{6,6^*}$ 13.0 Hz, H-6) 4.12 (dd, 1 H, $J_{\alpha,\beta}$ 4.6 Hz, $J_{\beta,\gamma}$ 6.4 Hz, Thr-β-H), 4.24 (d, 1 H, $J_{a,\beta}$ 4.6 Hz, Thr-α-H), 4.25 (dd, 1 H, $J_{a,\beta}$ 5.1 Hz, $J_{\beta,\gamma}$ 6.4 Hz, Thr-β-H), 4.25 (d, 1 H, $J_{\alpha,\beta}$ 5.1 Hz, Thr-α-H), 4.29 (dd, 1 H, $J_{5,6^*}$ 4.6 Hz, $J_{6,6^*}$ 13.0 Hz, H-6*), 4.69 (dd, 1 H, $J_{\alpha,\beta}$ 7.1 Hz, J_{α,β^*} 6.2 Hz, Asn-α-H), 4.77 (dd, 1 H, $J_{\alpha,\beta}$ 7.3 Hz, J_{a,β^*} 5.6 Hz, Asn-α-H), 4.96 (dd, 1 H, $J_{3,4}$ 9.2 Hz, $J_{4,5}$ 9.9 Hz, H-4), 5.12 (dd, 1 H, $J_{2,3}$ 10.4 Hz, $J_{3,4}$ 9.2 Hz, H-3). MS (FAB) Calcd for $C_{30}H_{46}N_6O_{17}$ $(M)^+$: 762.74. Found: $(M+Na)^+$ 785.8 (81%), $(M+H)^+$ 763.8 (100%), $(M–NH_2)^+$ 746.8 (37%).

N^{α}-*Acetyl*-L-*threonyl*-N^{γ}-*[1,5-anhydro-2-deoxy-glucitol-2]*-L-*asparagyl*-L-*aspartyl*-L-*threonylamide* (**11**).—The reaction time was 3 h. The final product was purified by HPLC [buffer A-buffer B, 100/0→85/15 (5 min)→60/40 (10 min)]. Yield: 4.0 mg (60% overall yield: 13%). ^{1}H NMR (D_2O, pD 3.5) δ 1.15 (d, 3 H, $J_{\beta,\gamma}$ 6.4 Hz, Thr-γ-H3), 1.16 (d, 3 H, $J_{\beta,\gamma}$ 6.1 Hz, Thr-γ-H_3), 2.05, (s, 3 H, Ac–H_3), 2.71 (dd, 1 H, $J_{\alpha,\beta}$ 6.2 Hz, J_{β,β^*} 15.5 Hz, Asn-β-H), 2.84 (dd, 1 H, J_{α,β^*} 6.1 Hz, J_{β,β^*} 15.5 Hz, Asn-β*-H), 2.84 (dd, 1H, $J_{\alpha,\beta}$ 7.6 Hz, $J\beta,\beta^*$ 16.5 Hz, Asn–β–H), 2.94 (dd, 1H J_{α,β^*} 5.3 Hz, J_{β,β^*} 16.5 Hz, Asn-β*-H), 3.12 (dd, 1 H, $J_{1a,1e}$ 10.4 Hz, $J_{1a,2}$ 10.7 Hz, H-1a), 3.25 (ddd, 1 H, $J_{4,5}$ 9.5 Hz, $J_{5,6}$ 2.2 Hz, $J_{5,6^*}$ 5.7 Hz, H-5), 3.32 (dd, 1 H, $J_{3,4}$ 9.0 Hz, $J_{4,5}$ 9.5 Hz, H-4), 3.43 (dd, 1 H, $J_{2,3}$ 9.4 Hz, $J_{3,4}$ 9.0 Hz, H-3), 3.61 (dd, 1 H, $J_{5,6}$ 2.2 Hz, $J_{6,6^*}$ 12.3 Hz, H-6) 3.73 (ddd, 1 H, $J_{1a,2}$ 10.7 Hz, $J_{1e,2}$ 5.1 Hz, $J_{2,3}$ 9.4 Hz, H-2), 3.78 (dd, 1 H, $J_{5,6^*}$ 5.7 Hz, $J_{6,6^*}$ 12.3 Hz, H-6*), 3.80 (dd, 1 H, $J_{1a,1e}$ 10.4 Hz, $J_{1e,2}$ 5.1Hz, H-1e), 4.14 (dd, 1 H, $J_{\alpha,\beta}$ 4.6 Hz, $J_{\beta,\gamma}$ 6.4 Hz, Thr-β-H), 4.26 (d, 1 H, $J_{\alpha,\beta}$ 4.6 Hz, Thr-α-H), 4.26 (dd, 1 H, $J_{\alpha,\beta}$ 6.1 Hz, $J_{\beta,\gamma}$ 6.1 Hz, Thr-β-H), 4.26 (d, 1 H, $J_{a,\beta}$ 6.1 Hz, Thr-α-H), 4.73 (dd, 1 H, $J_{\alpha,\beta}$ 6.2 Hz, J_{α,β^*} 6.1 Hz, Asn-α-H), 4.79 (dd, 1 H, $J_{\alpha,\beta}$ 7.6 Hz, J_{α,β^*} 5.3 Hz, Asn-α-H). MS (MALDI–TOF) Calcd for $C_{24}H_{40}N_6O_{14}$ $(M)^+$: 636.26. Found: $(M+Na)^+$ 658.3.

N^{α}-*Acetyl*-L-*seryl*-N^{γ}-*[3,4,6-tri*-O-*acetyl-1,5-anhydro-2-deoxy-glucitol-2]*-L-*asparagyl*-L-*asparagyl*-L-*threonyl*-L-*arginyl*-L-*lysyl*-L-*seryl*-L-*isoleucyl*-L-*histidyl*-L-*isoleucyl*-L-*glycyl*-L-*prolyl*-L-*glycyl*-L-*arginyl*-L-*alanyl*-L-*phenylalanylamide* (**12**).—Acylation times for all amino acids were 90 min, but in case of building block **9** 24 h. The cleavage of one third aliquot of the product **12** was followed by purification using HPLC [buffer A–buffer B, 100/0→80/20 (5 min)→70/30 (30 min)→50/50 (5 min)]. Yield: 7.6 mg (23%) (calculated on the substitution of the resin). ^{1}H NMR (D_2O, pD 3.5) δ 0.83 (dd, 3 H, $J_{\gamma^*a,\delta}$ 7.6 Hz, $J_{\gamma^*b,\delta}$ 7.9 Hz, Ile-δ-H_3), 0.83 (dd, 3 H, $J_{\gamma^*a,\delta}$ 7.4, $J_{\gamma^*b,\delta}$ 7.6 Hz, Ile-δ-H_3), 0.85 (d, 3H, $J_{\beta,\gamma}$ 6.9 Hz, Ile-γ-H_3), 0.90 (d, 3 H, $J_{\beta,\gamma}$ 6.9 Hz, Ile-γ-H_3), 1.13 (m, 1 H, J_{γ^*a,γ^*b} 20.3 Hz, $J_{\gamma^*b,\delta}$ 7.9 Hz, Ile-γ^*_b-H), 1.15 (m, 1 H, J_{γ^*a,γ^*b} 20.3 Hz, $J_{\gamma^*b,\delta}$ 7.6 Hz, Ile-γ^*_b-H), 1.22 (d, 3 H, $J_{\beta,\gamma}$ 6.4 Hz, Thr-γ-H), 1.28 (d, 1H, $J_{\alpha,\beta}$ 7.1 Hz, Ala-β-H), 1.38 (m, 1H, J_{γ^*a,γ^*b} 20.3 Hz, $J\gamma^*a,\delta$ 7.6 Hz, Ile-γ^*_a-H), 1.44 (m, 1 H, J_{γ^*a,γ^*b} 20.3 Hz, $J_{\gamma^*a,\delta}$ 7.4 Hz, Ile-γ^*_a-H), 1.57 (ddt, 2 H, $J_{\beta,\gamma}$ 8.6 Hz, $J_{\beta^*,\gamma}$ 5.7 Hz, $J_{\gamma,\delta}$ 6.9 Hz, Arg-γ-H_2), 1.57 (m, 2H, $J_{\gamma,\delta}$ 8.5 Hz, Lys-γ-H_2), 1.64 (ddt, 2 H, $J_{\beta,\gamma}$ 9.1 Hz, $J_{\beta^*\gamma}$ 6.0 Hz, $J_{\gamma,\delta}$ 6.9 Hz, Arg-γ-H_2), 1.68 (ddt, 2 H, $J_{\gamma,\delta}$ 8.5 Hz, $J_{\delta,\varepsilon}$ 7.6 Hz, J_{δ,ε^*} 8.1 Hz, Lys-δ-H_2), 1.75 (m, 1 H, $J_{\beta,\gamma}$ 8.6 Hz, Arg-β-H), 1.79 (m, 1 H, Lys-β-H), 1.81 (m, 1 H, $J_{\alpha,\beta}$ 7.4 Hz, $J_{\beta,\gamma}$ 6.9 Hz, Ile-β-H), 1.81 (m, 1 H, $J_{\beta,\gamma}$ 9.1 Hz, Arg-β-

Table 2
^{1}H NMR data (H_2O/D_2O 9:1, pH 3.5, 500 MHz) for glycopeptide **3** coupling constants (Hz) in parentheses

δ(ppm)	NH	Hα$_1$	Hα$_2$	Hβ$_1$	Hβ$_2$	Hγ$_{1a}$	Hγ$_{1b}$	Hγ$_2$	Hδ$_1$	Hδ$_2$	Hε$_1$	Hε$_2$	Ac/NH	H-1a	H-1b	H-2	H-3	H-4	H-5	H-6a	H-6b	Arom.
Ser-1	8.302 (6.3)	4.392 (5.7) (5.3)		3.847 (11.9)	3.804								2.044									
Asn-2	8.544 (7.8)	4.731 (5.4) (7.6)		2.850 (15.9)	2.741								8.160 (8.5)	3.870 (11.0) (5.4)	3.199 (10.8)	3.799 (10.1)	3.505 (8.7)	3.375 (9.7)	3.314 (2.2) (5.8)	3.855 (12.3)	3.374	
Asn-3	8.364 (7.3)	4.740 (6.0) (7.5)		2.842 (15.9)	2.746								7.493									
Thr-4	8.109 (7.5)	4.278 (4.5)		4.234 (6.4)		1.188																
Arg-5	8.265 (7.3)	4.310 (6.3) (8.5)		1.843 (7.5)	1.755	1.612 (6.6)		1.612 (6.9)	3.173 (6.0)	3.173			7.136									
Lys-6	8.250 (7.8)	4.303 (6.2) (8.0)		1.806 (15.9)	1.719	1.411 (8.6)		1.411 (5.8)	1.654 (7.4)	1.654 (7.4)	2.967	2.967										
Ser-7	8.252 (7.3)	4.431 (5.0) (5.3)		3.822 (11.5)	3.780																	
Ile-8	8.055 (7.7)	4.153 (7.5)		1.796 (7.1) (7.3)		1.314 (19.8) (7.3)	1.106 (7.5)	0.815	0.798													
His-9	8.559 (7.6)	4.718 (6.4) (8.9)		3.192 (15.3)	3.098											8.556 (1.4)		7.232				
Ile-10	8.210 (7.9)	4.191 (8.2)		1.793 (7.0) (6.7)		1.416 (20.2) (7.3)	1.127 (7.8)	0.871	0.808													
Gly-11	8.300 (5.9) (5.5)	4.114 (17.3)	4.033																			
Pro-12		4.350 (8.5) (4.6)		2.252 (13.5) (7.7)	1.961 (7.0)	2.000 (6.6)		2.000 (7.2)	3.645 (10.0)	3.594												
Gly-13	8.469 (5.9)	3.910	3.910																			
Arg-14	8.089 (6.7)	4.259 (6.4) (8.1)		1.750 (5.7)	1.663 (3.6)	1.532 (6.1)		1.532 (6.1)	3.131	3.131			7.106									
Ala-15	8.262 (6.6)	4.240 (7.2)		1.258																		
Phe-16	8.075 (7.3)	4.533 (6.0) (8.6)		3.123 (13.9)	2.990								7.463 7.041									7.36–7.22

H), 1.82 (m, 1 H, $J_{\beta^*\gamma}$ 5.7 Hz, Arg-β*-H), 1.84 (m, 1 H, $J_{\alpha,\beta}$ 7.9 Hz, $J_{\beta,\gamma}$ 6.9 Hz, Ile-β-H), 1.85 (m, 1 H, $J_{\beta^*,\gamma}$ 6.0 Hz, Arg-β*-H), 1.86 (m, 1 H, Lys-β*-H), 1.99 (ddt, 1 H, $J_{\alpha,\beta}$ 6.4 Hz, J_{β,β^*} 11.9 Hz, $J_{\beta,\gamma}$ 6.9 Hz, Pro-β-H), 2.04 (dddt, 2 H, $J_{\beta,\gamma}$ 6.9 Hz, $J_{\beta^*,\gamma}$ 7.2 Hz, $J_{\gamma,\delta}$ 6.4 Hz, J_{γ,δ^*} 9.4 Hz, Pro-γ-H_2), 2.05 (s, 3 H, Ac–H_3), 2.07 (s, 3 H, Ac–H_3), 2.08 (s, 3 H, Ac–H_3), 2.10 (s, 3 H, Ac–H_3), 2.28 (ddt, 1 H, J_{α,β^*} 9.2 Hz, J_{β,β^*} 11.9 Hz, $J_{\beta^*,\gamma}$ 7.2 Hz, Pro-β*-H), 2.68 (dd, 1 H, $J_{\alpha,\beta}$ 7.1 Hz, J_{β,β^*} 15.6 Hz, Asn-β-H), 2.76 (dd, 1 H, $J_{\alpha,\beta}$ 7.6 Hz, J_{β,β^*} 16.0 Hz, Asn-β-H), 2.80 (dd, 1 H, J_{α,β^*} 6.4 Hz, J_{β,β^*} 15.6 Hz, Asn-β*-H), 2.86 (dd, 1 H, J_{α,β^*} 6.0 Hz, J_{β,β^*} 16.0 Hz, Asn-β*-H), 2.99 (dd, 2 H, $J_{\gamma,\delta}$ 7.6 Hz, $J_{\gamma^*,\delta}$ 8.1 Hz, Lys-ε-H2), 3.02 (dd, 1 H, $J_{\alpha,\beta}$ 8.5 Hz, J_{β,β^*} 14.2 Hz, Phe-β-H), 3.10 (dd, 1 H, $J_{\alpha,\beta}$ 7.8 Hz, J_{β,β^*} 15.8 Hz, His-β-H), 3.15 (dd, 1 H, $J_{\alpha,\beta}$ 6.0 Hz, J_{β,β^*} 14.2 Hz, Phe-β*-H), 3.16 (t, 2 H, $J_{\gamma,\delta}$ 6.9 Hz, Arg-δ-H2), 3.20 (t, 2 H, $J_{\gamma,\delta}$ 6.9 Hz, Arg-δ-H2), 3.22 (dd, 1 H, J_{α,β^*} 7.1 Hz, J_{β,β^*} 15.8 Hz, His-β*-H), 3.47 (dd, 1 H, $J_{1a,1e}$ 11.4 Hz, $J_{1a,2}$ 11.4 Hz, H-1a), 3.62 (dt, 1 H, $J_{\gamma,\delta}$ 6.4 Hz, J_{δ,δ^*} 13.5 Hz, Pro-δ-H), 3.70 (dt, 1 H, J_{γ,δ^*} 9.4 Hz, $J_{\delta2,\delta^*}$ 13.5 Hz, Pro-δ*-H), 3.82 (t, 2 H, $J_{\alpha,\beta}$ 5.0 Hz, Ser-β-H_2), 3.83 (dd, 1 H, $J_{\alpha,\beta}$ 5.3 Hz, J_{β,β^*} 11.7 Hz, Ser-β-H), 3.85 (dd, 1 H, J_{α,β^*} 5.3 Hz, J_{β,β^*} 11.7 Hz, Ser-β*-H), 3.87 (ddd, 1 H, $J_{4,5}$ 9.7 Hz, $J_{5,6}$ 1.9 Hz, $J_{5,6^*}$ 6.0 Hz, H-5), 3.93 (s, 2 H, Gly-α-H_2), 3.98 (dd, 1 H, $J_{1a,1e}$ 11.4 Hz, $J_{1e,2}$ 7.0 Hz, H-1e), 4.05 (d, 1 H, $J_{\alpha2,\alpha^*}$ 16.4 Hz, Gly-α-H), 4.14 (d, 1 H, $J_{\alpha2,\alpha^*}$ 16.4 Hz, Gly-α*-H), 4.17 (ddd, 1 H, $J_{1a,2}$ 11.4 Hz, $J_{1e,2}$ 7.0 Hz, $J_{2,3}$ 9.9 Hz, H-2), 4.18 (d, 1 H, $J_{\alpha,\beta}$ 7.4 Hz, Ile-α-H), 4.19 (dd, 1 H, $J_{5,6}$ 1.9 Hz, $J_{6,6^*}$ 13.0 Hz, H-6), 4.22 (d, 1 H, $J_{\alpha,\beta}$ 7.9 Hz, Ile-α-H), 4.26 (m, 2 H, $J_{\alpha2,\beta}$ 6.4 Hz, Thr-α-H, Thr-β-H), 4.26 (d, 1 H, $J_{\alpha2,\beta}$ 7.1 Hz, Ala-α-H), 4.28 (m, 1 H, Lys-α-H), 4.32 (dd, 1 H, $J_{5,6^*}$ 6.0 Hz, $J_{6,6^*}$ 13.0 Hz, H-6*), 4.32 (m, 1 H, Arg-α-H), 4.34 (m, 1 H, Arg-α-H), 4.40 (t, 1 H, $J_{\alpha,\beta}$ 5.3 Hz, Ser-α-H), 4.46 (t, 1 H, $J_{\alpha,\beta}$ 5.0 Hz, Ser-α-H), 4.47 (dd, 1 H, $J_{\alpha,\beta}$ 6.4 Hz, J_{α,β^*} 9.2 Hz, Pro-α-H), 4.54 (dd, 1 H, $J_{\alpha,\beta}$ 8.5 Hz, J_{α,β^*} 6.0 Hz, Phe-α-H), 4.72 (dd, 1 H, $J_{\alpha,\beta}$ 7.1 Hz, J_{α,β^*} 6.4 Hz, Asn-α-H), 4.75 (dd, 1 H, $J_{\alpha,\beta}$ 7.8 Hz, J_{α,β^*} 7.1 Hz, His-α-H), 4.76 (dd, 1 H, $J_{\alpha,\beta2}$ 7.6 Hz, $J_{\alpha2,\beta2^*}$ 6.0 Hz, Asn-α-H), 4.99 (dd, 1 H, $J_{3,4}$ 9.4 Hz, $J_{4,5}$ 9.7 Hz, H-4), 5.17 (dd, 1 H, $J_{2,3}$ 9.9 Hz, $J_{3,4}$ 9.4 Hz, H-3), 7.2–7.4 (m, 5 H, Phe–Ar–H_5), 7.26 (d, 1 H, $J_{2,4}$ 1.4 Hz, His-4-H), 8.60 (d, 1 H, $J_{2,4}$ 1.4 Hz, His-2-H). MS (MALDI–TOF): Calcd for $C_{89}H_{142}N_{28}O_{29}$ $(M)^+$: 2067.05. Found: $(M+H)^+$ 2068.1.

N^{α}-*Acetyl*-L-*seryl*-N^{γ}-*[1,5-anhydro-2-deoxy-glucitol-2]*-L-*asparagyl*-L-*asparagyl*-L-*threonyl*-L-*arginyl*-L-*lysyl*-L-*seryl*-L-*isoleucyl*-L-*histidyl*-L-*isoleucyl*-L-*glycyl*-L-*prolyl*-L-*glycyl*-L-*arginyl*-L-*alanyl*-L-*phenylalanylamide* (**3**).—The reaction time was 4 h. The final product was purified by HPLC [buffer A–buffer B, 100/0→85/15 (5 min)→75/25 (40 min)→60/40 (5 min)]. Yield: 3.9 mg (55%, overall yield: 12%). MS (MALDI–TOF) Calcd for $C_{83}H_{136}N_{28}O_{26}$ $(M)^+$: 1941.02. Found: $(M+H)^+$ 1942.2

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