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Fmoc-protected iminosugar modified asparagine derivatives as building blocks for glycomimetics-containing peptides

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Abstract—CSF114(Glc) is the first synthetic Multiple Sclerosis Antigenic Probe able to identify autoantibodies in a statistically significant number of Multiple Sclerosis patients. The β-turn conformation of this glucopeptide is fundamental for a correct presentation of the epitope Asn(Glc). To verify the influence of sugar mimics in antibody recognition in Multiple Sclerosis, we synthesized Fmoc-protected Asn derivatives containing alkaloid-type sugar mimics. The corresponding glycomimetics-containing peptide derivatives of the CSF114-type sequence were tested in competitive and solid-phase non-competitive ELISA on Multiple Sclerosis patients' sera.

1. Introduction

The importance of carbohydrate recognition in biological events is well established on many experimental findings. How post-translational protein modifications, in particular glycosylation, can have a role in the origin of autoimmune responses is still not characterized but almost all of the key molecules involved in innate and adaptive immune responses are glycoproteins. Moreover, in the last years, a number of autoimmune diseases have been associated with glycosylation defects.[1]

Our interest was to further investigate the role of glycosyl moiety in autoantibody (auto-Ab) recognition in Multiple Sclerosis (MS) using different glycomimetic derivatives of CSF114 peptide sequence. CSF114(Glc) is a structure-based designed glucosylated peptide, characterized by a β-turn,[2] able to identify autoantibodies[3] in a statistically significant number of MS patients compared to healthy blood donors and other autoimmune diseases.[4] We demonstrated that the presence of a β-D-glucopyranosyl moiety on an Asn residue at position 7 of CSF114(Glc) is fundamental for auto-Ab recognition. In fact, no Abs could be identified by the corresponding unglycosylated peptide sequence. Moreover, the specific autoantibody recognition is most likely driven by direct interactions of the antibody binding site with the Asn-linked sugar moiety and not with the CSF114 peptide sequence. These data let us to assess that in MS, autoantibody recognition is strictly correlated with specific glycosylated epitopes.[3]

To extend auto-Ab recognition to glycosylated epitopes in MS and to verify the influence of sugar mimics, we

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synthesized glycomimetics-containing peptide derivatives of the CSF114-type sequence containing alkaloid-type sugar mimics having pyrrolidine and piperidine structures, well known as glycosidase inhibitors.[5]

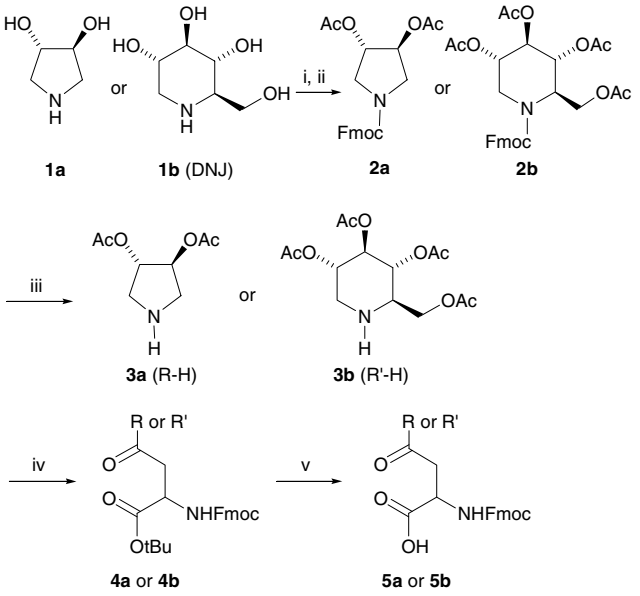
The activity of polyhydroxylated alkaloids found in plants and microorganisms is related to their ability of mimicking the pyranosyl or furanosyl structures of monosaccharides. These sugar mimics, in which the oxygen ring has been replaced by a nitrogen, are one of the most interesting discoveries in the field of natural products in recent years. Naturally occurring sugar mimics containing nitrogen are classified into several structural classes: polyhydroxylated piperidines and pyrrolidines, and polyhydroxylated alkaloids containing bicyclic skeletons that can be divided into fused compounds, such as pyrrolizidines and indolizidines (bearing a bridgehead nitrogen atom) and bridged bicyclic compounds such as nortropanes (possessing a secondary amine group). In these bicyclic alkaloids, the configuration of the stereogenic carbons bearing the hydroxyl groups relates to that of the corresponding carbohydrates.[6]

Iminosugars can be regarded as potential therapeutic agents and as tools for understanding biological recognition processes, because of the formation of specific bonds to the active sites of glycosidases.[7,8] Since the mode of action of glycosidases involves the cleavage of glycosidic bonds between sugar molecules, individual glycosidases show specificity for certain sugar molecules and for a specific anomeric configuration of the sugar.[9,10]

These enzymes are involved in the biosynthesis of the oligosaccharide portions of glycoproteins and glycolipids, which play a crucial role in mammalian cellular structures and functions. For instance, the oligosaccharide chains regulate the correct functioning of glycoproteins by stabilizing them and ensuring their correct conformation. In particular, 1-deoxynojirimycin [(2*S*-hydroxymethyl)-3*R*,4*R*,5*S*-piperidinetriol or 1,5-dideoxy-1,5-imino-D-glucitol, DNJ] has demonstrated interesting anti-diabetic, anti-cancer, and anti-HIV properties, and showed to possess potent inhibitory activity of glycosidase enzymes.[11–13] The iminosugar *N*-butyldeoxynojirimycin (NBDNJ) is a potent inhibitor of α-glucosidase I, a cellular enzyme removing terminal glucose residues from nascent oligosaccharide.[14,15]

In addition to their ability to inhibit processing of exoglycosidases, lysosomal glycosidases, and the intestinal disaccharidases involved in carbohydrate digestion, iminosugars appear to have additional activities, including immunomodulatory properties and inhibition of glycolipid synthesis, which continue to expand their range of potential uses.[16]

We were especially interested in preparing building blocks containing *N*-linked iminosugars and providing a general high yielding method to covalently bind them to the Asp side chain. The building blocks were protected for solid-phase peptide synthesis (SPPS), following the Fmoc/*t*-Bu strategy (Scheme 1).



Scheme 1. Reagents and conditions: (i) Fmoc-OSu, dry Py, N_2; (ii) Ac_2O, Py, N_2 (**2a** 70%; **2b** 84%); (iii) Pip 20%, THF (**3a** 64%; **3b** 80%); (iv) Fmoc-L-Asp-O*t*-Bu, HATU, NMM, DMF (**4a** 37%; **4b** 89%); (v) TFA/DCM (1:1) (**5a** 98%; **5b** 90%).

2. Chemistry

We undertook the synthesis of new asparagine Fmoc-protected building blocks bearing orthogonally protected polyhydroxylated iminosugars on the side chain: (*S*)-α-[[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[3*S*,4*S*-bis(acetyloxy)-1-pyrrolidine]butanoic acid [Fmoc-L-Asn(DHPyrAc2)-OH, **5a**], (*S*)-α-[[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[2*R*-[(acetyloxy)methyl]-3*R*,4*R*,5*S*-tris(acetyloxy)-1-piperidine]butanoic acid [Fmoc-L-Asn(DNJAc4)-OH, **5b**], (*S*)-α-[[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[2*R*-[2-deoxy-1,3,4,6-tetra-*O*-acetyl-2*R*-D-glucopyranosyl]-3*R*,4*R*-bis(1,1-dimethylethoxy)-1-pyrrolidine]butanoic acid 1-(pentafluorophenyl) ester [Fmoc-L-Asn(DHPyr*t*-Bu2-2-deoxy GlcAc4)-OPfp, **9a**], and (*S*)-α-[[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[2*S*-[2-deoxy-1,3,4-tri-*O*-acetyl-2*S*-L-rhamnopyranosyl]-3*S*,4*S*-bis(1,1-dimethylethoxy)-1-pyrrolidine]butanoic acid 1-(pentafluorophenyl) ester [Fmoc-L-Asn(DHPyr*t*-Bu2-2-deoxyRhaAc3)-OPfp, **9b**] (Fig. 1).

Since the iminosugars employed are not commercially available, it was necessary to produce polyhydroxylated nitrogen heterocycles, that is, 3,4-dihydroxypyrrolidine (DHPyr, **1a**),[17] deoxynojirimycin (DNJ, **1b**),[18] 2-deoxy-2-[(2*R*,3*R*,4*R*)-3,4-dimethylethoxy-2-pyrrolidinyl]-3,4,6-tri-*O*-acetyl-D-glucopyranose (**6a**), and 2-deoxy-2-[(2*S*,3*S*,4*S*)-3,4-dimethylethoxy-2-pyrrolidinyl]-3,4-di-*O*-acetyl-L-rhamnopyranose (**6b**).[19,20] Hydrogenolysis of (3*S*,4*S*)-1-benzylpyrrolidine over $Pd(OH)_2/C$ gave 3,4-dihydroxypyrrolidine **1a**.[21] Deoxynojirimycin **1b**, an iminosugar with the same number of hydroxyl functions and configuration of glucose, was prepared following the method of Matos et al.[18] monitoring the deprotection of the hydroxyl functions by electrospray ionization

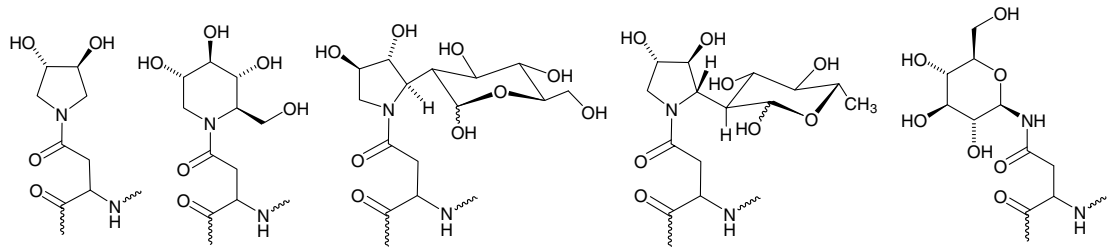


Figure 1. *N*-Linked derivatives of Asn: DHPyr, DNJ, DHPyr-2-deoxyGlc, DHPyr-2-deoxyRha, and Glc.

mass spectrometry (ESI-MS). Two pseudoimino-*C*-disaccharides containing a dihydroxypyrrolidine linked to deoxyglucose **6a** or deoxyrhamnose **6b** were obtained by an intermolecular 1,3-dipolar cycloaddition between an enantiopure pyrroline *N*-oxide and the appropriate 1,2-glycal that produces a tricyclic isoxazolidine.[19,20]

The target molecules were obtained by isoxazolidine ring-opening and sequential steps of protection, Fmoc-deprotection, and coupling with Fmoc-L-Asp-OH protected as *tert*-butyl or pentafluorophenyl ester. For temporary secondary amine protection, the iminosugars **1a** and **b** and **6a** and **b** were treated with *N*-(9*H*-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) in pyridine. Then, *O*-acetylation of hydroxyl functions was achieved in situ by addition of acetic anhydride, as reported by Meldal and Bock.[22] After purification by FCC, the fully protected iminosugars **2a** and **b** and **7a** and **b** were obtained in good yields. Treatment of the Fmoc-protected iminosugars with a solution of 20% piperidine in THF gave the corresponding Fmoc-deprotected compounds **3a** and **b** and **8a** and **b**.

Coupling between the amino acid and the iminosugar moieties was performed using in situ coupling reagents, or pre-activation of the α-carboxyl group. In the present work, both methods were employed in good yield. Fmoc-L-Asp-O*t*-Bu was coupled with monocyclic iminosugars **3a** and **b** using HATU as coupling reagent and NMM in DMF to obtain **4a** and **b**, while Fmoc-L-Asp(Cl)-OPfp was coupled with bicyclic iminosugars **8a** and **b** to obtain **9a** and **b** (Fig. 2 and Scheme 2) using dry THF and NMM. Fmoc-L-Asp(Cl)-OPfp was obtained as described by Meldal et al.[23] After deprotection of α-carboxyl group of asparagine derivatives **4a** and **b** with TFA, compounds **5a** and **b** were obtained (Fig. 2).

The strategy involving pre-activated Fmoc-Asp(Cl)-OPfp was chosen to not interfere with hydroxyl protection of the imino-*C*-disaccharides. The building blocks **5a** and **b** and **9a** and **b**, containing the sugar mimics, have been introduced in the CSF114 sequence, obtaining glycopeptides [Asn^7(DHPyr)]CSF114 (**10**), [Asn^7(DNJ)]CSF114 (**11**), and [Asn^7(DHPyr-2-deoxy-Glc)]CSF114 (**12**).

The synthesis of the peptide containing the amino sugar **9b** was unsuccessful possibly because of problems related to the steric hindrance of the rhamnose-containing imino-*C*-disaccharide. All the glycopeptides were synthesized following the Fmoc/*t*-Bu strategy and the

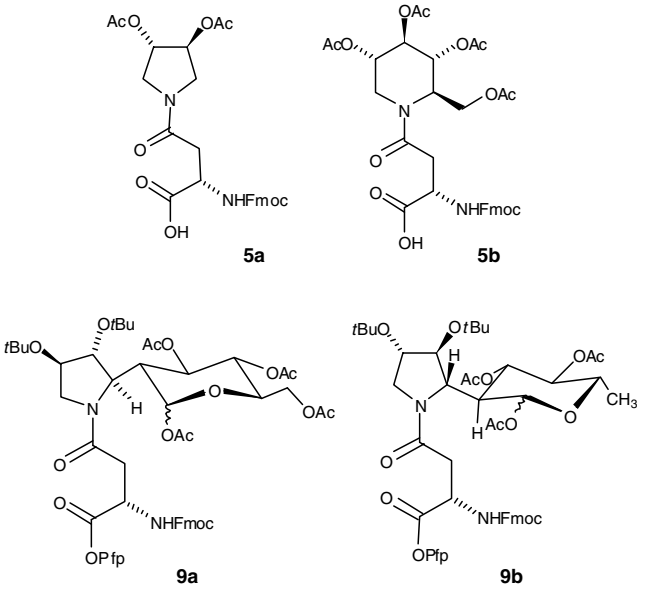
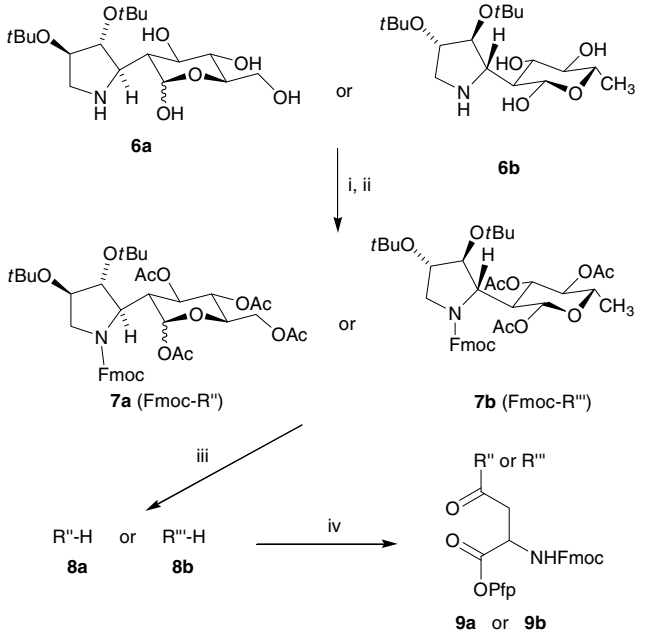


Figure 2. Building blocks **5a** and **b** and **9a** and **b**.



Scheme 2. Reagents and conditions: (i) Fmoc-OSu, dry Py, N_2; (ii) Ac_2O, Py, N_2 (**7a** 52%; **7b** 45%); (iii) Pip 20%, THF (**8a** 68%; **8b** 73%); (iv) Fmoc-L-Asp(Cl)-OPfp, NMM, dry THF (**9a** 41%; **9b** 44%).

Table 1. Chemical data for the synthesized CSF114-type glycopeptides **10–12**

Compound	Peptide	Gradient at 3 mL min^{-1} for semi-preparative HPLC	ESI-MS $[M+2H]^{2+}$: Found (Calcd)	HPLC[a] (t_R, min)
10	[Asn^7(DHPyr)]CSF114	25–50% B in 30 min	1265.9 (2529.3)	13.67
11	[Asn^7(DNJ)]CSF114	25–40% B in 30 min	1296.1 (2590.3)	10.23
12	[Asn^7(DHPyr-2-deoxyGlc)]CSF114	20–60% B in 30 min	1338.2 (2676.4)	10.25

[a] Analytical HPLC gradient at 1 mL min^{-1}: 20–60% B in 15 min.

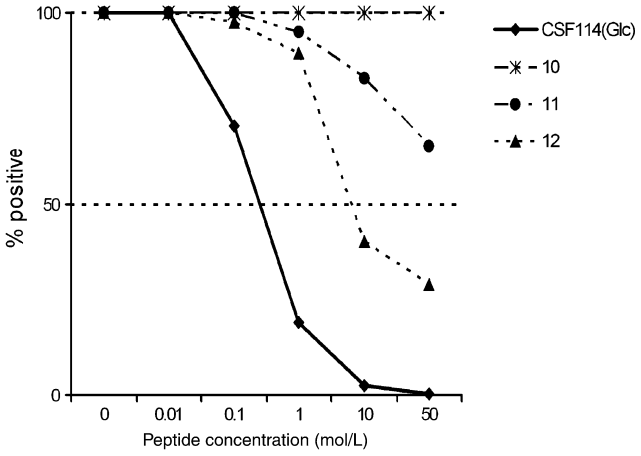


Figure 3. Inhibition test of antibodies binding to CSF114(Glc) with the glycopeptides **10–12**. The results are expressed as % of a representative MS positive serum (ordinates axis). The concentrations of the peptides are plotted on the *x*-axis.

standard synthetic protocol described in the general procedure. The glycopeptides **10–12** were synthesized by introducing Fmoc-L-Asn(DHPyrAc2)-OH (**5a**), Fmoc-L-Asn(DNJAc4)-OH (**5b**), and Fmoc-L-Asn(DHPyr*t*-Bu2-2-deoxyGlcAc4)-OPfp (**9a**) during the SPPS at position 7, as described in the general procedure. Peptide cleavage from the resin and deprotection of the amino acid side chains were carried out as described in the general procedure. After lyophilization, deprotection of the hydroxyl functions of the sugar linked to the peptide was accomplished by a methanolic solution of MeONa.

The crude products were purified and analyzed by RP-HPLC. Characterization of the products was performed with ThermoFinnigan LCQ Advantage LC-ESI-MS (Table 1).

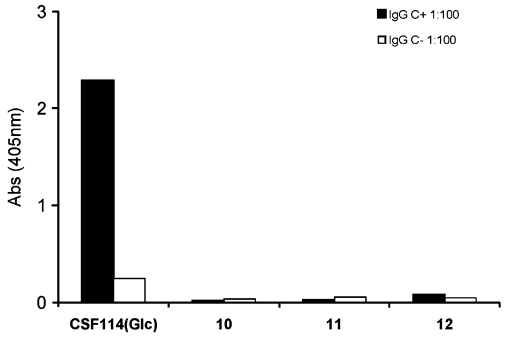


Figure 4. Abs titers of MS patients' sera and of blood donors' sera to CSF114(Glc) and to the glycopeptides **10–12**.

3. Immunoassays of CSF114-type glycopeptides 10–12

The autoantibody titer in MS patients' sera by CSF114-type glycopeptides was evaluated by competitive and solid-phase non-competitive ELISA.[3]

The inhibition curves (Fig. 3) showed that the glycopeptides **11** and **12** display inhibitory activity only at higher concentration, while the glycopeptide **10** showed no activity at all. CSF114(Glc) is the glycopeptide with the lowest IC_{50} value. None of the CSF114-type glycomimetics-containing peptides was able to inhibit anti-CSF114(Glc) autoantibodies in MS patients.

In solid-phase non-competitive ELISA (Fig. 4), only CSF114(Glc) detected increased IgG antibodies in MS patients' sera compared to healthy blood donors.

In conclusion, we have described an efficient method to synthesize new asparagine derivatives orthogonally protected for Fmoc/*t*-Bu SPPS, bearing alkaloid-type sugar mimics containing pyrrolidine and piperidine structures. The building blocks were successfully introduced in the type I′ β-turn structure CSF114. The CSF114-type glycopeptides were tested in MS patients' sera both by competitive and solid-phase non-competitive ELISA. Biological data obtained with the new alkaloid-type sugar mimics containing peptides supported by our previous results[3] confirmed that Asn(Glc) is up to now the unique minimal and fundamental epitope recognizing auto-Abs in a relapsing-remitting form of MS.

4. Experimental

4.1. General

THF was distilled over sodium/benzophenone and DCM over CaH_2. Flash column chromatographies (FCC) were performed according to Still et al.[24] on SiO_2 (Merck, Silica Gel 60, 40–63 μm). Thin layer chromatographies (TLC) were carried out on SiO_2 (Merck, Silica Gel 60 F plastic plates) and spots located with: UV light (254 and 366 nm), methanolic ninhydrin, Fluram® (Fluka; fluorescamine, 4-phenyl-spyro[furan-2(3*H*),1′(3′*H*)-isobenzofuran]-3,3′-dione) in acetone, and ethanolic *p*-anisaldehyde (EtOH/*p*-anisaldehyde/AcOH/H_2SO_4, 90:2:1:3). Elemental analyses were performed on a Perkin-Elmer 240 C Elemental Analyzer. ^1H and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, on a Varian spectrometer. Glycopeptides were analyzed by analytical RP-HPLC (Waters Alliance, 2695 separation module equipped with a 2996

diode array detector) using a Jupiter C18 (5 μm, 250 × 4.6 mm) column (Phenomenex) at 1 mL min^{-1}. The solvent system used was A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). Glycopeptides were purified by preparative RP-HPLC (model 600, Waters) on a Jupiter C18 column (10 μm, 250 × 10 mm) at 4 mL min^{-1} using the same solvent systems reported above. Characterization of the products was performed with the LCQ Advantage liquid chromatography electrospray ionization mass spectrometer (ThermoFinnigan). Glycopeptides were lyophilized with an Edwards Modulyo apparatus.

4.1.1. 3*S*,4*S*-Pyrrolidinediol (1a). To a solution of 3*S*,4*S*-1-benzylpyrrolidinediol (1 g, 5.2 mmol) in MeOH (15 mL) was added $Pd(OH)_2/C$ (1 mmol). The mixture was stirred for 2 days at room temperature under H_2 (1 atm), filtered through Celite, washed with MeOH, and evaporated to dryness. The product **1a** was dissolved in water and lyophilized (498 mg, 93%). R_f [DCM/MeOH, 10:1; ninhydrin] = 0.1. ^1H NMR (DMSO-d_6) δ 3.76 (pdd, 2H, 3-H and 4-H), 3.4 (br s, 2H, 2× OH), 2.9 (dd, J = 4.4, 11.6 Hz, 2H, 2-H, and 5-H), 2.47 (dd, J = 4.2, 11.4 Hz, 2H, 2-H and 5-H). ^{13}C NMR (DMSO-d_6) δ 77.3 (CHOH), 52.9 (CH_2). ESI-MS (*m*/*z*) $[M+H]^+$: found 104.1. Anal. Calcd for $C_4H_9NO_2$: C, 46.59; H, 8.80; N, 13.58. Found: C, 46.78; H, 8.78; N, 13.42.

4.1.2. 2*R*-(Hydroxymethyl)-3*R*,4*R*,5*S*-piperidinetriol (1b). To a solution of 2,3,4,6-tetra-*O*-benzyl-1,5-dideoxy-1,5-D-glucitol (1 g, 1.73 mmol) in MeOH (10 mL) was added $Pd(OH)_2/C$ (0.35 mmol). The mixture was stirred at room temperature under H_2 (1 atm) for 3 weeks. The deprotection reaction was controlled by ESI-MS until the main signal was $[M+H]^+$ = 164.9. Then the mixture was filtered through Celite, washed with MeOH, and the solvent evaporated to dryness. The product **1b** was recrystallized from EtOH/H_2O giving a pale yellow solid (245 mg, 86%). Spectra are in accordance with the literature.[18]

4.2. Fmoc-protection of amino group: general procedure

Fmoc-OSu (1.1 equiv) was added to the various iminosugars (1 equiv) dissolved in dry pyridine under nitrogen. The mixture was stirred at room temperature overnight. To the pyridine solution was added Ac_2O (8 equiv), and the reaction mixture was stirred for 16 h at room temperature under nitrogen in the dark. The solvent was removed by co-evaporation with toluene. The crude products were purified by FCC to provide the protected iminosugars **2a** and **b** and **7a** and **b**.

4.2.1. *N*-Fmoc-3*S*,4*S*-pyrrolidinediol diacetate (2a). 3*S*,4*S*-Pyrrolidinediol (**1a**) (480 mg, 0.46 mmol) yielded **2a** as a white solid (132 mg, 70%). R_f [AcOEt/hexane, 1:2; UV] = 0.2. ^1H NMR ($CDCl_3$) δ 7.75 (d, J = 7.4 Hz, 2H, Fmoc 4-H and 5-H), 7.60 (d, J = 7.4 Hz, 2H, Fmoc 1-H and 8-H), 7.34–7.27 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 5.16 (d, J = 3.6 Hz, 2H, CH_2–O), 4.23 (dd, J = 1.4, 7.6 Hz, 2H, Pyr 3-H and 4-H), 4.24 (*p*t, J = 6.6 Hz, 1H, Fmoc 9-H), 4.23 (dd, J = 4.4, 12.8 Hz, 2H, Pyr 2-H and 5-H), 3.53 (*p*dd, J = 12.4 Hz, 2H, Pyr 2′-H and 5′-H), 2.08 (s, 3H, Ac), 2.07 (s, 3H, Ac). ^{13}C NMR ($CDCl_3$) δ 169.5 ($COCH_3$), 169.0 ($COCH_3$), 154.6 (CONH), 143.7, 141.2, 127.7, 127.0, 125.0 and 120.0 (Fmoc C_{arom}), 74.9 (Pyr CH), 74.0 (Pyr CH), 67.4 (Fmoc CH_2), 50.2 (Pyr CH_2), 49.9 (Pyr CH_2), 47.2 (Fmoc CH), 20.8 ($COCH_3$). ESI-MS (*m*/*z*) $[M+H]^+$: found 410.2. Anal. Calcd for $C_{23}H_{23}NO_6$: C, 67.47; H, 5.66; N, 3.42. Found: C, 67.58; H, 5.78; N, 3.12.

4.2.2. *N*-Fmoc-2*R*-[(acetyloxy)methyl]-3*R*,4*R*,5*S*-piperidinetriol triacetate (2b). 1-Deoxynojirimycin (**1b**) (270 mg, 1.65 mmol) yielded **2b** as a white solid (766 mg, 84%). R_f [AcOEt/hexane, 1:1; UV, vanilline] = 0.4. ^1H NMR ($CDCl_3$) δ 7.74 (d, J = 7 Hz, 2H, Fmoc 4-H and 5-H), 7.54 (d, J = 7 Hz, 2H, Fmoc 1-H and 8-H), 7.36–7.27 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 4.93 (*p*t, 1H, DNJ 4-H), 4.84–4.79 (m, 2H, Fmoc CH_2–O), 4.43–4.33 (m, 6H, DNJ CH_2OAc, 2-H, 3-H, 5-H and Fmoc 9-H), 3.55–3.50 (m, 2H, DNJ 6-H), 2.11, 2.09, 2.07 and 2.05 (4 s, 12H, 4× Ac). ^{13}C NMR ($CDCl_3$) δ 165.5–169.6 ($COCH_3$, CONH), 155.7 (urethane CO), 143.7, 141.2, 127.7, 127.0, 125.0 and 120.0 (Fmoc C_{arom}), 67.2–67.6 (C-3, C-5 and C-4), 67.1 (Fmoc CH_2–O), 60.1 (2-CH_2–OAc), 53.2 (C-2), 47.0 (C-6), 39.6 (Fmoc C-9), 17.4 (CH_3). ESI-MS (*m*/*z*) $[M+H]^+$: found 554.2. Anal. Calcd for $C_{29}H_{31}NO_{10}$: C, 62.92; H, 5.64; N, 2.53. Found: C, 62.84; H, 5.56; N, 2.73.

4.2.3. *N*-Fmoc-2-deoxy-2*R*-[3*R*,4*R*-bis(1,1-dimethylethoxy)-2*R*-pyrrolidinyl]-1,3,4,6-tetra-*O*-acetyl-D-glucopyranose (7a). Compound **6a** (930 mg, 2.46 mmol) yielded **7a** as a solid (976 mg, 52%). R_f [AcOEt/hexane, 1:3; UV, *p*-anisaldehyde] = 0.32. ^1H NMR ($CDCl_3$) δ 7.74 (d, J = 7 Hz, 2H, Fmoc 4-H and 5-H), 7.54 (d, J = 7 Hz, 2H, Fmoc 1-H and 8-H), 7.36–7.27 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 5.69–5.63 (m, 2H, 5′-H_2), 4.99–4.94 (m, 1H, 1-H_α), 4.38–3.82 (m, 11H, 3′-H, 4′-H, and 2′-H, deoxyGlc 3-H, 4-H, 5-H and 6-H_2, Fmoc CH_2O and 9-H), 3.13–3.09 (m, 1H, deoxyGlc 2-H), 2.06, 2.05, 2.03 and 2.00 (4 s, 12H, 4× Ac), 1.26–1.12 (m, 18H, 2× *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 170.6–169.1 ($COCH_3$), 156.0 (CONH), 143.7, 141.2, 127.7, 127.0, 125.0, and 120.0 (Fmoc C_{arom}), 92.3 (C-1), 80.7 (C-4), 76.4 (C-3′), 74.0 (C-4′), 72.1 (3-C), 71.9 and 69.3 (CMe_3), 67.4 (Fmoc CH_2), 62.9 (C-6), 62.1 (C-5), 53.6 (Pyr C-5), 47.0 (Fmoc CH and C-2), 43.8 (C-2′), 34.0 (CH_2O), 29.2 and 28.3 [$C(CH_3)_3$], 21.1 and 20.6 ($COCH_3$). ESI-MS (*m*/*z*) $[M+Na]^+$: found 790.4. Anal. Calcd for $C_{41}H_{53}NO_{13}$: C, 64.13; H, 6.96; N, 1.82. Found: C, 64.43; H, 6.78; N, 1.89.

4.2.4. *N*-Fmoc-2-deoxy-2*S*-[3*S*,4*S*-bis(1,1-dimethylethoxy)-2*S*-pyrrolidinyl]-1,3,4-tri-*O*-acetyl-L-rhamnopyranose (7b). Compound **6b** (1.22 g, 3.37 mmol) yielded **7b** as a solid (1.07 g, 45%). R_f [AcOEt/hexane, 1:3; UV, *p*-anisaldehyde] = 0.48. ^1H NMR ($CDCl_3$) δ 7.73 (d, J = 7.4 Hz, 2H, Fmoc 4-H and 5-H), 7.54 (d, J = 8 Hz, 2H, Fmoc 1-H and 8-H), 7.36–7.27 (m, 4H, Fmoc 2-H, 7-H, 3-H and 6-H), 5.73–5.60 (m, 2H, 5′-H_2), 4.76–4.62 (m, 3H, 1-H_α, 3′-H and 4′-H), 4.45–3.76 (m, 7H, 2′-H, deoxyRha 3-H, 4-H and 5-H, Fmoc CH_2O and 9-H), 3.14–3.10 (m,

1H, deoxyRha 2-H), 2.05, 2.04 and 2.01 (3 s, 9H, 3× Ac), 1.27–1.12 (m, 21H, CH_3 and 2× *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 170.3–169.8 ($COCH_3$), 157.0 (CONH), 143.7, 141.2, 127.7, 127.0, 125.0, and 120.0 (Fmoc C_{arom}), 92.1 (C-1), 81.0 (C-4), 76.2 (C-3′), 74.9 (C-4′), 72.1 (C-3), 70.1 and 70.0 (CMe_3), 67.4 (Fmoc CH_2), 61.1 (C-5), 52.0 (C-5′), 47.0 (Fmoc CH and C-2), 45.9 (C-2′), 35.0 (CH_2O), 29.3 and 28.4 [$C(CH_3)_3$], 21.2 and 20.8 ($COCH_3$), 17.6 (CH_3). ESI-MS (*m*/*z*) $[M+Na]^+$: found 732.3. Anal. Calcd for $C_{39}H_{51}NO_{11}$: C, 65.99; H, 7.24; N, 1.97. Found: C, 65.69; H, 7.14; N, 2.05.

4.3. Deprotection of amino group: general procedure

Fmoc-iminosugars **2a** and **b** and **7a** and **b** were treated with 20% piperidine in THF at room temperature for 30 min. The solvent was removed and the residues were purified from THF/hexane to provide compounds **3a** and **b** and **8a** and **b**.

4.3.1. 3*S*,4*S*-Pyrrolidinediol diacetate (3a). Compound **2a** (1 g, 2.44 mmol) yielded **3a** as an oil (292 mg, 64%). R_f [AcOEt/hexane, 1:2; UV, ninhydrin] = 0.15. ^1H NMR ($CDCl_3$) δ 5.06 (d, J = 3.6 Hz, 2H, 3-H and 4-H), 3.68 (dd, J = 9.6, 13.6 Hz, 2H, 2-H and 5-H), 3.56 (*p*dd, J = 16.2 Hz, 2H, 2-H and 5-H), 2.13 (s, 3H, Ac), 2.08 (s, 3H, Ac). ^{13}C NMR ($CDCl_3$) δ 169.8 ($COCH_3$), 75.2 (CHOAc), 50.4 (CH_2), 20.8 ($COCH_3$). ESI-MS (*m*/*z*) $[M+H]^+$: found 188.1. Anal. Calcd for $C_8H_{13}NO_4$: C, 51.33; H, 7.00; N, 7.48. Found: C, 51.08; H, 6.90; N, 7.54.

4.3.2. 2*R*-[(Acetyloxy)methyl]-3*R*,4*R*,5*S*-piperidinetriol triacetate (3b). Compound **2b** (760 mg, 1.37 mmol) yielded **3b** as a white solid (363 mg, 80%). R_f [isopropanol/AcOEt/H_2O, 6:1:3; UV, ninhydrin] = 0.67. ^1H NMR ($CDCl_3$) δ 5.08 (*p*d, J = 9.6 Hz, 1H, 4-H), 4.86 (*p*t, J = 8.6 Hz, 2H, 3-H and 5-H), 4.05 (br s, 2H, CH_2OAc), 3.77–3.74 (m, 1H, 2-H), 3.43 (*p*dt, J = 9, 12.6 Hz, 2H, 6-H_2), 2.05 (s, 12H, 4× Ac). ^{13}C NMR ($CDCl_3$) δ 169.8 (CO), 75.1 (C-3), 51.2 (C-2), 20.8 ($COCH_3$). ESI-MS (*m*/*z*) $[M+H]^+$: found 332.1. Anal. Calcd for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39; N, 4.23. Found: C, 50.82; H, 6.43; N, 4.42.

4.3.3. 2-Deoxy-2*R*-[3*R*,4*R*-bis(1,1-dimethylethoxy)-2*R*-pyrrolidinyl]-1,3,4,6-tetra-*O*-acetyl-D-glucopyranose (8a). Compound **7a** (750 g, 0.98 mmol) yielded **8a** as an oil (363 mg, 68%). R_f [DCM/MeOH, 10:1; vanillin] = 0.46. ^1H NMR ($CDCl_3$) δ 5.65–5.60 (m, 2H, 5′-H_2), 5.09 (*p*d, 1H, 1-H_α), 4.94–4.86 (m, 2H, 3′-H and 4′-H), 4.70 (d, J = 8.9 Hz, 1H, 1-H_β), 4.26–3.94 (m, 3H, 2′-H, 2-H and 3-H), 3.76–3.74 (m, 1H, 4-H), 3.49–3.38 (m, 3H, 5-H and 6-H_2), 2.05, 2.03, 2.00 and 1.99 (4 s, 12H, 4× Ac), 1.27–1.09 (m, 18H, 2× *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 170.8–170.4 ($COCH_3$), 92.6 (C-1), 80.4 (C-4), 76.4 (C-3′), 74.6 (C-4′), 72.5 (C-3), 70.9 and 70.3 (CMe_3), 65.1 (C-6), 62.6 (C-5), 54.5 (C-5′), 46.4 (C-2), 44.5 (C-2′), 29.6 and 28.4 [$C(CH_3)_3$], 22.6 and 20.7 ($COCH_3$). ESI-MS (*m*/*z*) $[M+Na]^+$: found 569. Anal. Calcd for $C_{26}H_{43}NO_{11}$: C, 57.23; H, 7.94; N, 2.57. Found: C, 57.45; H, 7.76; N, 2.61.

4.3.4. 2-Deoxy-2*S*-[3*S*,4*S*-bis(1,1-dimethylethoxy)-2*S*-pyrrolidinyl]-1,3,4-tri-*O*-acetyl-L-rhamnopyranose (8b). Compound **7b** (840 mg, 1.18 mmol) yielded **8b** as an oil (422 mg, 73%). R_f [DCM/MeOH, 10:1; vanillin] = 0.31. ^1H NMR ($CDCl_3$) δ 5.62–5.52 (m, 2H, 5′-H_2), 5.02–4.98 (m, 1H, 1-H_α), 4.68–4.56 (m, 2H, 3′-H and 4′-H), 4.23–3.91 (m, 2H, 2′-H and 3-H), 3.76–3.74 (m, 1H, 4-H), 3.47–3.39 (m, 1H, 5-H), 2.75 (*p*dt, J = 11.0, 2.2 Hz, 1H, 2-H), 2.06, 2.04 and 2.00 (3× s, 9H, 3× Ac), 1.25–1.09 (m, 21H, CH_3 and 2× *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 170.5–170.2 ($COCH_3$), 92.4 (C-1), 80.5 (C-4), 76.6 (C-3′), 75.9 (C-4′), 73.9 (C-3), 72.5 and 68.8 (CMe_3), 63.5 (C-5), 54.5 (C-5′), 49.4 (C-2), 44.7 (C-2′), 28.6 and 28.2 [$C(CH_3)_3$], 22.6 and 20.8 ($COCH_3$), 17.8 (CH_3). ESI-MS (*m*/*z*) $[M+Na]^+$: found 510.33. Anal. Calcd for $C_{24}H_{41}NO_9$: C, 59.12; H, 8.48; N, 2.87. Found: C, 59.31; H, 8.34; N, 2.81.

4.4. Coupling reaction: general procedure I

A solution of **3a** or **b** (1 equiv) in DMF was added to a solution of Fmoc-L-Asp-O*t*-Bu (1 equiv), HATU (1 equiv), and NMM (1 equiv) in DMF. The reaction mixture was stirred for 2 h at room temperature. Evaporation of the solvent yielded the crude products, which were purified with FCC to obtain **4a** or **b**.

4.4.1. (*S*)-α-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[3*S*,4*S*-bis(acetyloxy)-1-pyrrolidine]butanoic acid *tert*-butyl ester (4a). Compound **3a** (290 mg, 1.53 mmol) yielded **4a** as a white solid (418 mg, 47%). R_f [AcOEt/hexane, 1:2; UV, ninhydrin] = 0.23. ^1H NMR ($CDCl_3$) δ 7.75 (d, J = 6.8 Hz, 2H, Fmoc 4-H and 5-H), 7.60 (d, J = 7 Hz, 2H, Fmoc 1-H and 8-H), 7.34–7.25 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 6.2 (d, J = 6.2 Hz, 1H, Asn NH), 5.17–5.10 (m, 2H, Pyr 3-H and 4-H), 4.6 (m, 1H, Asn α-H), 4.24 (*p*t, J = 6.7 Hz, 1H, Fmoc 9-H), 3.86–3.75 (m, 4H, Pyr 2-H_2 and 5-H_2), 3.04 (dd, J = 17.1, 4.4 Hz, 1H, Asn β-H), 2.68 (dd, J = 17.2, 4.4 Hz, 1H, Asn β′-H), 2.07 (s, 3H, Ac), 2.03 (s, 3H, Ac), 1.45 (s, 9H, *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 170.0 (COO*t*-Bu), 169.2–169.6 ($COCH_3$), 156.3 (CONH), 143.7, 141.2, 127.7, 127.0, 125.0, and 120.0 (Fmoc C_{arom}), 81.9 (CMe_3), 75.0–73.4 (Pyr CH), 67.1 (Fmoc CH_2), 51.0 (Asn C-α), 50.5–49.7 (Pyr CH_2), 47.1 (Fmoc CH), 35.6 (CH_2O), 28.1 [$C(CH_3)_3$], 20.8 ($COCH_3$). ESI-MS (*m*/*z*) $[M+H]^+$: found 581.3. Anal. Calcd for $C_{31}H_{36}N_2O_9$: C, 64.13; H, 6.25; N, 4.82. Found: C, 64.76; H, 6.65; N, 4.67.

4.4.2. (*S*)-α-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[2*R*-[(acetyloxy)methyl]-3*R*,4*R*,5*S*-tris(acetyloxy)-1-piperidine]butanoic acid *tert*-butyl ester (4b). Compound **3b** (360 mg, 1.09 mmol) yielded **4b** as a white solid (702 mg, 89%). R_f [AcOEt/hexane, 1:1; UV, vanillin] = 0.29. ^1H NMR ($CDCl_3$) δ 7.74 (d, J = 7 Hz, 2H, Fmoc 4-H and 5-H), 7.54 (d, J = 7 Hz, 2H, Fmoc 1-H and 8-H), 7.36–7.20 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 6.07 (br d, J = 8 Hz, 1H, Asn NH), 4.71–4.65 (m, 2H, Fmoc OCH_2), 4.57–4.34 (m, 2H, Asn α-H and Fmoc 9-H), 4.25–4.06 (m, 4H, DNJ 3-H, 5-H, and CH_2OAc), 3.01 (dd, J = 16.4, 4.4 Hz, 1H, Asn β-H), 2.85 (dd, J = 16.8, 4 Hz, 1H, Asn β′-H), 2.79–2.70

(m, 2H, DNJ 6-H_2), 2.04 (br s, 12H, 4× Ac), 1.48 (s, 9H, *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 177.2 (C-γ), 169.6 (COO*t*-Bu), 156.3 (urethane CO), 143.7, 141.2, 127.7, 127.0, 125.0, and 120.0 (Fmoc C_{arom}), 82.6 (*C*Me_3), 76.2 (C-4), 67.1 (Fmoc CH_2–O), 66.9–66.4 (C-3 and C-5), 64.0 (CH_2OAc), 54.4 (Asn C-α), 47.2 (Fmoc C-9), 44.7 (C-2), 42.5 (C-6), 35.9 (Asn C-β), 14.04 [C(*C*H_3)$_3$]. ESI-MS (*m*/*z*) [M+Na]$^+$: found 747.3. Anal. Calcd for $C_{37}H_{44}N_2O_{13}$: C, 61.32; H, 6.12; N, 3.87. Found: C, 61.44; H, 6.36; N, 3.68.

4.5. Coupling reaction: general procedure II

Fmoc-L-Asp(Cl)-OPfp (1 equiv) dissolved in dry THF was added to a solution containing the appropriate iminosugar **8a** or **b** (1 equiv) and NMM (1 equiv) in dry THF at 0 °C and stirred at room temperature for 30 min. Then, filtration of the precipitate and concentration under reduced pressure afforded an oil that was purified from THF/hexane to afford compound **9a** or **b**.

4.5.1. (*S*)-α-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[2*R*-[2-deoxy-1,3,4,6-tetra-*O*-acetyl-2*R*-D-glucopyranosyl]-3*R*,4*R*-bis(1,1-dimethylethoxy)-1-pyrrolidine]butanoic acid 1-(pentafluorophenyl) ester (9a). Compound **8a** (600 mg, 1.10 mmol) yielded **9a** (473 mg, 41%). R_f [AcOEt/hexane, 1:1; vanillin] = 0.52. ^{1}H NMR ($CDCl_3$) δ 7.75 (d, *J* = 6.6 Hz, 2H, Fmoc 4-H and 5-H), 7.58 (d, *J* = 6.4 Hz, 2H, Fmoc 1-H and 8-H), 7.41–7.30 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 6.22 (d, *J* = 8.8 Hz, 1H, α-NH), 5.67–5.54 (m, 2H, 2-H and 5′-H), 5.14 (d, *J* = 2.6 Hz, 1H, 1-H_α), 4.95–4.84 (m, 2H, 3′-H and 4′-H), 4.71 (d, *J* = 8.8 Hz, 1H, 1-H_β), 4.68–4.62 (m, 1H, Asn α-H), 4.39–4.34 (m, 1H, Fmoc 2′-H), 4.27–4.16 (m, 3H, Fmoc CH_2O and 9-H), 4.13–4.03 (m, 2H, deoxyGlc 2-H and 3-H), 3.79–3.67 (m, 1H, deoxyGlc 4-H), 3.53–3.21 (m, 5H, Asn β-H, deoxyGlc 5-H and 6-H and 6′-H), 2.08, 2.06, 2.02 and 1.98 (4 s, 12H, 4× Ac), 1.20–1.09 (m, 18H, 2× *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 170.8–170.0 (*C*OCH_3), 168.3 (COOPfp), 156.0 (CONH), 143.7, 141.2, 127.7, 127.0, 125.0 and 120.0 (Fmoc C_{arom}), 92.5 (C-1), 80.3 (C-4), 76.4 (C-3′), 74.7 (C-4′), 72.3 (C-3), 70.9 and 70.3 (*C*Me_3), 67.7 (Fmoc CH_2), 65.1 (C-6), 62.6 (C-5), 54.6 (Asn C-α), 52.6 (C-5′), 48.9 (Fmoc CH), 46.6 (C-2), 42.9 (C-2′), 35.6 (CH_2O), 29.6 and 28.5 [C(*C*H_3)$_3$], 21.8 and 20.6 (CO*C*H_3). ESI-MS (*m*/*z*) [M+Na]$^+$: found 1049.4. Anal. Calcd for $C_{51}H_{57}F_5N_2O_{16}$: C, 58.39; H, 5.48; F, 9.06; N, 2.67. Found: C, 58.19; H, 5.51; F, 8.98; N, 2.73.

4.5.2. (*S*)-α-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[2*S*-[2-deoxy-1,3,4-tri-*O*-acetyl-2*S*-L-rhamnopyranosyl]-3*S*,4*S*-bis(1,1-dimethylethoxy)-1-pyrrolidine]butanoic acid 1-(pentafluorophenyl) ester (9b). Compound **8b** (750 mg, 1.53 mmol) yielded **9b** (671 mg, 44%). R_f [AcOEt/hexane, 1:1; vanillin] = 0.60. ^{1}H NMR ($CDCl_3$) δ 7.75 (d, *J* = 6.6 Hz, 2H, Fmoc 4-H and 5-H), 7.58 (d, *J* = 6.4 Hz, 2H, Fmoc 1-H and 8-H), 7.41–7.30 (m, 4H, Fmoc 2-H, 7-H, 3-H and 6-H), 5.96 (d, *J* = 8 Hz, 1H, α-NH), 5.63–5.53 (m, 2H, 5-H, and 5′-H), 5.05 (d, *J* = 2.2 Hz, 1H, 1-H_α), 4.69–4.57 (m, 3H, 3′-H, 4′-H, and Asn α-H), 4.40–4.34 (m, 3H, Fmoc 2′-H and CH_2O), 4.23–4.18 (m, 3H, deoxyRha 2-H, 3-H, and Fmoc 9-H), 3.75–3.60 (m, 1H, deoxyRha 4-H), 3.54–3.43 (m, 2H, Asn β-H_2), 3.47–3.20 (m, 1H, deoxyRha 5-H), 2.08, 2.01, and 1.98 (3× s, 9H, 3× Ac), 1.24–1.15 (m, 21H, CH_3 and 2× *t*-Bu). ^{13}C NMR ($CDCl_3$) δ 170.5–169.7 (*C*OCH_3), 168.3 (COOPfp), 160.0 (CONH), 143.7, 141.2, 127.7, 127.0, 125.0, and 120.0 (Fmoc C_{arom}), 94.6 (C-1), 80.1 (C-4), 76.5 (C-3′), 75.5 (C-4′), 74.3 (C-3), 73.7 and 72.2 (*C*Me_3), 68.6 (Fmoc CH_2), 63.5 (C-5), 55.2 (Asn C-α), 52.4 (C-5′), 49.0 (Fmoc CH), 46.8 (C-2), 43.6 (C-2′), 36.3 (CH_2O), 29.4 and 28.3 [C(*C*H_3)$_3$], 22.2 and 20.6 (CO*C*H_3), 17.6 (CH_3). ESI-MS (*m*/*z*) [M+Na]$^+$: found 991.4. Anal. Calcd for $C_{49}H_{55}F_5N_2O_{14}$: C, 59.39; H, 5.59; F, 9.59; N, 2.83. Found: C, 59.51; H, 5.80; F, 9.51; N, 2.89.

4.6. *tert*-Butyl-deprotection of Fmoc-protected amino acids: general procedure

The pure *t*-Bu-protected monomers **4a** or **b** were dissolved in a mixture of TFA and DCM (1:1). The resulting mixture was stirred for 3 h at room temperature. The solvents were evaporated off. After dissolution in water and lyophilization, Fmoc-protected amino acids **5a** and **b** were obtained.

4.6.1. (*S*)-α-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[3*S*,4*S*-bis(acetyloxy)-1-pyrrolidine]butanoic acid (5a). *tert*-Butyl ester **4a** (460 mg, 0.79 mmol) yielded **5a** as a white solid (408 mg, 98%). R_f [AcOEt/hexane 2:1; UV] = 0.08. ^{1}H NMR ($CDCl_3$) δ 7.75 (d, *J* = 6.8 Hz, 2H, Fmoc 4-H and 5-H), 7.60 (d, *J* = 7 Hz, 2H, Fmoc 1-H and 8-H), 7.34–7.27 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 6.2 (d, *J* = 6.2 Hz, 1H, Asn NH), 5.18 (m, 2H, Pyr 3-H and 4-H), 4.23–4.19 (m, 4H, Asn α-H, Fmoc OCH_2 and 9-H), 3.80–3.75 (m, 4H, Pyr 2-H_2 and 5-H_2), 3.04 (dd, *J* = 16.8, 4.4 Hz, 1H, Asn β-H), 2.7 (dd, *J* = 16.8 4.4 Hz, 1H, Asn β′-H), 2.07 (s, 3H, OAc), 2.04 (s, 3H, OAc). ^{13}C NMR ($CDCl_3$) δ 172.6 (COOH), 169.5–169.7 (*C*OCH_3), 155.9 (CONH), 143.7, 141.2, 127.7, 127.0, 125.0, and 120.0 (Fmoc C_{arom}), 74.6–73.3 (Pyr CH), 67.2 (Fmoc CH_2), 50.9 (Asn C-α), 50.3–50.0 (Pyr CH_2), 47.0 (Fmoc CH), 36.7 (CH_2O), 20.8 (CO*C*H_3). ESI-MS (*m*/*z*) [M+Na]$^+$: found 547.2. Anal. Calcd for $C_{27}H_{28}N_2O_9$: C, 61.83; H, 5.38; N, 5.34. Found: C, 61.46; H, 54.97; N, 5.29.

4.6.2. (*S*)-α-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]-γ-oxo-[2*R*-[(acetyloxy)methyl]-3*R*,4*R*,5*S*-tris(acetyloxy)-1-piperidine]butanoic acid (5b). *tert*-Butyl ester **4b** (80 mg, 0.11 mmol) yielded **5b** as a white solid (66 mg, 90%). R_f [AcOEt/Esano, 2:1; UV] = 0.1. ^{1}H NMR ($CDCl_3$) δ 7.74 (d, *J* = 7 Hz, 2H, Fmoc 4-H and 5-H), 7.54 (d, *J* = 7 Hz, 2H, Fmoc 1-H and 8-H), 7.36–7.28 (m, 4H, Fmoc 2-H, 7-H, 3-H, and 6-H), 6.07 (d, *J* = 8 Hz, 1H, Asn NH), 4.71–4.66 (m, 2H, Fmoc OCH_2), 4.57–4.34 (m, 2H, Asn α-H, Fmoc 9-H), 4.25–4.06 (m, 4H, DNJ 3–H, 5–H and CH_2OAc), 3.01 (dd, *J* = 16.4, 4.4 Hz, 1H, Asn β-H), 2.85 (dd, *J* = 16.8, 4 Hz, 1H, Asn β′-H), 2.79–2.76 (m, 1H, DNJ 6-H), 2.04 (br s, 12H, 4× OAc). ^{13}C NMR ($CDCl_3$) δ 176.0 (C-γ), 173.0 (COOH), 156.3 (urethane CO), 143.7, 141.2, 127.7, 127.6, 125.0, and 120.0 (Fmoc C_{arom}), 74.6 (C-4), 67.1 (Fmoc CH_2–O), 66.9–66.4 (C-3 and

C-5), 64.0 (CH_2OAc), 54.4 (Asn C-α), 47.2 (Fmoc C-9), 44.7 (C-2), 42.5 (C-6), 35.9 (Asn CH_2O). ESI-MS (*m*/*z*) $[M+Na]^+$: found 691.2. Anal. Calcd for $C_{33}H_{36}N_2O_{13}$: C, 59.28; H, 5.43; N, 4.19. Found: C, 59.04; H, 4.98; N, 4.10.

4.7. General procedure for the solid-phase peptide synthesis (SPPS): automated synthesis

Glycopeptides were synthesized on an automatic batch synthesizer (APEX 396, Advanced ChemTech) equipped with a 40-well reaction block, using a Wang resin preloaded with the C-terminal amino acid of the sequence, following the Fmoc/*t*-Bu SPPS strategy. Fmoc-amino acids and resin were purchased from Novabiochem AG (Laufelfingen, Switzerland). Fmoc deprotections were performed in 30 min with 20% piperidine in DMF. Coupling reactions (repeated twice) were performed for 45 min by using a 0.5 M solution of the Fmoc-protected amino acids and HOBt in DMF (2.5 equiv), a 0.5 M solution of TBTU in DMF (2.5 equiv), and 4 M NMM in DMF (5 equiv). Peptide cleavage from the resin and deprotection of the amino acid side chains were carried out in 3 h with TFA/thioanisole/EDT/phenol/H_2O (82.5:5:2.5:5:5) (vol:vol:vol:vol:vol). The resin was filtered off, and the solution was concentrated. The crude products were precipitated with cold Et_2O, centrifuged, and lyophilized. Deprotection of the sugar moiety was performed by adding 0.1 M MeONa to a solution of the crude material in dry MeOH to pH 12. After 2 h of stirring, the reaction was quenched with dry CO_2 to neutrality, the solvent was evaporated to dryness, and the residue was lyophilized.

4.7.1. Parallel synthesis of H-Thr-Pro-Arg-Val-Glu-Arg-Asn(DHPyr)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-OH [Asn^7(DHPyr)]CSF114 (10); H-Thr-Pro-Arg-Val-Glu-Arg-Asn(DNJ)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-OH [Asn^7(DNJ)]-CSF114 (11); H-Thr-Pro-Arg-Val-Glu-Arg-Asn(DHPyr-2-deoxyGlc)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-OH [Asn^7(DHPyr-2-deoxyGlc)]CSF114 (12). Glycopeptides **10**–**12** were synthesized using Fmoc-L-Lys(Boc)-Wang resin (0.57 mmol/g, 100 mg). The introduction of sugar moieties was performed by using a 2-fold excess (0.114 mmol) of the building blocks Fmoc-L-Asn(DHPyrAc2)-OH (**5a**) or Fmoc-L-Asn(DNJAc4)-OH (**5b**) dissolved in DMF, HATU (2 equiv), and NMM (3.5 equiv) for 1.5 h. Coupling with Fmoc-L-Asn(DHPyr*t*-Bu2-2-deoxyGlcAc4)-OPfp (**9a**) was performed by using 2-fold excess (0.114 mmol) of the building block, HOBt (2 equiv), and NMM (3.5 equiv) dissolved in DMF for 1.5 h. All glycopeptides were cleaved and side chains deprotected at room temperature, and then deacetylated as described in the general procedure. Glycopeptides **10**–**12** were purified by semi-preparative HPLC. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized. Characterization of the products was performed using analytical HPLC and ESI-MS spectrometry. The analytical data are reported in Table 1.

4.8. Immunological assays: general procedure

Antibody titers were determined in solid-phase ELISA (SP-ELISA).[25] 96-Well activated Polystyrene ELISA plates (Limbro Titertek, ICN Biomedicals, Inc., Aurora, Ohio, USA) were coated with 1 μg/100 μL/well of peptides or glycopeptides in pure carbonate buffer 0.05 M (pH 9.6) and incubated at 4 °C overnight. After five washes with saline containing 0.05% Tween 20, nonspecific binding sites were blocked by fetal calf serum (FCS), 10% in saline Tween (100 μL/well) at room temperature for 60 min. Sera diluted from 1:100 were applied at 4 °C for 16 h in saline Tween 10% FCS. After five washes, we added 100 μL/well of alkaline phosphatase conjugated anti-human IgM or IgG Fab2-specific affinity-purified antibodies (Sigma, St. Louis, Missouri, USA) diluted 1:500 in saline Tween/FCS. After an overnight incubation and five washes, 100 μL of substrate solution consisting of 2 mg/mL *p*-nitrophenylphosphate (Sigma, St. Louis, Missouri, USA) in 10% diethanolamine buffer was applied. After 30 min, the reaction was blocked with 50 μL of 1 M NaOH and the absorbance read in a multichannel ELISA reader (SUNRISE, TECAN, Austria) at 405 nm. ELISA plates, coating conditions, reagent dilutions, buffers, and incubation times were tested in preliminary experiments. Each serum was individually titrated to check for parallellism of antibody absorbances in dilutions. Within-assays and between-assays coefficients of variation were below 10%. The antibody levels revealed by SP-ELISA are expressed as absorbance value at a dilution of 1:100 as a ratio of positive controls in the same experiment. Positive samples were analyzed twice to evaluate the differences between the two determinations. The reference values were set as the mean + 2SD of the control groups. Within- and between-assays coefficients of variation were below 10%.

Antibody affinity and antibody affinity heterogeneity were measured by competitive ELISA following the methods previously published.[26] In preliminary titration curves the semi-saturation dilution was calculated (absorbance 0.7). At this dilution, antibody was preincubated with increasing antigen concentration (6 h at 25 °C). Unblocked antibodies were revealed by ELISA, and the absorbance was graphically represented in relation to the antigen concentration. Data are expressed as % of absorbance of positive serum in reception to peptide concentration.

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