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Chemoenzymatic synthesis of a sialylated diantennary N-glycan linked to asparagine

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Abstract

A partial structure of many glycoproteins, a glycosylated asparagine carrying a complex type undecasaccharide N-glycan (Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Manα1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)]Man(β1-4)GlcNAc(β1-4)GlcNAc-Asn) was obtained by total synthesis. As a starting material served a chemically synthesized diantennary heptasaccharide azide which was deprotected in a three-step sequence in high yield. The reduction of the anomeric azide was accomplished with propanedithiol in methanol–ethyldiisopropylamine. Coupling of the glycosyl amine to an activated aspartic acid gave the benzyl protected asparagine conjugate. After removal of the six benzyl functions the resulting free heptasaccharide asparagine was elongated enzymatically in the oligosaccharide part. The use of β-1,4-galactosyltransferase and α-2,6-sialyltransferase in the presence of alkaline phosphatase allowed the efficient transfer of four sugar units to the acceptor resulting in a full length N-glycan, a sialylated diantennary undecasaccharide-asparagine of the complex type. © 1998 Elsevier Science Ltd.

Keywords: Galactosyltransferase; Sialyltransferase; Alkaline phosphatase; N-glycan; Glycoprotein

1. Introduction

Nearly all proteins found on the cell surface and in the blood serum of vertebrates are glycosylated [1]. The finding that the asparagine-linked oligosaccharides (N-glycans) influence the properties of the entire glycoprotein has stimulated glycobiologists to investigate structure–activity relationships [2]. These studies, however, are generally hampered by the poor availability of pure glycoproteins or their oligosaccharides which is caused by the microheterogeneity of the natural material. The interest in defined model compounds has prompted us to take on the synthesis of entire N-glycans. The groups of Lönngren [3], Ogawa et al. [4], Paulsen [5] have pioneered chemical synthesis of N-glycans and reported numerous syntheses of partial structures, however, only one example has been published where a sialylated diantennary N-glycan was synthesized chemically and could be successfully deprotected [4]. An analysis of the difficulties encountered in the chemical synthesis of complex N-glycans revealed three key reactions. The construction of the central β-mannosyl unit, the

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introduction of sialic acid and the deprotection of the sialylated N-glycan. To circumvent the latter two problems, we chose a new strategy to obtain complete N-glycans by combining chemical synthesis [4–6] with the ease of enzymatic carbohydrate chain elongation using glycosyltransferases [7,8]. A highly efficient method to introduce galactose and sialic acid enzymatically in the presence of alkaline phosphatase was developed earlier [8] and used as a basis of a chemoenzymatic approach [9]. When this work was started the most generally applicable method for β-mannosylation appeared to be the procedure introduced by the group of Kunz and Günther [10]. The presence of a single benzylidene protective group in the β-mannoside part of an appropriately functionalized core-trisaccharide led to the development of a double regioselective glycosylation strategy [11]. A temporary 4,6-*O*-benzylidene acetal on the β-mannoside permits the regio- and stereoselective introduction of the $(1 \rightarrow 3)$ and the $(1 \rightarrow 6)$ arm of N-glycans. Following this approach, not only diantennary N-glycans were obtained, but also triantennary and tetraantennary N-glycans were synthesized chemically [12].

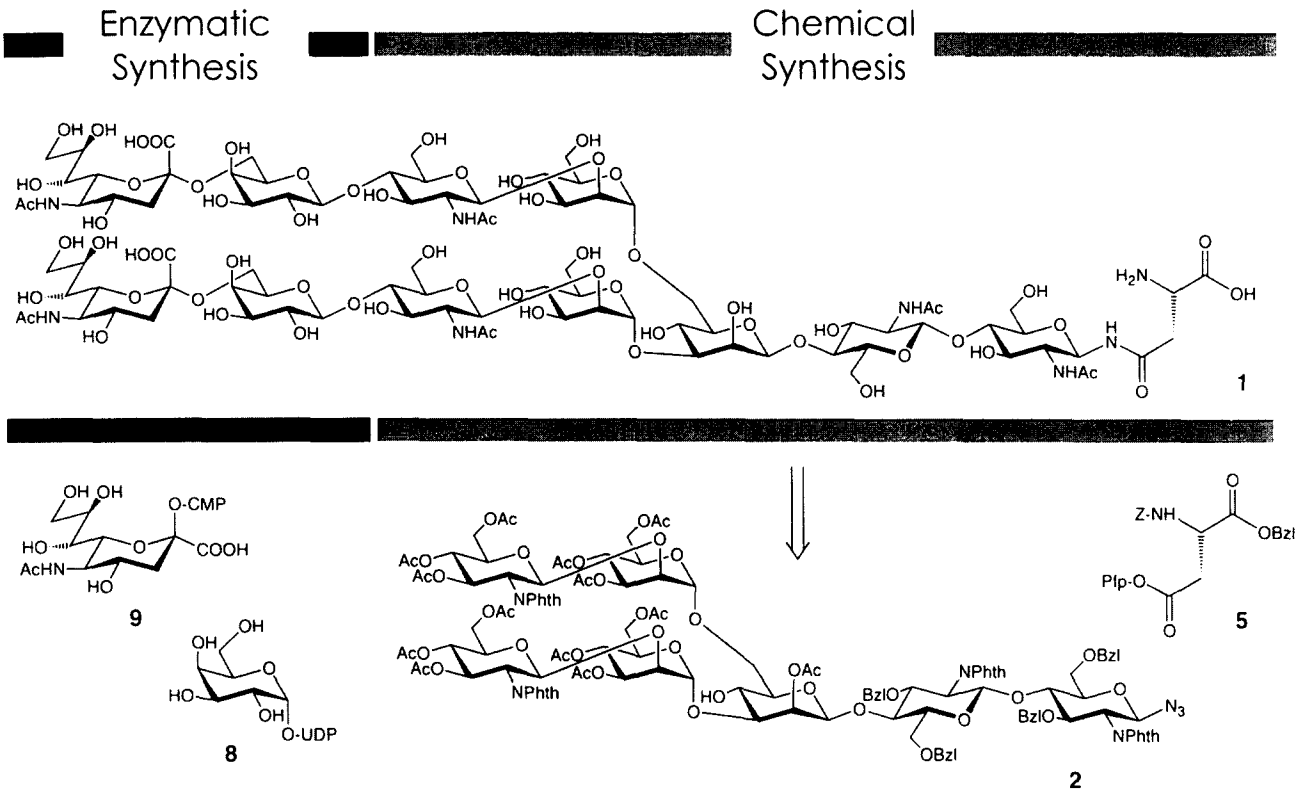
2. Results and discussion

The synthesis of the complex glycoprotein fragment **1** [13] typically found in transferrin and many other glycoproteins was accomplished using a combination of modern oligosaccharide chemistry and a highly efficient enzymatic glycosylation method. With the building blocks **2**, **5**, **8** and **9** shown in the retrosynthetic analysis (Scheme 1) the overall number of synthetic steps could be significantly reduced compared to an all-chemical approach.

A suitable starting material for diantennary N-glycans was found in the protected heptasaccharide **2** which was synthesized in multigram amounts following the double regioselective glycosylation method [13]. [1] The heptasaccharide **2** is equipped with an anomeric azido group introduced at an early stage of the synthesis. This azide function can be reduced to an amine and coupled, e.g., to an activated aspartic acid derivative giving rise to the stable N-glycosidic bond [14]. However, depending on the protective group pattern, some of the conditions applied for selective deprotection may not be compatible with all parts of the molecule. In the case of compound **2** the harshest conditions were required for the removal of the phthalimido functions. All attempts to cleave the phtalimido groups from an asparagine conjugate derived from the glycosylamine of heptasaccharide **2** were not successful. This approach was thought to be advantageous because the 2-phthalimido groups assist the β-amine to retain the desired configuration in the coupling reaction.

Dephthaloylation was thus carried out prior to the coupling to aspartic acid (Scheme 2). The typical procedure for the liberation of amino groups from phthalimides is the treatment with hydrazine hydrate which works well on small oligosaccharide azides [14]. However, for the heptasaccharide **2** the yields obtained reached only 50%. TLC-monitoring of the reaction revealed the appearance of polar side-products indicating that under the reaction conditions the azido group was attacked. This was likely to be caused by small amounts of the reducing agent diimine [15] generated from hydrazine upon heating. The assumption that *N*-methylhydrazine may be less prone to reduce azides was confirmed in the experiment but the yields improved only slightly (70%). The best conditions for the removal of the four phthaloyl groups from the heptasaccharide azide **2** were found in a method that was introduced recently by Kanie et al. [16]. Heating of the protected oligosaccharide in a mixture of ethylene diamine and *n*-butanol gave the deacetylated heptasaccharide tetraamine without the side reactions encountered before. The heptasaccharide tetraamine was isolated in high yield. To simplify the synthesis we decided to *N*-acetylate the intermediate amine by a two-step reaction involving peracetylation (acetic anhydride–pyridine) followed by basic hydrolysis of the O-acetates with aqueous methylamine. All three reactions were carried out subsequently in one flask and were facilitated by convenient removal of the volatile reagents and TLC control. The final product **3** is water-soluble and bears four hydrophobic benzyl groups. This allowed the rapid purification of **3** by solid phase extraction on SepPak-RP 18 cartridges (Waters). A diluted aqueous solution of the reaction mixture was passed through the cartridges that retained only hydrophobic compounds. The adsorption was followed by a step gradient to remove bound side products that eluted faster. The desired deprotected heptasaccharide **3** was obtained in high yield (98%) which reflects the quantitative conversion in

[1] Full experimental details of the protected heptasaccharide azide will be reported elsewhere.



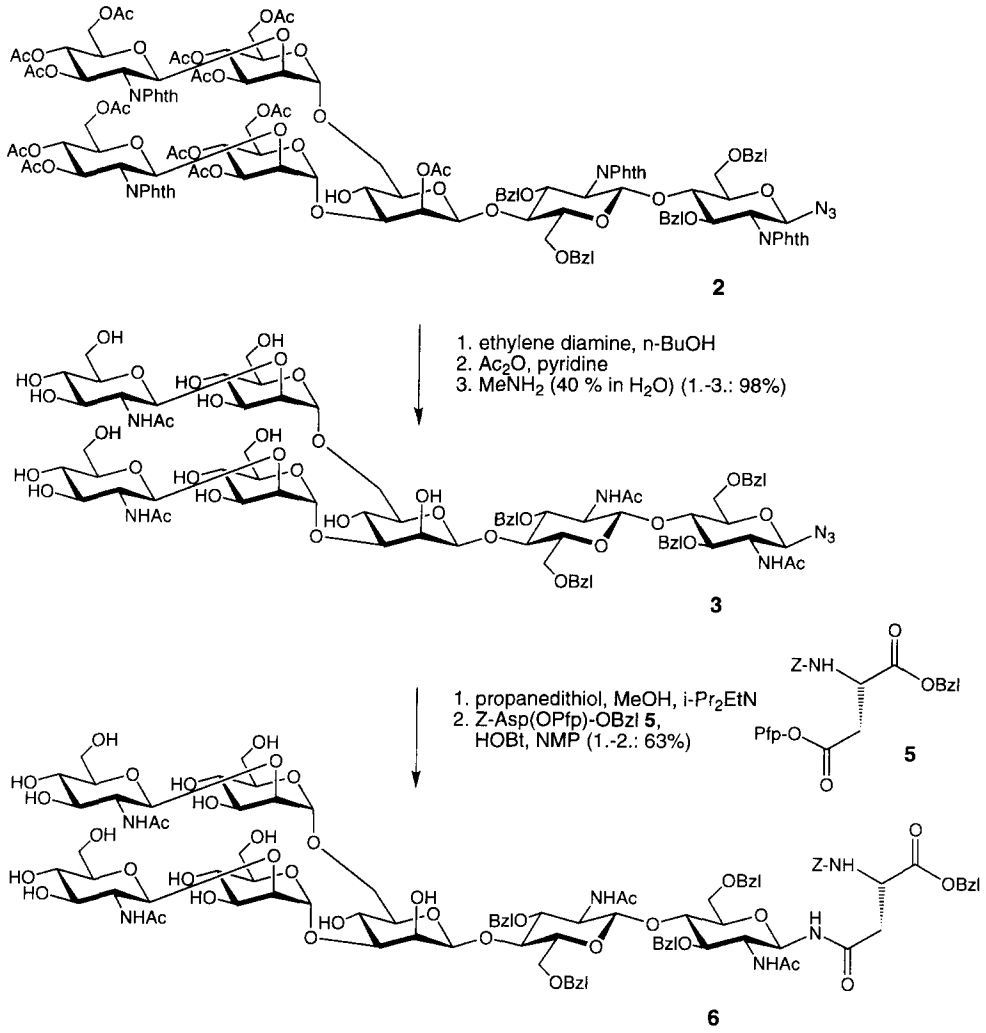
Scheme 1.

all three steps according to TLC analysis. This protocol is very favourable for complex oligosaccharides containing azido groups and has also been successfully applied to tetraantennary N-glycans [12].

The coupling of the heptasaccharide to aspartic acid may be conducted at the level of the acetylated precursor of **3**. However, this strategy may lead to side reactions in the peptide part during deacetylation of the glycopeptide. Therefore the selectively protected heptasaccharide **3** was chosen for the coupling step.

Commercially available *Z*-Asp(OH)-OBzl **4** was converted to the pentafluorophenyl ester **5** [17] using pentafluorophenyl trifluoroacetate. The heptasaccharide azide **3** was reduced to the amine and coupled with the activated aspartic acid **5** in the presence of *N*-hydroxybenzotrigzol (HOBT) and ethyldiisopropylamine. When the reduction was carried out as a hydrogenation over palladium on calcium carbonate or Raney-nickel the resulting glycopeptide showed a 1:1 ratio of the β-linked compound **6** and its α-isomer. Hydrogenation under basic conditions still gave over 30% of the nonnatural α-linked product. We assumed that in our case the noble metal was causing the anomerization of the glycosyl amine and looked for an alternative coupling method [18]. This was found in a procedure published by Bayley at al. [19] using excess propanedithiol in dry methanol–ethyldiisopropylamine. The reduction proceeded fast and after distilling off the reagents the glycosyl amine was coupled with **5**. Traces of thiol remained in the reaction mixture requiring several equivalents of coupling reagent, a side effect that can be avoided using polymer bound azides [20]. After RP-HPLC purification over a polystyrene column, the desired glycosylamino acid **6** was isolated in 63% yield.

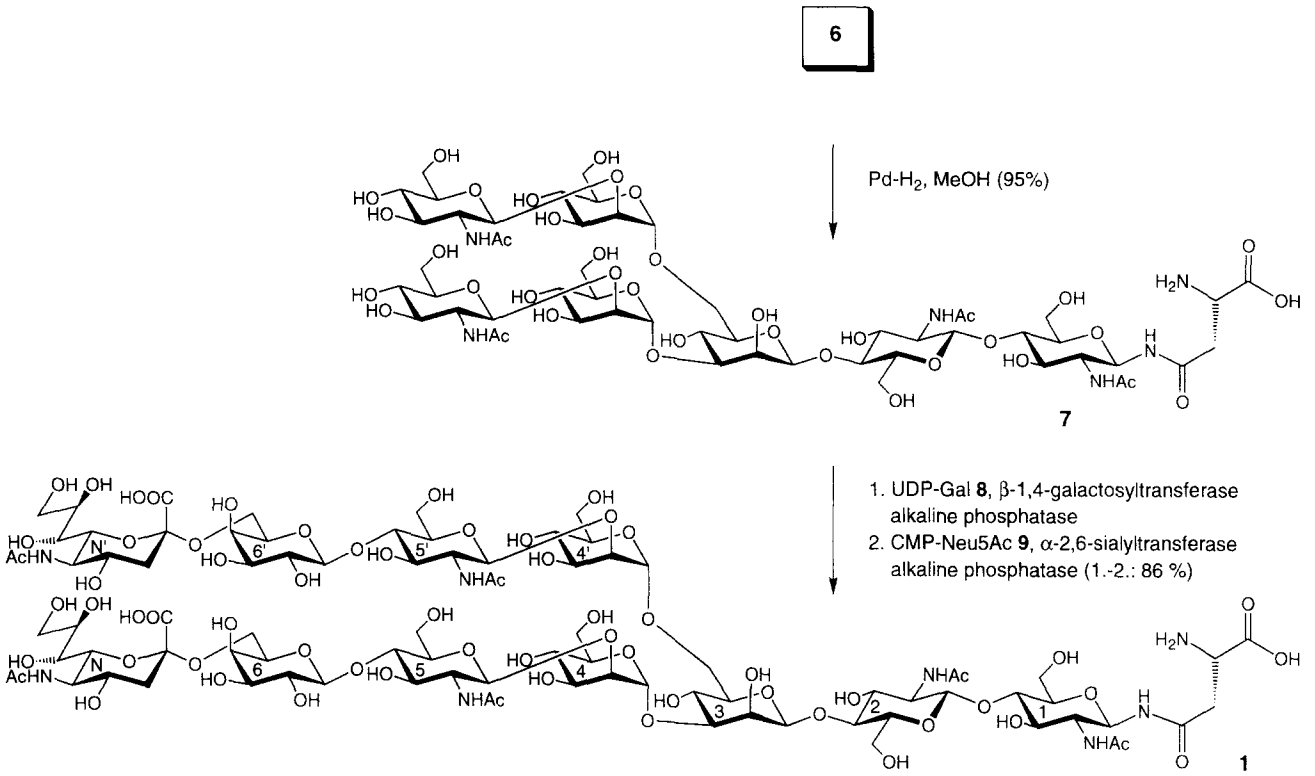
Prior to the envisioned enzymatic elongation the remaining six protecting groups of compound **6** were removed in a single step by catalytic hydrogenation over palladium black (Scheme 3). The resulting free heptasaccharide asparagine **7** was efficiently separated from the catalyst by repeated washing with a methanol–acetic acid mixture and purified by size-exclusion chromatography (95%). The two GlcNAc acceptor sites in the diantennary N-glycanyl asparagine **7** were galactosylated using the alkaline phosphatase-enhanced glycosylation method [8]. During the enzymatic elongation of the acceptor substrate **7** with galactosyltransferase [21], the inhibitor uridine diphosphate (UDP) is generated from the donor substrate UDP-galactose **8**. Alkaline phosphatase of high purity degrades UDP to uridine which does not affect



Scheme 2.

the glycosyltransferase in the concentrations used for enzymatic synthesis. In consequence this increases the reaction velocity and the yields of the final products. Two galactose residues were efficiently transferred onto the heptasaccharide **7** giving a galactosylated nonasaccharide intermediate. Double sialylation of the nonasaccharide was affected by incubation of the reaction mixture with CMP-*N*-acetylneuraminic acid **9**, α-2,6-sialyltransferase and alkaline phosphatase. Good yields were only obtained when the sialyltransferase and the donor substrate were added in two portions. The transfer of the four terminal sugar units was performed stepwise to ensure complete conversion and conducted as a one-pot reaction. Multiantennary N-glycans [22] show higher substrate and product inhibition during glycosyltransferase reactions than their monovalent partial structures. Also the enzymatic elongation of N-glycans was shown to prefer the (1 → 3)-arm [1,22] for the transfer of the first sugar in the case of both glycosyltransferases used. The second transfer onto the (1 → 6)-arm is significantly slower. Despite those difficulties the chosen reaction conditions led to nearly quantitative enzymatic glycosylation and furnished the diantennary undecasaccharide-asparagine **1** in 86% yield after gel filtration. The structures of the target molecule **1** and its precursors were confirmed by TOCSY, NOESY, HMQC, HMQC-COSY, HMQC-DEPT and HMQC-TOCSY experiments [23], reference spectra [24] and mass spectrometry.

The complex glycoconjugate **1**, which up to now could only be isolated from natural sources [25] has been prepared by combined chemical and enzymatic synthesis for the first time. With the development of this methodology chemoenzymatic synthesis of natural N-glycans and their conjugates has become an



Scheme 3.

advantageous alternative to the isolation [26] or semisynthesis [27] of this class of ubiquitous natural products.

3. Experimental

General methods.—TLC was performed on silica gel plates ($60F_{254}$; E. Merck, Darmstadt) and visualized by spraying with 0.5 M H_2SO_4 in EtOH containing 0.1% orcinol. UDP-galactose **8**, cytidine-5′-monophospho-*N*-acetylneuraminic acid **9**, bovine galactosyltransferase (EC 2.4.1.22.), α-2,6-sialyltransferase (EC 2.4.99.1.) and bovine serum albumin were obtained from Sigma Chemical. Calf intestinal alkaline phosphatase (molecular biology grade) (EC 3.1.3.1.) was obtained from Boehringer Mannheim. HPLC-separations were performed on a Pharmacia LKB gradient system 2249 equipped with a Pharmacia LKB Detector VWM 2141 (Freiburg, Germany). For size-exclusion chromatography a Pharmacia Hi Load Superdex 30 column (600 × 16 mm) was used and RP-HPLC was performed on a Macherey–Nagel Nucleogel RP 100-8 column (300 × 7.7 mm). NMR spectra were recorded on a Bruker AMX 500 instrument. For spectra recorded in D_2O the HOD signal (4.81 ppm) was used as a reference. ESI–MS spectra were recorded on a Finnigan TSQ mass spectrometer using 1:1 MeOH–water as eluent. FABMS spectra were recorded on a Finnigan MAT 95Q instrument using a thioglycerine–HOAc matrix (MB). MALDI-TOF mass spectra were recorded on a Voyager Biospectrometry workstation (Vestec/Perseptive) MALDI-TOF mass spectrometer, using 2,5-dihydroxybenzoic acid (DHB) as a matrix.

(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 2)-α-D-mannopyranosyl-(1 → 3)-[(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 2)-α-D-mannopyranosyl-(1 → 6)]-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-glucopyranosylazide (3).—Compound **2** (100 mg, 38.4 mmol) was dissolved in a mixture of *n*-BuOH (10 mL) and ethylene diamine (2.5 mL) and heated for 16 h at 90 °C (TLC, 2:1 iPrOH–1 M NH_4OAc). The reagents were distilled off and the residue was dried in high vacuo. When the overall weight remained constant, pyridine (10 mL) and Ac_2O (5 mL) were added. After 1 h at ambient temperature (TLC, 10:1 CH_2Cl_2–MeOH) the mixture was concentrated in vacuo (45 °C bath temperature) followed by co-evaporation of toluene for three times. The remainder was dried in high vacuo and dissolved in aq 40% $MeNH_2$ (5 mL). Deacetylation was complete after 2

h (TLC, 4:1 iPrOH–1 M NH_4OAc) and the solution was concentrated in vacuo. Solid-phase extraction was performed by dissolving the remainder in water (25 mL) and passing the solution through three connected SepPak-RP18-cartridges (Waters). The cartridges were washed with water (30 mL) and impurities were washed off with 1:4 acetonitrile–water (15 mL). The product was eluted with 2:3 acetonitrile–water (15 mL) followed by lyophilization. Yield: 64 mg (97.8%) fluffy solid; R_f amine 0.5 (2:1 iPrOH–1 M NH_4OAc); R_f peracetate 0.44 (10:1 CH_2Cl_2–MeOH); R_f **2** 0.55 (4:1 iPrOH–1 M NH_4OAc); $[\alpha]_D^{23}$ −9.9° (*c* 0.5, MeOH); $C_{78}H_{107}N_7O_{35}$ (1702.73) ESI–MS: M_{calc} = 1701.6; M_{found} = 1702.6 (M + 1), 1724.7 (M + Na). ^1H NMR (Me_2SO-d_6, 500 MHz): δ 7.99 (m, 2 H, NH-2^1, 2^2), 7.57 (m, 2 H, NH-2^5,$2^{5'}$), 7.36–7.18 (m, 20 H, Ar), 5.00–4.95 (m, 8 H, H-1^4, OH-3^5,$3^{5'}$,4^3,4^5,$4^{5'}$, CH_2O), 4.75 (d, $J_{OH,4}$ 4.9 Hz, 1 H, OH-4^4), 4.71 (d, $J_{OH,4}$ 4.9 Hz, 1 H, OH-$4^{4'}$), 4.66 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-$1^{4'}$), 4.62–4.42 (m, 12 H, H-$1^{1'}$,1^2,1^3, OH-2^3,6^4,6^5,$6^{5'}$, CH_2O), 4.36 (d, $J_{1,2}$ 8.2 Hz, 1 H, H-1^5), 4.31 (d, J_{gem} 12.2 Hz, 1 H, CH_2O), 4.23 (d, $J_{1,2}$ 8.3 Hz, 1 H, H-$1^{5'}$), 4.19 (m, 1 H, OH-$6^{4'}$), 3.99–3.95 (m, 2 H, H-2^3, OH-3^4), 3.91 (d, $J_{OH,3}$ 7.9 Hz, 1 H, OH-$3^{4'}$), 3.88–3.82 (m, 2 H, H-4^1,2^4), 3.14–3.02 (m, 6 H, H-4^5,$4^{5'}$,5^2,5^3,5^5,$5^{5'}$), 1.80, 1.79, and 1.77 (12 H, 4 NAc). ^{13}C NMR (Me_2SO-d_6, 125 MHz): δ 169.7, 169.6, and 169.3 (C=O), 139.3, 139.0, 138.5, and 138.4 (C–i Ar), 128.2–127.0 (C–Ar), 101.5 (C-$1^{5'}$), 101.1 (C-1^5), 100.2 (C-1^3), 100.0 (C-1^2), 99.9 (C-1^4), 97.6 (C-$1^{4'}$), 88.0 (C-1^1), 81.5 (C-3^3), 81.0 (C-3^2), 80.4 (C-3^1), 79.3 (C-$2^{4'}$), 78.8 (C-2^4), 77.0 (C-5^5), 76.9 (C-$5^{5'}$), 76.3 (C-5^1), 75.8 (C-4^2), 75.5 (C-5^3), 74.9 (C-4^1), 74.4 (C-5^2), 74.2 (C-5^4), 74.0 (C-3^5,$3^{5'}$), 73.8 (CH_2O), 73.6 (C-$5^{4'}$), 73.2, 72.2, and 71.9 (CH_2O), 70.5 (C-4^5), 70.4 (C-$4^{5'}$), 70.0 (C-3^4), 69.9 (C-$3^{4'}$), 69.2 (C-2^3), 68.7 (C-6^2), 68.2 (C-6^1), 67.6 (C-4^4), 67.0 (C-$4^{4'}$), 66.0 (C-6^3), 65.0 (C-4^3), 61.5 (C-6^4), 61.1 (C-$6^{4'}$), 61.0 (C-6^5), 60.8 (C-$6^{5'}$), 55.6 (C-2^5,$2^{5'}$), 55.2 (C-2^2), 53.6 (C-2^1), 22.2, 23.0, and 22.8 (NAc).

*N-benzyloxycarbonyl-*L*-aspartic acid-α-benzyl ester - β - pentafluorophenyl ester* (**5**).—*N*-Benzyloxycarbonyl-L-aspartic acid-α-benzyl ester **4** (610 mg, 1.7 mmol) was reacted with pentafluorophenyl trifluoroacetate (344 μL, 2 mmol) according to the published procedure [17]. The crude product was recrystallized from EtOAc–hexane. Yield: 750 mg (84%); mp 95 °C; $[\alpha]_D^{23}$ −16.3° (*c* 1, acetone).

N^4*-[(2-Acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 2)-α-*D*-mannopyranosyl-(1 → 3)-[(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 2)-α-*D*-mannopyranosyl-(1 → 6)]-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)]-*N^2*-benzyloxy-carbonyl-*L*-asparagine benzyl ester* (**6**).—Heptasaccharide **3** (20 mg, 11.7 μmol) was dissolved in a mixture of dry MeOH (600 μL) and ethyldiisopropylamine (20 μL). The flask was flushed with Ar followed by addition of propanedithiol (60 μL). Reduction to the amine was completed after 4 h (TLC, 4:1 iPrOH–1M NH_4OAc). Subsequently, the mixture was concentrated and dried in high vacuo to remove the majority of the reagents. To the remainder was added a solution of **5** (40 mg, 76 μmol), *N*-hydroxybenzotriazol (HOBT) (10 mg, 65.3 μmol) and ethyldiisopropylamine (20 μL, 118 μmol) in 450 μL *N*-methylpyrrolidone (NMP). After complete reaction of the amine (TLC, 4:1 iPrOH–1 M NH_4OAc) the mixture was concentrated in high vacuo and the remainder was purified by RP-HPLC (column: Macherey–Nagel Nucleogel RP 100-8, 300 × 7.7 mm; mobile phase: gradient of 32–45% acetonitrile over 27 min; flow rate: 2 mL/min; detection at 220 and 260 nm). The pooled fractions were lyophilized. Yield: 15 mg (63.3%); R_f amine 0.36 (4:1 isopropanol–1 M ammonium acetate); R_f **6** 0.58 (4:1 iPrOH–1 M NH_4OAc); $[\alpha]_D^{23}$ −0.3° (*c* 0.5, MeOH). $C_{97}H_{126}N_6O_{40}$ (2016.08) FABMS (MB): M_{calc} = 2014.8; M_{found} = 2017 ± 1 (M + 1). ^1H NMR (Me_2SO-d_6, 500 MHz): δ 8.44 (d, $J_{NH,1}$ 8.8 Hz, 1 H, γNH Asn), 7.99 (d, $J_{NH,2}$ 7.1 Hz, 1 H, NH-2^2), 7.83 (d, $J_{NH,2}$ 8.9 Hz, 1 H, NH-2^1), 7.58 (m, 3 H, NH-2^5,$2^{5'}$, NH-urethane), 7.37–7.18 (m, 20 H, Ar), 5.11 and 5.06 (2 d, J_{gem} 12.8 Hz, 2 H, CH_2O), 5.05–4.94 (m, 11 H, H-1^1,1^4, OH-3^5,$3^{5'}$,4^3,4^5,$4^{5'}$, CH_2O), 4.75 (d, $J_{OH,4}$ 4.8 Hz, 1 H, OH-4^4), 4.71 (d, $J_{OH,4}$ 4.8 Hz, 1 H, OH-$4^{4'}$), 4.66 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-$1^{4'}$), 4.62–4.40 (m, 12 H, αCH Asn, H-1^2,1^3, OH-2^3,6^4,6^5,$6^{5'}$, CH_2O), 4.36 (d, $J_{1,2}$ 8.1 Hz, 1 H, H-1^5), 4.31 (d, J_{gem} 12.2 Hz, 1 H, CH_2O), 4.23 (d, $J_{1,2}$ 8.2 Hz, 1 H, H-$1^{5'}$), 4.19 (m, 1 H, OH-$6^{4'}$), 3.99–3.94 (m, 2 H, H-2^3, OH-3^4), 3.90 (d, $J_{OH,3}$ 8.0 Hz, 1 H, OH-$3^{4'}$), 3.88–3.83 (m, 2 H, H-2^4,4^1), 3.14–3.02 (m, 5 H, H-4^5,$4^{5'}$,5^3,5^5,$5^{5'}$), 2.67 (dd, J_{gem} 16.1 Hz, J_{vic} 5.0 Hz, 1 H, βCHa Asn), 2.50 (dd, J_{vic} 7.2 Hz, 1 H, βCHb Asn), 1.80, 1.77, and 1.70 (12 H, 4 NAc). ^{13}C NMR (Me_2SO-d_6, 125 MHz): δ 171.2 (C=O ester), 169.7, 169.5, 169.4, and 169.3 (C=O NAc), 155.8 (C=O urethane), 139.2, 138.5, 138.4, 136.7, and 135.9 (C-i Ar), 128.3–126.9 (C–Ar), 101.5 (C-$1^{5'}$), 101.1 (C-1^5), 100.2 (C-1^3), 99.8 (C-1^4), 99.6

(C-1^2), 97.6 (C-$1^{4'}$), 81.5 (C-3^3), 81.4 (C-3^1), 80.8 (C-3^2), 79.2 (C-$2^{4'}$), 78.8 (C-2^4), 78.5 (C-1^1), 77.0 (C-5^5), 76.9 (C-$5^{5'}$), 76.1 (C-5^1), 75.9 (C-4^2), 75.5 (C-5^3), 74.6 (C-4^1), 74.4 (C-5^2), 74.2 (C-5^4), 73.9 (C-3^5,$3^{5'}$), 73.8 (CH_2O), 73.6 (C-$5^{4'}$), 73.2, 72.2, and 71.8 (CH_2O), 70.5 (C-$4^{5'}$), 70.3 (C-4^5), 69.9 (C-3^4,$3^{4'}$), 69.1(C-2^3), 68.7 (C-6^2), 68.3 (C-6^1), 67.6 (C-4^4), 67.1 (C-$4^{4'}$), 66.0 (CH_2O, C-6^3), 65.6 (CH_2O), 64.9(C-4^3), 61.5 (C-6^4), 61.1 (C-$6^{4'}$), 61.0 (C-6^5), 60.8 (C-$6^{5'}$), 55.6 (C-$2^{5'}$,$2^{5'}$), 55.3 (C-2^2), 53.2 (C-2^1), 50.4 (αC Asn), 36.8 (βC Asn), 23.2, 22.9, and 22.7 (NAc).

N^4-*{(2-Acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 2)-α-*D*-mannopyranosyl-(1 → 3)-[(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 2)-α-*D*-mannopyranosyl-(1 → 6)-β-*D*-mannopyranosyl-(1 → 4)-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 4)-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)]}-*L*-asparagine* (**7**).—A suspension of $PdII_2$ (40 mg) in MeOH (10 mL) was converted to palladium black by stirring under H_2. After removal of the solvent the catalyst was washed twice with MeOH. Subsequently, a solution was added containing the benzylated glycopeptide **6** (20 mg, 9.92 μmol) and HoAc (0.3 mL) in MeOH (2.7 mL). The suspension was stirred under H_2 for 16 h (TLC, 2:1 iPrOH–1 M NH_4OAc). The catalyst was removed by centrifugation and washed repeatedly with 9:1 MeOH–HoAc. After concentration of the combined supernatant the remainder was purified by gel filtration (column: Pharmacia Hi Load Superdex 30,600 × 16 mm; mobile phase: 100 mM NH_4HCO_3; flow rate: 750 μL/min; detection: 220 and 260 nm) and lyophilized. Yield: 13.5 mg (95.1%); R_f 0.18 (TLC, 2:1 iPrOH–1 M NH_4OAc); $[\alpha]_D^{23}$ −0.3° (*c* 1, water); $C_{54}H_{90}N_6O_{38}$ (1431.32) MALDI-TOF–MS (DHB in 9:1 water–EtOH): M_{calc} = 1430.53; M_{found} = 1453.96 (M + Na). ^{1}H NMR (D_2O, 500 MHz): δ 5.17 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-1^4), 5.12 (d, $J_{1,2}$ 9.7 Hz, 1 H, H-1^1), 4.97 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-$1^{4'}$), 4.82 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-1^3), 4.66 (d, $J_{1,2}$ 7.6 Hz, 1 H, H-1^2), 4.60 (m, 2 H, H-1^5,$1^{5'}$), 4.30 (dd, $J_{2,3}$ 1.9 Hz, 1 H, H-2^3), 4.24 (dd, $J_{2,3}$ 1.9 Hz, 1 H, H-2^4), 4.16 (dd, $J_{2,3}$ 1.9 Hz, 1 H, H-$2^{4'}$), 2.92 (dd, J_{gem} 16.6, J_{vic} 4.1 Hz, 1 H, βCHa Asn), 2.79 (dd, J_{vic} 7.3 Hz, 1 H, βCHb Asn), 2.13, 2.10, and 2.06 (12 H, 4 NAc). ^{13}C NMR (D_2O, Me_2SO-d_6 as internal standard, 125 MHz): δ 177.0, 176.4, 176.3, 176.2, and 174.8 (C=O), 102.8 (C-1^2), 102.0 (C-1^3), 101.2 (C-1^5,$1^{5'}$), 101.1 (C-1^4), 98.6 (C-$1^{4'}$), 82.0 (C-3^3), 81.1 (C-4^2), 80.4 (C-4^1), 79.7 (C-1^1), 78.1 (C-2^4), 78.0 (C-$2^{4'}$), 77.8 (C-5^1), 77.4 (C-5^5,$5^{5'}$), 76.0 (C-5^2), 75.9 (C-5^3), 75.1 (C-5^4), 75.0 and 74.9 (C-3^5,$3^{5'}$), 74.44 (C-$5^{4'}$), 74.39 (C-3^1), 73.6 (C-3^2), 71.8 (C-2^3), 71.5 (C-4^5,$4^{5'}$), 71.1 (C-$3^{4'}$), 71.0 (C-3^4), 68.93 (C-$4^{4'}$), 68.90 (C-4^4), 67.4 (C-6^3), 67.3 (C-4^3), 63.3 and 63.2 (C-6^4,$6^{4'}$), 62.2 (C-6^5,$6^{5'}$), 61.54 (C-6^2), 61.48 (C-6^1), 56.9 (C-2^5,$2^{5'}$), 56.5 (C-2^2), 55.2 (C-2^1), 53.2 (αC Asn), 38.5 (βC Asn), 23.9, 23.8, and 23.7 (NAc).

N^4-*{(5-Acetamido-3,5-dideoxy-*D*-glycero-α-*D*-galacto-non-2-ulopyranosylonic acid)-(2 → 6)-β-*D*-galactopyranosyl-(1 → 4)-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 2)-α-*D*-mannopyranosyl-(1 → 3)-[(5-acetamido-3,5-dideoxy-*D*-glycero-α-*D*-galacto-non-2-ulopyranosylonic acid)-(2 → 6)-β-*D*-galactopyranosyl-(1 → 4)-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 2)-α-*D*-mannopyranosyl-(1 → 6)]-β-*D*-mannopyranosyl-(1 → 4)-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 4)-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)}-*L*-asparagine* (**1**).—Compound **7** (4 mg, 2.79 μmol) was dissolved in 20 mM sodium cacodylate buffer (pH 7.4, 1400 μL) containing bovine serum albumin (1.0 mg), NaN_3 (2.5 μmol), manganese chloride (1.4 μmol), uridine-5′-diphosphogalactose **8** (5.6 mg, 8.4 μmol), alkaline phosphatase (EC 3.1.3.1, 6 U) and GlcNAc-β-1,4-galactosyltransferase (EC 2.4.1.22, 120 mU). The reaction mixture was incubated for 24 h at 37 °C maintaining the pH at 7.0 by addition of 1 M NaOH. After complete reaction (TLC, 2:1 iPrOH–1 M NH_4OAc) cytidine-5′-monophospho-*N*-acetylneuraminic acid **9** (4.8 mg, 6.2 μmol) and β-galactoside-α-2,6-sialyltransferase (EC 2.4.99.1, 25 mU) were added. Incubation was continued for 24 h at 37 °C (pH 7.0) followed by another addition of cytidine-5′-monophospho-*N*-acetylneuraminic acid **9** (4.8 mg, 6.2 μmol) and β-galactoside-α-2,6-sialyltransferase (25 mU). After 24 h of reaction time, the precipitate was removed by centrifugation. The supernatant was concentrated to 400 μL and purified by gel filtration (column: Pharmacia Hi Load Superdex 30, 600 × 16 mm; mobile phase: 100 mM NH_4HCO_3; flow rate: 750 μL/min; detection: 220 and 260 nm). The positive fractions were collected and lyophilized. Yield: 5.6 mg (85.7%); R_f nonasaccharide (digalactoside) 0.13 (2:1 isopropanol–1 M NH_4OAc); R_f **1** 0.08 (2:1 iPrOH–1 M NH_4OAc); $[\alpha]_D^{23}$ −9.1° (*c* 0.5, water); $C_{88}H_{144}N_8O_{64}$ (2338.12) ESI–MS (50% 0.01 M NH_4OAc–50% MeOH): M_{calc} = 2336.8; M_{found} = 2337.8 (M + 1). ^{1}H NMR (D_2O, 500 MHz): δ 5.19 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-1^4), 5.12 (d, $J_{1,2}$ 9.7 Hz, 1 H, H-1^1), 5.01 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-$1^{4'}$), 4.83 (d, $J_{1,2}$ < 1.0 Hz, 1 H, H-1^3), 4.69–4.65 (m, 3 H, H-1^2,1^5,$1^{5'}$), 4.505 and 4.502 (2 d, $J_{1,2}$ 7.8 Hz, 2

H, H-1^6,$1^{6'}$), 4.31 (dd, $J_{2,3}$ 1.9 Hz, 1 H, H-2^3), 4.25 (dd, $J_{2,3}$ 1.9 Hz, 1 H, H-2^4), 4.17 (dd, $J_{2,3}$ 1.9 Hz, 1 H, H-$2^{4'}$), 3.00 (dd, J_{gem} 17.2, J_{vic} 4.2 Hz, 1 H, βCHa Asn), 2.92 (dd, J_{vic} 7.0 Hz, 1 H, βCHb Asn), 2.76–2.71 (m, 2 H, H-$3eq^N$,$3eq^{N'}$), 2.14, 2.13, 2.09, 2.07, and 2.06 (18 H, 6 NAc), 1.78 (dd, J_{gem}, J_{vic} 12.1 Hz, 2 H, H-$3ax^N$,$3ax^{N'}$). ^{13}C NMR (D_2O, Me_2SO-d_6 as internal standard, chemical shifts were determined by a HMQC spectrum, 125 MHz): δ 104.8 (C-1^6,$1^{6'}$), 102.6 (C-1^2), 101.8 (C-1^3), 100.7 (C-1^4), 100.6 (C-$1^{5'}$,$1^{5'}$), 98.1 (C-$1^{4'}$), 81.9 (C-4^5,$4^{5'}$), 81.8 (C-3^3), 80.9 (C-4^2), 80.2(C-4^1), 79.4 (C-1^1), 77.8 (C-2^4), 77.6 (C-$2^{4'}$), 77.5 (C-5^1), 75.7 (C-5^2,5^3,5^5,$5^{5'}$), 75.0 (C-5^6,$5^{6'}$), 74.9 (C-5^4), 74.1 (C-$5^{4'}$), 74.0 (C-3^1), 73.8 (C-3^6,$3^{6'}$,3^N,$3^{N'}$), 73.3 (C-3^2,3^5,$3^{5'}$), 73.0 (C-8^N,$8^{N'}$), 72.0 (C-2^6,$2^{6'}$), 71.4 (C-2^3), 70.7 (C-3^4,$3^{4'}$), 69.7 (C-7^N,$7^{N'}$), 69.6 (C-4^6,$4^{6'}$), 69.4 (C-4^N,$4^{N'}$), 68.6 (C-4^4,$4^{4'}$), 67.1 (C-6^3), 67.0 (C-4^3), 64.6 C-6^6,$6^{6'}$), 64.0 (C-9^N,$9^{N'}$), 62.9 (C-6^4,$6^{4'}$), 61.5 (C-6^5,$6^{5'}$), 61.2 (C-6^2), 61.1 (C-6^1), 56.2 (C-2^2), 55.9 (C-2^5,$2^{5'}$), 54.9 (C-2^1), 53.2 (C-5^N,$5^{N'}$), 52.1 (αC Asn), 39.0 (C-3^N,$3^{N'}$), 36.3 (βC Asn), 23.9, 23.8, and 23.7 (NAc).

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