Novel sequential solid-phase synthesis of N-linked glycopeptides from natural sources

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In the present report a practical and versatile procedure for the solid-phase synthesis of N-linked glycopeptides from natural sources has been demonstrated. The approach is based on the mild hydrazinolysis procedure to release N-linked oligosaccharides in their intact unreduced form from the natural glycoproteins, *e.g.* fetuin and ribonuclease B and subsequent formation of the corresponding glycosylamines. Treatment of the reducing sugars 1–7 with a saturated solution of ammonium hydrogen carbonate in either water or dimethyl sulfoxide (DMSO) gives in almost quantitative yields the glycosylamines 8–14. Coupling of the unprotected glycosylamines 8–14 to the side-chain-activated aspartic acid derivative Fmoc-Asp(ODhbt)-OBu^t 16 affords the N-glycosylated asparagine derivatives 17–23. Subsequent acetylation of the carbohydrate hydroxy groups and cleavage of the *tert*-Bu ester by trifluoroacetic acid (TFA) treatment yields the glycosylated N-linked building blocks 31–37. The building blocks 31–37 are then incorporated into the multiple-column peptide-synthesis protocol of the glycopeptide T-cell epitope analogues 40–46 of the mouse haemoglobin-derived decapeptide Hb (67–76), VITAFNEGLK. The decapeptide sequence VITAFNEGLK binds well to the MHC Class II E^k molecule and is non-immunogenic in CBA/J mice. Syntheses of several natural and unnatural glycosylations, *e.g. N*-acetylglucosamine, *N*,*N*′-diacetylchitobiose, glucose, maltotriose, maltoheptose and di- and tri-antennary complex oligosaccharides on the decapeptide Hb (67–76) affording the N-linked glycopeptides 40–46 are described. The N-linked glycopeptides 40–46 have been fully characterised by 1D- and 2D-^{1}H and ^{13}C NMR spectroscopy and by ES-MS.

Introduction

Many secreted and cell-surface proteins are heavily glycosylated with both N- and O-linked oligosaccharides. Asparagine-linked glycosylation of proteins is the predominant modification in eukaryotic cells. The N-linked oligosaccharides are covalently attached to the asparagine (Asn, N) side-chain *via* a β-N-glycosidic linkage. The structures of N-linked oligosaccharides fall into three categories: high mannose, complex and hybrid type.[1,2] All N-linked glycoproteins share the common pentasaccharide core structure $(Man)_3$-$(GlcNAc)_2$ but do vary in the nature of the outer residues. The effects of these sugars on the physical properties,[3] immunogenicity,[4] folding[5] and stability of the glycoproteins are important issues. Protein glycosylations can effect pharmacological parameters including circulating life-time,[6] solubility,[3] proteolytic stability[7] and immunogenicity.[4] The *N*-glycopeptides are often used as models for studying the interactions between carbohydrates and peptides.[8,9] Owing to the difficulties in expressing or isolating well defined glycoproteins, a convenient synthetic route to these glycopeptides would be of great value. Investigations of protein–carbohydrate interactions involving complex N-linked glycopeptides have been performed with only little success due to difficulties in their preparation.

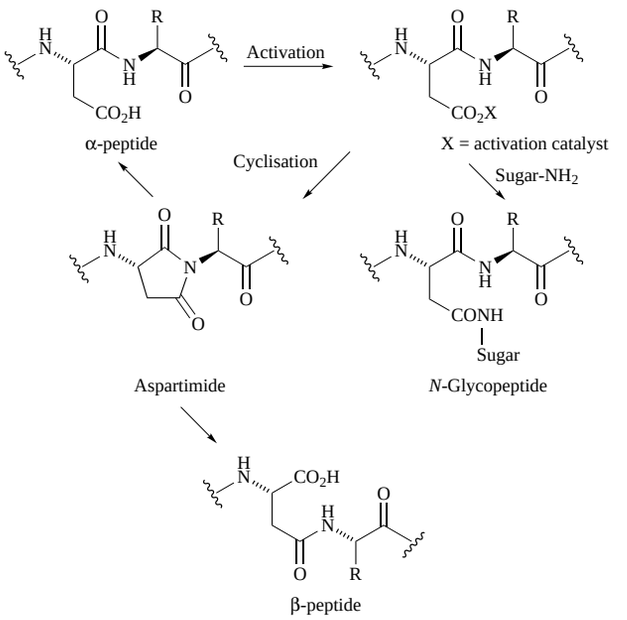
Owing to the biological importance of protein glycosylation, the solid-phase synthesis of N-linked glycopeptides has frequently been attempted. Currently, there are two approaches to synthesise N-linked glycopeptides: (i) The convergent approach[10,11] and (ii) the building-block approach.

The former is based on the coupling of a glycosylamine to a fully protected peptide containing an activated aspartic acid side-chain either in solution or on a solid-phase. An orthogonal side-chain-protecting group compatible with the fluoren-9-ylmethoxycarbonyl (Fmoc) methology was required during peptide assembly for the aspartyl β-carboxy group. The protecting group was selectively removed to obtain the free β-carboxy group which could be activated and subsequently coupled with a simple glycosylamine to give the desired *N*-glycopeptide. The major problem which has not been solved efficiently yet is the intramolecular aspartimide formation with the C-terminal amide group upon activation of the aspartic acid side-chains, particularly when the active ester is N-terminal to a small residue like glycine or alanine (Scheme 1). This side-reaction competes with glycosylation and practically limits the efficacy of the convergent approach to very small glycan structures. Several approaches have been described which reduce aspartimide formation.[12,13] However, all of these methods remain problematic.

Alternatively, the versatile and general building block approach[14,15] has been found to be advantageous in many respects. It involves the incorporation of preformed glycosylated N^{α}-Fmoc-protected asparagine derivatives to which the target sugar is N-linked through the β-carboxamide in standard or multiple-synthesis protocols. This method currently offers the most versatile and general approach for the preparation of a large variety of complex homogeneous glycopeptides with well defined and predetermined structures[15] and even larger libraries of biologically active glycopeptides.

The release of N-linked oligosaccharides in small amounts has previously been accomplished by enzymic[16,17] and chemical methods. Enzymic methods have a high degree of specificity;

however, the enzyme must be validated for every new application. An important endoglycosidase which has been very useful in the analysis of N-linked glycans was identified in *Flavobacterium meningosepticum*. However, success of the enzymic method for preparative purposes is unfortunately highly dependent on the substrate specificity and on the availability of the enzyme. Chemical methods have the advantage of broad specificity towards the substrate and include a large variety of chemical reagents, *e.g.* hydrazine,[18] β-elimination by mild alkaline treatment or under strongly alkaline conditions,[19] and lithium aluminium borohydride.[20] The reducing agent converts the reducing sugars to a sugar alditol residue. On the other hand, hydrazine[18] as cleaving reagent offers the possibility of obtaining the released oligosaccharides in their unreduced hemiacetal form. It can be used to release O- and N-glycans from proteins, and the method can be optimized to permit the selective removal of only O-linked residues.



Scheme 1 Aspartimide formation during convergent *N*-glycopeptide synthesis. This side-reaction is depending on the chemical structure of the adjacent amino acid towards the C-terminal. For large oligosaccharides, aspartimide formation is predominant.

Bovine fetuin, the major glycoprotein in foetal calf serum, is an α-globulin of M_r 48 000.[21] It is a globular 341-amino acid protein with six internal disulfide bridges and six carbohydrate moieties per molecule, three O-glycosidically linked to Ser/Thr and three N-glycosidically linked to Asn.[22] The majority of the Asn-linked oligosaccharides (80%) on fetuin have three peripheral branches[23] with the desialylated structure presented in Fig. 1a. A minor population of dibranched Asn-linked oligosaccharides, lacking the branch arising from the peripheral β-1,4-linked GlcNAc residue, has also been detected.

The isolation of high-mannose structures was achieved by hydrazinolysis of bovine ribonuclease B.[24,25] Bovine ribonuclease B is a glycoprotein with a relative molecular mass of 15 500 daltons. It contains 124 amino acid residues and one N-glycosylation site at Asn 34.[24] Ribonuclease B contains high-mannose type oligosaccharides with five to nine mannosyl residues [$(Man)_5(GlcNAc)_2 \rightarrow (Man)_9(GlcNAc)_2$] as presented in Fig. 1b.

The N-linked synthetic target has been selected to allow the investigation of the activation of T-cells by glycopeptides bound to MHC-class II molecules as an important event in the triggering of the immune response. Owing to lack of avail-

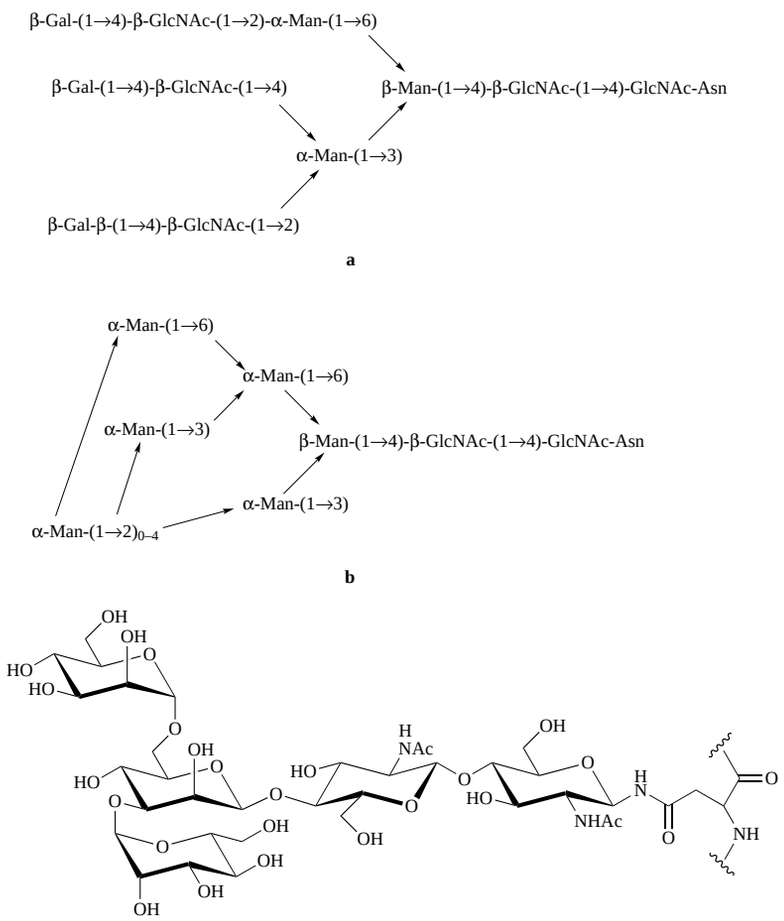


Fig. 1 (a) Desialylated triantennary complex-type oligosaccharide structure from bovine fetuin. (b) Branched high-mannose-type oligosaccharide structure from ribonuclease B. (c) The common core of N-linked oligosaccharides.

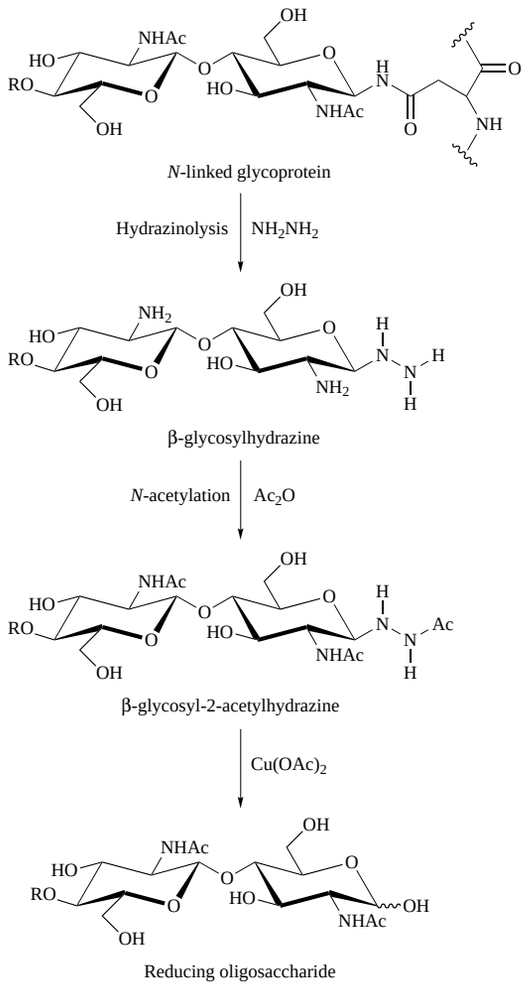
ability the glycan-specific immunogenicity of glycoproteins and glycopeptides is described in only a few reports.[26,27] Studies of glycopeptide epitopes, mimicking post-translational modification of proteins, is therefore of significant interest. Recently it was found that O-glycosylation, as found in mucins, can be specifically recognized by T-cells when linked to the MHC-Class II binding CBA/J mouse haemoglobin Hb (67–76) peptide.[26] This decapeptide, VITAFNEGLK, binds well to the MHC-Class II E^k molecule and is itself non-immunogenic in CBA/J mice.[28]

The aim has been to release and recover N-linked oligosaccharides from the natural sources fetuin and ribonuclease B in their non-reduced form by employing the mild conditions of hydrazinolysis[18,29] in order to synthesise N-linked glycosylasparagine building blocks for use in the multiple-column peptide syntheses (MCPS) of N-linked glycopeptides. Both fetuin and ribonuclease B are commercially available, well characterised, affordable and offer the complex-type (fetuin) or the high-mannose (ribonuclease B) oligosaccharide structures, respectively. The N-linked glycopeptides **40**–**46** were prepared as putative immunogenic epitopes binding to the Major Histocompatibility Complex (MHC) class II E^k molecule in CBA/J mice and eliciting a T-cell response.

Results and discussion

In order to investigate the fine specificity of T-cell response against synthetic glycopeptides further, a series of *N*-glycopeptide analogues of immunogenic epitope structures has been synthesised. In the present paper, the synthesis of N-glycosylated asparagine building blocks with extended triantennary chains was demonstrated and they were applied in the solid-phase synthesis of N-linked glycopeptides derivatised at the asparagine in the decapeptide VITAFNEGLK. The versatility of the present method was demonstrated by variation of the glycan structure to include glucose, maltotriose, maltoheptose, *N*-acetylglucosamine and chitobiose as model compounds as well as high-mannose and the di- and tri-antennary complex oligosaccharide structures.

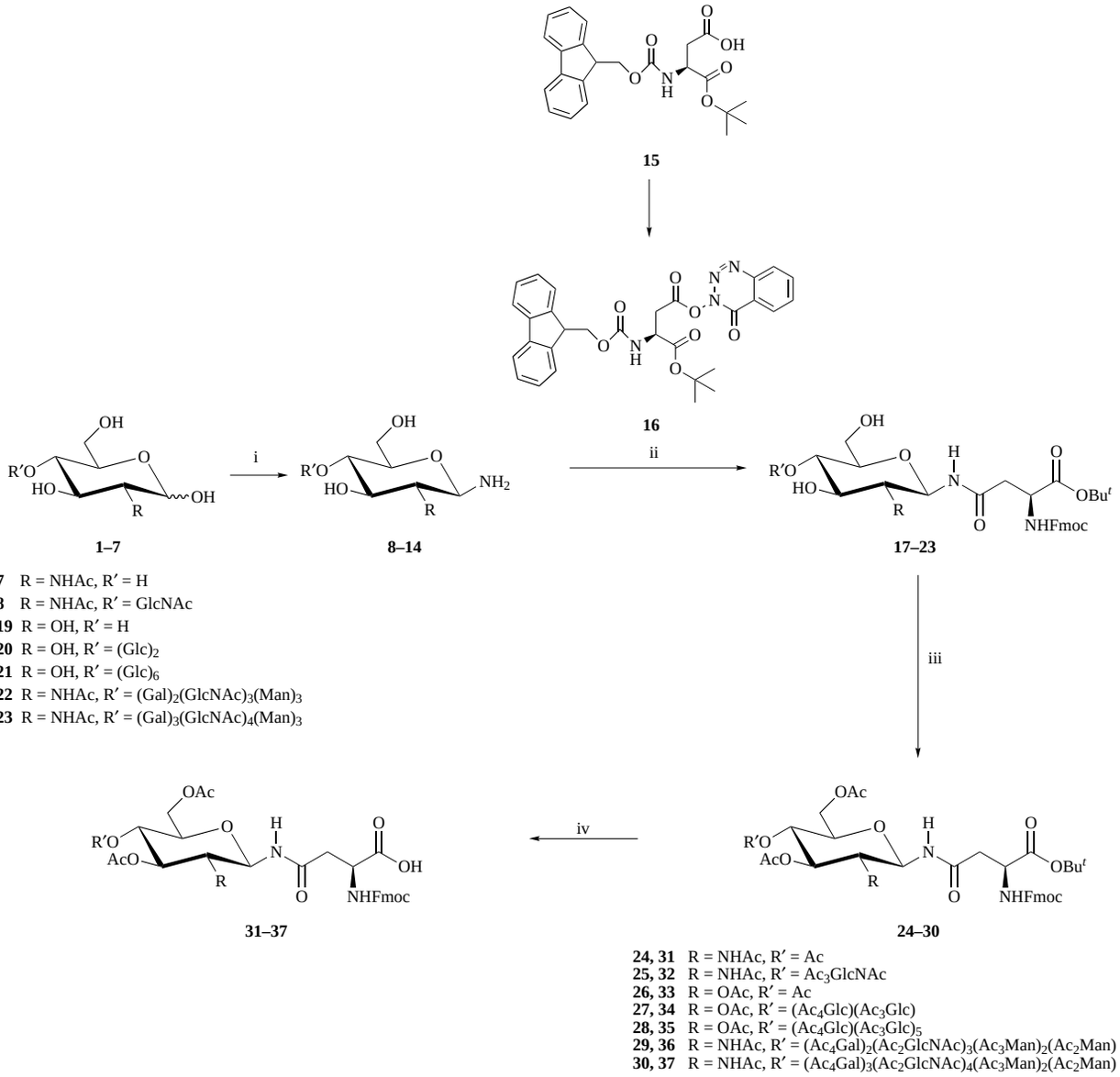
The results of preparative hydrazinolysis of fetuin and ribonuclease B indicate that hydrazinolysis performed under controlled and optimised conditions can be used to release intact, unreduced N-linked oligosaccharides on a preparative scale (100–500 mg) in high overall yields. In the case of fetuin hydrazonolysis the N-linked oligosaccharides could be separated from the small O-linked disaccharides by simple separation on cellulose. The construction of the N-glycosyl linkage by the present method proceeds through conversion of the isolated oligosaccharides into their corresponding glycosylamines followed by coupling with the 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt)-activated side-chain Fmoc-Asp(ODhbt)-OBu^t **16**. The hydroxy groups of the product were O-acetylated and the Bu^t ester protecting group was removed by TFA treatment. The purified N-linked asparagine building blocks **31**–**37** have been directly employed in MCPS. MCPS was performed in a Teflon block with 20 synthesis columns utilizing standard Fmoc-based solid-phase peptide-synthesis procedures. This procedure has previously been shown to be suitable for successful *N*-glycopeptide synthesis. The *N*-glycopeptide syntheses were performed either on Macrosorb or preferably on poly(ethylene glycol)–poly(dimethylacrylamide) copolymer (PEGA) resin.[30] The resin was derivatised by the acid-labile hydroxymethylphenoxyacetic acid (HMPA) by activation with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 4-ethylmorpholine (NEM). The first amino acid, lysine, was coupled as Fmoc-Lys(Boc)-OH by activation with 1-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) and *N*-methylimidazole. Other amino acids were coupled as (pentafluorophenol) OPfp esters with addition of Dhbt-OH as a catalyst.



Scheme 2 Hydrazinolysis to obtain N-linked oligosaccharides as the reducing sugars from the natural glycoprotein sources, bovine fetuin and ribonuclease B

Hydrazinolysis procedure

Hydrazinolysis of glycoproteins on an analytical scale has become a popular method for the isolation of N-linked oligosaccharides.[29,31] The large-scale hydrazinolysis for the release and preparative use of N-linked oligosaccharides in the synthesis of *N*-glycopeptides has not yet been reported. The use of hydrazinolysis on a large scale for the isolation of N-linked oligosaccharides from glycoproteins according to Scheme 2 offers a possibility to obtain larger quantities of N-linked oligosaccharides to be used in synthetic reaction sequences. The hydrazinolysis procedure was performed under controlled and optimised conditions. After exhaustive dialysis and lyophilization of fetuin and ribonuclease B, respectively, 5 g portions were heated with anhydrous hydrazine in a sealed tube by an optimised hydrazinolysis procedure[29] at 85 °C for 12 h. Hydrazine was then removed by repeated condensation to dryness, with several additions of toluene, under reduced pressure. After subsequent N-acetylation with acetic anhydride in a saturated DMSO or aq. sodium hydrogen carbonate solution and ion-exchange chromatography on a Dowex AG-50 X12 column the pool of oligosaccharides was lyophilised. Subjecting the crude product to cellulose column chromatography separated the N-linked oligosaccharides from the peptides and amino acid contaminants and smaller O-linked saccharides (disaccharides). To cleave the formed oligosaccharide hydrazides and residual sialic acids the oligosaccharide pool was first treated with copper(II) acetate for 30 min and then with 25 mM sulfuric acid at 80 °C for 1 h. After column purification the neutral desialyted oligosaccharides were filtered off and lyophilized. Exhaustive ^{1}H NMR spectroscopy, laser-desorption mass spectrometry



Scheme 3 Procedure for the synthesis of the oligosaccharide–asparagine building blocks **31**–**37**. *Reagents and conditions:* i, $(NH_4)HCO_3$; ii, **16** (2 mol equiv.), DIPEA (1.2 mol equiv.), DMSO; iii, Ac_2O–pyridine; iv, TFA (1 h).

(LD-MS) and P-4 gel chromatography studies proved the presence of two structures: the diantennary-complex structure **6** (20%) and the triantennary-complex structure **7** (80%). The hydrazinolysis procedure with ribonuclease B afforded the isolation of a mixture of exclusively high-mannose-type structures from Man_5 to Man_9. P-4 gel chromatography afforded five different high-mannose structures (Man_5 to Man_9) in sufficient quantities for ^1H NMR characterisation. Owing to capacity problems the P-4 gel chromatography method is inadequate for preparative purposes. Therefore the synthesis was continued with the mixture of Man_5–Man_9 high-mannose structures. The isolation and purification of the specific high-mannose structures was performed after coupling to Fmoc-Asp(ODhbt)-OBu^t **16** by semi-preparative high-performance liquid chromatography (HPLC) purification. This provided high overall yields of the desired glycosylated building blocks.

Synthesis of the glycosylated blocks 31–37

The conversion of the unprotected mono- and oligosaccharides **1**–**7** to their corresponding β-glycosylamines **8**–**14** was carried out by modification of the Kochetkov method.[32] The reducing sugars *N*-acetylglucosamine **1**, *N*,*N*-diacetylchitobiose **2**, glucose **3**, maltotriose **4**, maltoheptose **5**, the asialo-fetuin-diantennary structure **6**, the corresponding triantennary structure **7** and the high-mannose oligosaccharide structures (Man_5 to Man_9) of ribonuclease B, were treated with saturated aq. ammonium hydrogen carbonate to produce the corresponding β-D-glycosylamines **8**–**14**. A solution of the saccharide (1%, w/v) in saturated aq. NH_4HCO_3 was kept for 24 h at 45 °C. Conversion of the reducing sugars **1**–**7** into the corresponding β-glycosylamines **8**–**14** was followed by ^1H and ^{13}C NMR spectroscopy in D_2O and the reducing sugar was converted stereoselectively into the β-anomer in all cases. Alternatively, DMSO could be used as a solvent, which increased the yield. The typical strong upfield shift displacement of the anomeric proton H-1 signal for the reducing terminus upon amination was observed. The H-1 of the glycans were all detected at δ ~4.3–4.1. The oligosaccharides were converted into the glycosylamines in high yields (95%) with no detectable formation of di-glycosylamine. After the conversion, the samples were diluted with water, evaporated and this procedure was repeated five times to remove all residual ammonium hydrogen carbonate. The samples were frozen and lyophilized for 24 h. Owing to the instability of the glycosylamines, purification by size-exclusion chromatography is not desirable and, thus, the lyophilized crude products were used directly for coupling to the Fmoc-Asp(ODhbt)-OBu′ derivative **16**. Compound **16** can be easily synthesised by esterification of the commercially

Table 1 Selected ^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) of building blocks measured at 250 MHz on solutions in $CDCl_3$ or [$^2H_6$]DMSO at 300 K

	31[a]	**32**[a]	**33**	**34**	**35**
Asn					
NH	7.53 (8.0)	7.69 (8.2)	6.18	6.21	6.20
H-α	4.78	4.59	5.06	5.05	5.03
H-β	2.88 (6.8, 16.0)	2.89	3.11	3.08	3.07
H′-β	2.70 (7.0)	2.72	2.91	2.89	2.92
Fmoc					
CH_2	4.38 (6.8, 10.5)	4.32	4.53	4.52	4.51
CH_2'	4.31	4.26	4.42	4.38	4.39
H-9	4.22	4.18	4.28	4.41	4.38
NH	8.61 (9.0)	8.70 (9.2)	6.44	6.39	6.41
Glc or GlcNAc					
H-1	5.18 (9.4)	5.3 (9.3)	5.30 (9.5)	5.28 (0.5)	5.29 (9.5)
H-2	3.88 (9.8, 9.4)	4.02	4.96 (9.5)	4.86 (9.2)	4.86 (9.0)
H-3	5.10 (9.6)	5.20	5.38 (9.4)	5.34 (9.5)	5.32 (9.4)
H-4	4.81 (10.0)	3.94	5.11 (9.4)	4.01 (9.5)	4.07 (9.5)
H-5	3.84 (4.0, 2.5)	3.77	3.85 (4.5, 2.0)	3.82 (3.5, 2.0)	3.81 (4.0, 2.0)
H^a-6	4.16 (12.5)	4.24	4.36 (12.5)	4.48 (12.0)	4.41 (12.5)
H^b-6	3.95	3.94	4.12	4.28	4.28
H-1′		4.90 (8.3)		5.38 (3.8)	5.40 (4.0)
H-2′		3.76 (9.2)		4.9 (10.0)	4.92 (10.0)
H-3′		5.37 (9.0)		5.38 (9.8)	5.41 (9.8)
H-4′		5.04 (9.0)		4.15 (10.0)	4.07
H-5′		3.77		3.92	3.89
H^a-6′		4.02		4.28	4.19
H^b-6′		3.87		4.10	4.04
NH	8.21 (9.2)	8.19 (9.0), 8.02 (9.2)			
Ac	1.89 (NHAc), 1.95, 1.98, 200	1.90 (NHAc), 1.94 (NHAc), 2.11, 2.12, 2.15, 2.17, 2.19	1.95, 1.97, 2.00, 2.04	1.92 (2×), 1.95 (2×), 1.97 (3×), 2.00, 2.01, 2.04	1.95 (2×), 1.97 (4×), 1.99 (2×), 2.01 (3×), 2.02 (3×), 2.04 (3×), 2.05 (3×), 2.07 (3×)

[a] ^{1}H NMR spectrum was recorded in [$^2H_6$]DMSO.

available amino acid Fmoc-Asp-OBu′ **15** with Dhbt-OH and *N*,*N*-dicyclohexylcarbodiimide (DCCI) in dry tetrahydrofuran (THF) as previously described for the α-esters.[33] The lyophilized glycosylamines **8**–**14** were dissolved in DMSO, two mol equiv. of *N*,*N*-diisopropylethylamine (DIPEA) were added and the glycosylamines **8**–**14** were then selectively N-acylated with 2 mol equiv. of Fmoc-Asp(ODhbt)-OBu′ **16** at room temp. yielding the glycosylated asparagine derivatives **17**–**23** (Scheme 3). The development of the reaction was followed by TLC and analytical reversed-phase (RP) HPLC. It was found that all the glycosylamines **8**–**14**, even the triantennary glycan from asialofetuin **14**, coupled almost quantitatively to the activated asparagine derivative **16**. The crude coupling mixtures were lyophilised and purified by RP-HPLC. The free hydroxy groups of the sugar moieties were O-acetylated with acetic anhydride–pyridine (1 : 2) for 12 h at 22 °C to afford the peracetylated derivatives. Simple chromatographic filtration on silica gel 60 resulted in the purification of the building blocks **24**–**30**. The Bu′ protection were removed by treatment with neat TGA for 1 h and the final building blocks **31**–**37** were purified by preparative HPLC. They were obtained in overall yields of 45–75%. The yields and the ^{1}H and ^{13}C NMR spectroscopy data of the glycosylated building blocks **31**–**37** are presented in Tables 1 and 2 and in the Experimental section. The unprotected building blocks **38** and **39** were obtained by omission of the O-acetylation procedure. Compounds **31**–**39** were subsequently successfully used as building blocks for the solid-phase synthesis of the *N*-glycopeptides **40**–**46**. The results demonstrated further the stability of the N- and O-glycosidic bonds in the extended sugar chains during the TFA treatment. No cleavage of N- or O-glycosidic bonds in the oligosaccharides was observed during analytical HPLC, ^{1}H NMR and MS studies. It should be mentioned that Fuc-α-(1–6) linkages previously observed not to be stable were not examined. However, in the O-acetylated form of glycans the Fuc moiety is known to be more stable, and glycopeptides containing Fuc can therefore be prepared by the present method.

A similar procedure was performed with the high-mannose structures from ribonuclease B; however, although separated analytically, problems in the preparative separation of the more-than-5 glycoform building blocks were observed as will be reported elsewhere.

Solid-phase synthesis of the *N*-glycopeptides 40–46 by the building-block approach

Building blocks **31**–**39** were used in an efficient and practical solid-phase synthesis of the *N*-glycopeptides **40**–**46** starting from the reducing oligosaccharides **1**–**7**. The building blocks **31**–**39** can be used directly in MCPS of *N*-glycopeptides omitting further chemical transformation on the solid phase. The MCPS was carried out on either Macrosorb or PEGA resin modified with the acid-labile HMPA linker utilizing preactivated Fmoc-amino acid-OPfp esters with Dhbt-OH catalysis and subsequent Fmoc deprotection with 20% piperidine in dimethylformamide (DMF). The glycosylated Fmoc-Asn-OH building blocks were coupled using the coupling reagents TBTU or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and only a small excess of the building blocks (1.1–1.6 mol equiv.) was used. The coupling reagents TBTU and HBTU were both evaluated and it was found that, compared with TBTU, HBTU afforded an overall insignificantly higher coupling yield between the glycosylated Fmoc-Asn-OH building blocks **31**–**39** and the resin-bound peptide. Despite the large size of the heptasaccharide building

Table 2 Selected ^{13}C NMR chemical shifts (ppm) of the building blocks **31**–**35** measured at 62.9 MHz at 300 K on solutions in [$^2H_6$]DMSO or $CDCl_3$

	31[a]	**32**[a]	**33**	**34**	**35**
Asn					
C-α	49.8	50.9	50.2	50.1	50.2
C-β	36.7	37.6	37.7	37.6	37.6
Glc or GlcNAc					
C-1	78.1	78.7	78.2	77.9	77.8
C-2	52.2	53.0	70.8	71.3	71.4
C-3	73.3	74.8	73.7	74.7	74.8
C-4	68.3	71.4	67.8	72.4	71.9
C-5	72.2	76.3	72.5	74.3	74.2
C-6	61.8	62.8	61.4	62.7	62.8
C-1′		100.94		95.8	96.0
C-2′		54.5		70.1	70.2
C-3′		73.1		68.9	69.2
C-4′		69.2		70.9	70.8
C-5′		74.2		68.6	68.4
C-6′		61.9		61.5	61.2
Fmoc					
C-9	47.6	47.5	47.1	47.0	47.0
CH_2	65.9	66.5	67.4	67.5	67.5
C=O	155.8	155.9	155.9	156.0	156.0
Ar	144.5, 141.5, 128.4, 127.8, 126.0, 120.9	144.6, 141.6, 128.5, 127.9, 126.1, 120.9	144.1, 141.7, 127.8, 127.1, 125.0, 120.2	144.2, 141.6, 127.9, 127.3, 125.2, 120.4	144.1, 141.7, 127.8, 127.2, 125.1, 120.3

[a] ^{13}C NMR spectrum was recorded in [$^2H_6$]DMSO.

Table 3 Results of the SPPS of the N-linked glycopeptides **40**–**46** utilising the building blocks **31**–**39**. Comparison of expected and observed molecular mass for the individual glycopeptides **40**–**46** analysed by MALDI and ES-MS

Compound	Molecular formula	Relative molecular mass	ES-MS[a]	Yields (%)[b]
40	$C_{58}H_{95}N_{13}O_{20}$	1294.47	1295.00	76
41	$C_{66}H_{108}N_{14}O_{25}$	1497.68	1498.10	62
42	$C_{56}H_{92}N_{12}O_{20}$	1253.41	1254.17	78
43	$C_{68}H_{112}N_{12}O_{30}$	1577.70	1578.01	62
44	$C_{92}H_{152}N_{12}O_{50}$	2226.27	2227.73	46
45	$C_{112}H_{184}N_{16}O_{60}$	2714.76	2715.41	n.d.
46	$C_{126}H_{207}N_{17}O_{70}$	3080.10	3080.56[c]	35

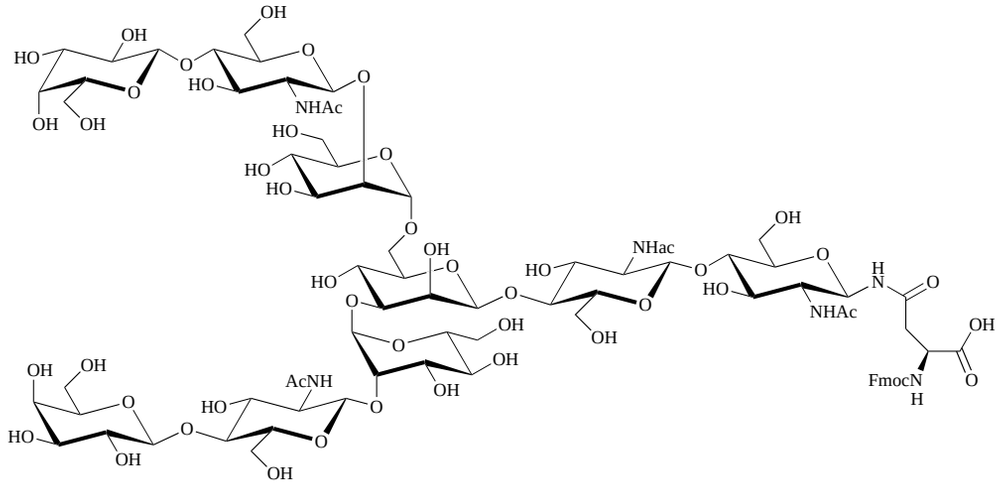
[a] Glycopeptides were detected as [glycopeptide + H]$^+$ ions. [b] Yields were determined based on the loading of the resin (0.2 mmol g^{-1}) and obtained after semipreparative RP-HPLC. [c] Glycopeptide was detected as [glycopeptide + H]$^+$ ions by MALDI-MS. n.d. = not determined.

block **35**, coupling yields were not significantly lower than observed with the smaller building blocks **31**–**34**. In the case of large building blocks **36**–**39** the PEGA resin was used because of its good swelling capacity in DMF and adequate pore size, which provides good access for the large building blocks **36**–**39** to the resin-bound peptide.

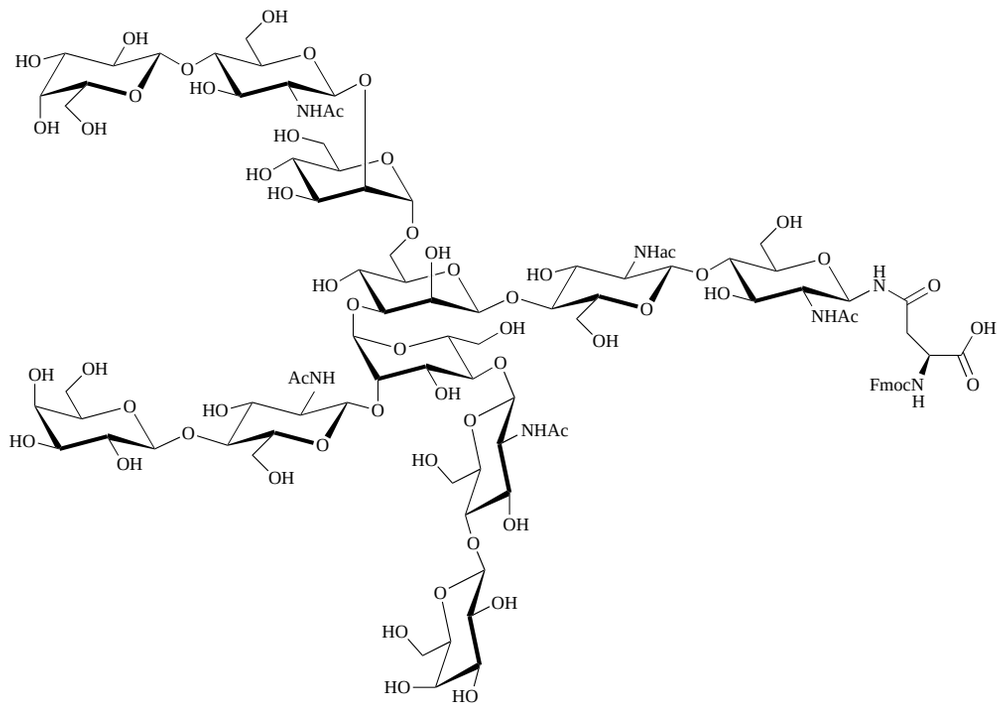
After successful coupling of the glycosylated building blocks **31**–**39** and Fmoc cleavage employing 20% piperidine in DMF, synthesis was completed using Fmoc-amino acid-OPfp esters and Dhbt-OH as an auxiliary nucleophile. The glycopeptide was deprotected and cleaved from the resin by treatment with TFA–water (96 : 5). Removal of the *O*-acetyl groups from the carbohydrate part was achieved by Zemplen O-de-acetylation with sodium methoxide in methanol at pH 8–9. The crude *N*-glycopeptides **40**–**46** were directly subjected to semipreparative RP-HPLC purification. Compounds **40**–**46** were characterised by matrix-assisted laser-desorption time-of-flight (MALDI-TOF), ES-mass spectrometry and 1D and 2D ^1H NMR spectroscopy. After lyophilization the pure *N*-glycopeptides were obtained in good yields from 35% in the case of the tribranched complex oligosaccharide derivative **46** up to 78% for the *N*-acetylglucosamine derivative **42**. The yields are based on the loading of the resin (0.2 mmol g^{-1}) (see Table 3). The solid-phase synthesis of the *N*-glycopeptides **40**–**46** proceeded without problems even with the large building blocks **35**–**39** and no deletion peptides were observed.

Conclusions

In the present work an efficient and practical MCPS protocol which allows the simultaneous and parallel synthesis of the N-linked glycopeptides **40**–**46** with a significant variety of carbohydrate sizes and structures has been described. The large oligosaccharides presented in Fig. 1 were obtained by hydrazinolysis (Scheme 2) of fetuin and ribonuclease B, respectively. The results indicate that hydrazinolysis performed under controlled and optimised conditions can be used to release intact, non-reduced, N-linked oligosaccharides from naturally occurring glycoproteins in overall high yields and purity. The N-linked glycosylated building blocks **31**–**39** can be synthesised by use of the four-step procedure presented in Scheme 3. Starting with the oligosaccharides **1**–**7**, aminolysis, coupling of the glycosylamines to the side-chain of activated Fmoc-Asp(ODhbt)-OBu′, O-acetylation of the carbohydrate moiety, and finally cleavage of the *tert*-Bu ester afforded the building blocks **31**–**39** after final HPLC purification with high purity and yields. The building blocks **31**–**39** have been shown to be well suited for the MCPS of the N-linked glycopeptides **40**–**46**. Furthermore, we have demonstrated the efficient solid-phase assembly of *N*-glycopeptides **40**–**46** by coupling the glycosylated asparagine building blocks **31**–**39** to the resin-bound peptide. TBTU and HBTU were efficient coupling reagents. The N-linked building blocks **31**–**39** were fully compatible with Fmoc-based solid-



38



39

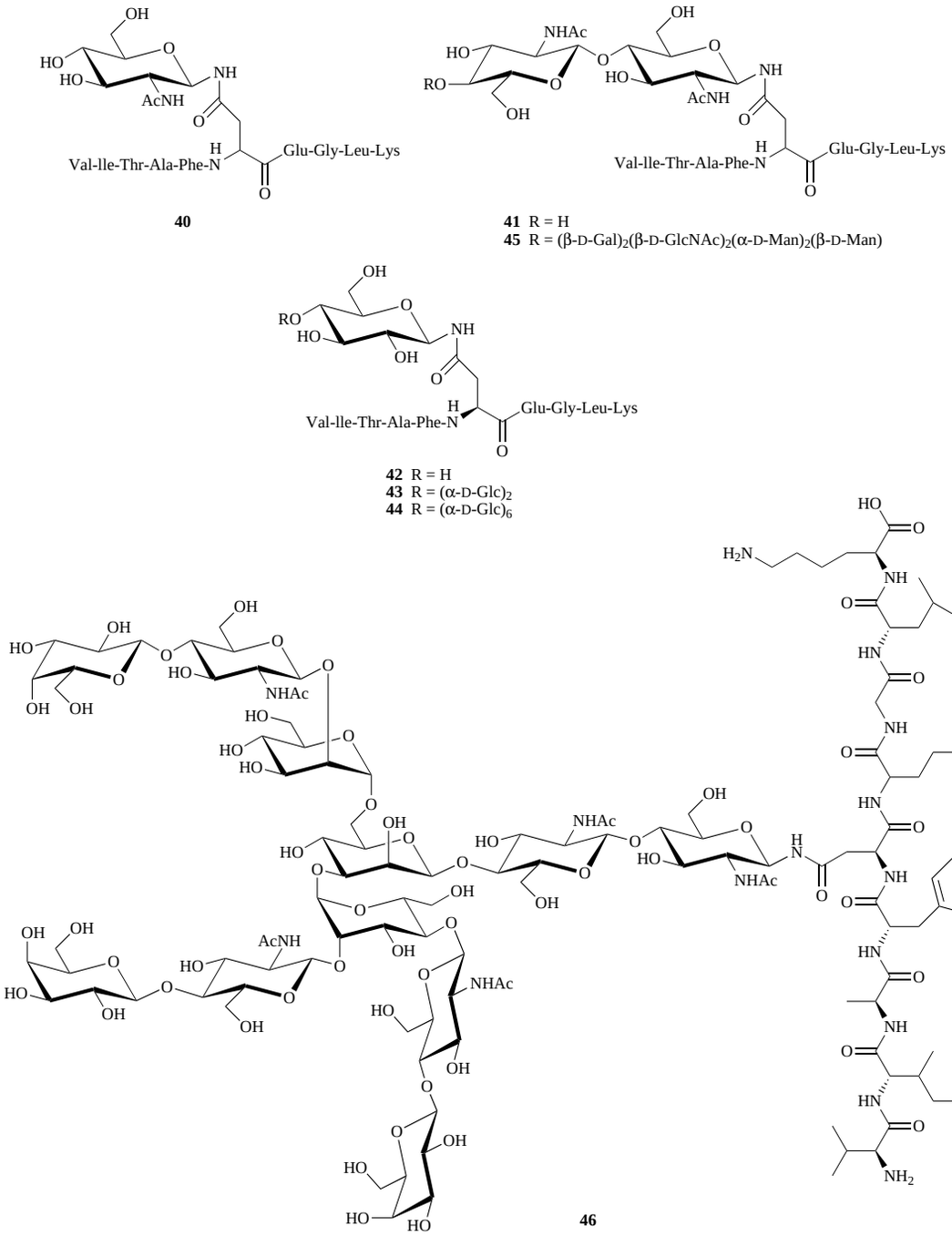
Scheme 4 Structures of di- and tri-antennary building blocks **38** and **39** ready for use in solid-phase peptide synthesis (SPPS)

phase chemistry. The difference in size of the building blocks **31**–**39** did not significantly influence the yields in the solid-phase coupling reactions. PEGA resins with appropriate pore size provide equal access for both small and large building blocks to the site of reaction. Applying the multiple-column glycopeptide synthesis protocol, the *N*-glycopeptides **40**–**46** could be obtained in good yields and with high purity (see Tables 3 and 4). Immunological studies of the *N*-glycopeptides **40**–**46** will be reported elsewhere.

Experimental

General procedures

All solvents were purchased from Labscan Ltd. (Dublin, Ireland). Vacuum liquid chromatography (VLC) was performed on Merck silica gel 60 H (0.040–0.060 mm) and chromatography under dry conditions was performed on dried silica gel (120 °C; 24 h) with solvents dried over molecular sieves. TLC was performed on Merck Silica Gel 60 F254 aluminium sheets with detection by charring with sulfuric acid and by UV light, when applicable. DMF was freshly distilled by fractional distillation at reduced pressure and kept over molecular sieves (3 Å). Dichloromethane was distilled from phosphorus pentaoxide and kept over molecular sieves (3 Å). Pyridine was distilled and kept over molecular sieves (3 Å). Light petroleum was the 60–80 °C fraction. Concentrations were performed under reduced pressure at <40 °C. Suitable protected N^{α}-Fmoc-amino acid-OPfp esters were purchased from Bachem (Bubendorf, Switzerland), the coupling reagents TBTU and HBTU from NovaBiochem (Switzerland) and MSTN from Fluka. Macrosorb SPR 250 resin was from Sterling Organics, NEM, *N*-acetylglucosamine, octa-acetylchitobiose, maltotriose, maltoheptose, fetuin and ribunuclease B from Sigma, Fmoc-Asp-OBu′ from Bachem, Dhbt-OH from Fluka and DIPEA from Sigma. Dowex AG50X 12 (H^+), Dowex AG 3X 4A (OH^-), Chelex 100 (Na^+) and Biogel P4 (400 mesh) were purchased from Bio-Rad (UK). ES-MS was performed in the positive mode on a VG Fisons Quattro Instrument. MALDI-TOF MS was performed on a Finnigan MAT 2000 spectrometer using a matrix of α-cyano-4-hydroxycinnamic acid. ^{1}H and ^{13}C NMR



Scheme 5 N-Linked glycopeptides synthesised by the present method. Peptides were assembled using Fmoc-amino acid-OPfp esters. The products **45** and **46** containing complex glycans could be used as substrates for 1–6-sialyl transferase.

spectra were recorded on Bruker AMX 600 or AM 500 spectrometers. Chemical shifts are given in ppm and referenced to internal $SiMe_4$ (δ_H 0.00) or to external 1,4-dioxane (δ_H 3.76 and δ_C 67.40, respectively) for solutions in D_2O at 300 K. For spectra recorded in CD_3CO_2D–water the HOAc signal at δ_H 2.03 was used as internal reference. For the assignment of signals ^1H, ^1H–^1H chemical-shift correlation spectroscopy (COSY), ^1H–^1H double quantum-filtered phase-sensitive COSY, nuclear Overhauser effect (NOE) in rotating frame (ROESY) and ^1H–^{13}C correlation spectroscopy experiments were used. Analytical and semi-preparative RP-HPLC separations were performed on a Waters HPLC system using switchable analytical RCM (8 × 100 mm) and Delta PAK (15 μm; 300 Å; 25 × 200 mm) C-18 columns with a flow rate of 1 cm^3 min^{-1} and 10 cm^3 min^{-1}, respectively. Detection was at 215 and 280 nm with a photodiode array detector (Waters M 991). Solvent System A: 0.1% TFA in water; B: 0.1% TFA in 90% acetonitrile–10% water.

Synthesis of Fmoc-Asp(ODhbt)-OBu^t 16

Fmoc-Asp-OBu^t **15** (1.5 g, 3.65 mmol) was dissolved in freshly distilled THF (20 cm^3) and the solution was cooled to −35 °C. DCCI (0.752 mg, 3.65 mmol) was added and the mixture was stirred for 10 min. Dhbt-OH (595 mg, 3.65 mmol) was added and the mixture was stirred for 2 h at −35 °C before being allowed to warm up to ambient temperature, filtered through Celite and concentrated. The residue was redissolved in THF (15 cm^3), filtered through Celite and concentrated. Chromatography on silica gel [ethyl acetate–light petroleum (1:4)] yielded the title compound **16** (1.78 g, 88.1%) [Found: $(M + Na)^+$ ES-MS, 579.22. $C_{30}H_{28}N_4O_7$ requires M, 556.7]; δ_H(250 MHz; $CDCl_3$) 8.35–7.15 (H, Fmoc, Dhbt), 6.05 (1 H, NH, NHFmoc), 4.65 (1 H, ddd, H^α), 4.33 (2 H, t, Fmoc CH_2), 4.18 (1 H, m, Fmoc CH), 3.32 (2 H, m, H_2-β), 1.46 (9 H, s, Bu^t); δ_C 135.93, 133.24, 129.48, 127.50, 126.23, 125.66, 120.35 (arom. Fmoc and Dhbt carbons), 67.83 (Fmoc CH_2), 51.16 (C-α), 47.51 (Fmoc CH), 35.02 (C-β) and 28.23 (s, Bu^t).

Table 4 ^{1}H NMR data of the N-linked glycopeptides **40**–**46** measured in CD_3CO_2D–D_2O (1 :) at 600 MHz and 300 K

	40	41	42	43	44	45	46
Val							
NH							
α-H	3.97	3.92	3.93	3.92	3.92	3.93	3.92
β-H	2.24	2.22	2.19	2.18	2.17	2.18	2.23
γ-H_3	1.00,	1.00,	1.00,	1.00,	1.01,	1.01,	1.01,
	1.02	0.99	0.99	0.99	0.99	0.99	1.00
Ile							
NH	8.37	8.40	8.41	8.41	8.40	8.39	8.39
α-H	4.34	4.32	4.32	4.31	4.30	4.32	4.33
β-H	1.82	1.83	1.81	1.80	1.82	1.83	1.83
γ-H_2	1.52/	1.51/	1.50/	1.50/	1.51/	1.52/	1.52/
	1.14	1.14	1.14	1.14	1.15	1.14	1.13
γ-H_3	0.86	0.89	0.88	0.88	0.86	0.89	0.88
δ-H_3	0.91	0.84	0.83	0.84	0.84	0.84	0.85
Thr							
NH	8.21	8.19	8.16	8.17	8.14	8.20	8.21
α-H	4.47	4.41	4.39	4.39	4.38	4.39	4.36
β-H	4.24	4.20	4.18	4.18	4.17	4.21	4.24
γ-H_3	1.14	1.12	1.15	1.14	1.13	1.15	1.14
Ala							
NH	8.05	8.08	8.09	8.08	8.07	8.09	8.02
α-H	4.36	4.41	4.32	4.31	4.35	4.38	4.35
β-H_3	1.29	1.27	1.28	1.28	1.28	1.29	1.27
Phe							
NH	7.99	8.01	8.01	8.02	8.01	7.98	8.00
α-H	4.60	4.56	4.56	4.57	4.57	4.59	4.60
β-H_2	3.14/	3.11/	3.11/	3.12/	3.13/	3.12/	3.11/
	2.99	2.98	2.99	2.99	3.00	2.98	2.99
Asn							
NH	8.23	8.22	8.24	8.23	8.22	8.24	8.21
α-H	4.73	4.67	4.68	4.69	4.69	4.72	4.68
β-H/	2.87/	2.84/	2.91/	2.90/	2.91/	2.90	2.91/
β-H′	2.75	2.71	2.81	2.82	2.82		2.80
N′H	8.47	8.40	8.68	8.68	8.67	8.61	8.58
Glu							
NH	7.98	7.98	8.09	8.09	8.07	8.06	8.08
α-H	4.39	4.34	4.36	4.32	4.36	4.38	4.39
β-H_2	2.18/	2.16/	2.13/	2.13/	2.12/	2.18/	2.12/
	2.00	1.97	1.96	1.95	1.97	1.96	1.95
γ-H_2	2.47	2.45	2.46	2.47	2.49	2.45	2.48
Gly							
NH	8.21	8.22	8.24	8.22	8.23	8.26	8.24
α-H_2	3.97	3.94	3.96	3.92	3.96	3.98	3.99
Leu							
NH	7.87	7.86	7.87	7.88	7.88	7.86	7.88
α-H	4.43	4.39	4.36	4.38	4.36	4.41	4.42
β-H_2	1.62	1.60	1.60	1.61	1.60	1.62	1.61
γ-H	1.63	1.62	1.59	1.58	1.60	1.58	1.60
δ-H_3	0.87/	0.85/	0.90/	0.90/	0.90/	0.90/	0.90/
	0.92	0.90	0.86	0.85	0.87	0.88	0.87
Lys							
NH	8.11	8.15	8.17	8.16	8.17	8.11	8.14
α-H	4.45	4.42	4.38	4.38	4.40	4.46	4.40
β-H_2	1.94/	1.91/	1.90/	1.90/	1.90/	1.90/	1.92/
	1.79	1.76	1.77	1.77	1.75	1.78	1.79
γ-H_2	1.47	1.43	1.42	1.40	1.44	1.46	1.48
δ-H_2	1.72	1.68	1.68	1.68	1.68	1.71	1.70
ε-H_2	3.05	3.01	3.00	2.99	3.01	3.00	3.02
NH_2	7.45	7.47	7.46	7.47	7.46	7.51	7.48
GlcNAc							
1-H	5.07	5.05				5.02	5.04
N-H	7.95	8.01					
2-H	3.88	3.86				3.84	3.88
3-H	3.69	3.77				3.72	3.75
4-H	3.51	3.52				n.d.	3.55
5-H	3.51	3.52				n.d.	n.d.
6-H	3.91	3.81				n.d.	n.d.
6-H′	3.75	3.81				n.d.	n.d.
GlcNAc							
1-H		4.58				4.64	4.64
N-H		8.17					
2-H		3.79				n.d.	n.d.
3-H		3.602				n.d.	n.d.
4-H		3.50				n.d.	n.d.
5-H		3.50				n.d.	n.d.
6-H		3.94				n.d.	n.d.
6-H′		3.74				n.d.	n.d.
Glc							
1-H			4.97	4.98	4.97		
$J_{1,2}$			(8.5)	(8.5)	(8.4)		
2-H			3.43	3.45	3.47		
3-H			3.60	3.59	3.59		
4-H			3.42	3.44	3.41		
5-H			3.51	3.53	3.51		
6-H			3.90	3.88	3.89		
6-H′			3.72	3.71	3.73		
other 1′-H				5.30	5.31		
				(2×)	(6×)		
Man-3						4.77	4.79
Man-4						5.12	5.12
Man-4′						4.94	4.91
GlcNAc-5						4.58	4.58
Gal-6						4.48	4.47
Gal-6′						4.47	4.47
GlcNAc-7							4.55
Gal-8							4.47
NH*Ac*	2.02	2.03,				2.04,	2.04
		2.02				2.05(2),	2.05(2),
						2.07	2.07(2)
ArH	7.30,	7.31,	7.30,	7.31,	7.31,	7.30,	7.31,
	7.25,	7.25,	7.24,	7.25,	7.25,	7.26,	7.26,
	7.22	7.21	7.21	7.21	7.24	7.24	7.22

n.d. = not determined.

Isolation of the N-linked oligosaccharides from fetuin and ribonuclease B by hydrazinolysis

Bovine fetuin or ribonuclease B (5 g) was exhaustively dialysed with a 5–10-kDa-cutoff dialysis membrane against 0.1% (v/v) aq. TFA and was then lyophilised followed by drying *in vacuo* for 96 h in a lyophilisation flask. The sample was then suspended in freshly distilled anhydrous hydrazine (80 cm^3; triple-vacuum-distilled at room temp.) with a water content of <1% v/v [as determined by direct GLC analysis with free induction decay (FID) quantitation]. The hydrazinolysis was performed under controlled conditions of time and temperature[29] in a sealed (melted) glass vessel under argon. The reaction temperature was raised to 85 °C at 10 °C h^{-1} and was then maintained at 85 °C for a further 12 h. Hydrazine was removed by concentration under reduced pressure at 25 °C followed by repeated co-concentration with anhydrous toluene. N-Acetylation of primary amino groups was performed by the addition of a five-fold excess of acetic anhydride (25 cm^3; 0.5 M final concentration) in saturated aq. $NaHCO_3$ (200 cm^3) first at 0 °C for 20 min, followed by further addition of acetic anhydride (25 cm^3) and reaction at ambient temperature for 30–60 min. Sodium ions were removed by passage through a Dowex AG-50 X12 (H^+, 200–400 mesh) column. The water eluate was reduced in volume by rotary evaporation and was then exhaustively lyophilized.

Purification of the oligosaccharides

The combined and freeze-dried fractions were applied to a column of microcrystalline cellulose (E. Merck, Darmstadt, Germany) (1.5 × 12 cm) equilibrated in butan-1-ol–ethanol–water (4:1:1 by volume). The oligosaccharide sample was applied to the cellulose column by dissolution in water (1 vol) and mixing with ethanol (1 vol) on the surface of the cellulose matrix. Butan-1-ol (4 vol) was subsequently added and mixed with the sample components. The peptide impurities were first eluted with 10 column volumes of butan-1-ol–ethanol–water (4:1:1 by vol). Oligosaccharides larger than disaccharides do not elute in the solvent system under these conditions. The oligosaccharide pool was recovered by elution with water (100 cm^3) after using methanol as transient solvent (1 vol). The oligosaccharide samples, determined by the phenol/sulfuric acid assay, were then filtered (0.45 μm Teflon filter) and then lyophilised. To cleave residual oligosaccharide hydrazides the oligosaccharide pool was dissolved in a fivefold molar excess of 22 mM copper(II) acetate in 0.1 M acetic acid and incubated at ambient temperature for 30 min. After passage through a tandem column of Chelex 100 (Na^+) and Dowex AG 50W-X12 (H^+), the oligosaccharide pool was then filtered through a 0.45 μm Teflon filter (Millex SR) and lyophilized. For the cleavage of the residual sialic acids on the fetuin-oligosaccharides the oligosaccharide pool was dissolved in a small amount of 25 mM sulfuric acid and kept for 1 h at 80 °C. This cleavage step was not necessary in the case of the purification of the neutral ribonuclease B oligosaccharide pool. To separate the cleaved sialic acid monosaccharides from neutral oligosaccharides the pool was passed through a column composed of Chelex 100 (Na^+), Dowex AG50X-12 (H^+), Dowex AG3-X4A (OH^-) and QAE Sephadex A-25 (four-layer column). The pool of neutral asialo-fetuin oligosaccharides was finally filtered through a 0.45 μm filter and lyophilized. Samples for ^{1}H NMR spectroscopy were prepared by repeated dissolution in D_2O followed by concentration. Finally, the samples were dissolved in 0.5 cm^3 of D_2O and transferred into an NMR tube. 1D ^{1}H NMR spectra were recorded at 500 MHz at a probe temperature of 300 K.

General method for the preparation of the glycosylamines 8–14

The glycosylamines **8**–**14** were prepared using a modification of the method by Likhosherstov *et al.*[32] The parent saccharides **1**–**7** were dissolved in saturated aq. ammonium hydrogen carbonate (10 mg/1 ml) and the solution was stirred at 45 °C for 1–2 days. Solid NH_4HCO_3 was added in fractions during the course of glycosylamine formation to ensure saturation. The amine formation was followed both by TLC (propan-1-ol–ethyl acetate–water, 6:1:3, detection with ninhydrin reagent) and ^{1}H and ^{13}C NMR spectroscopy in D_2O. In all cases conversions were found in the range of 80–90% by comparison of the anomeric 1-H signal of the exclusively β-glycosylamine with the anomeric 1-H signals of the reducing half acetals. After the successful conversion into the glycosyl amines **8**–**14** it was diluted with water and concentrated to half the volume. This procedure was repeated 5 times until no further ammonia could be detected. The glycosylamines **8**–**14** were repetitively frozen, lyophilized and dissolved. Gravimetrically determined yields were almost 100%. According to ^{1}H NMR spectroscopy yields were around 80–90%; however, yields determined in D_2O may be artificially low due to hydrolysis of the glycosyl amines **8**–**14** in aqueous solution. Formation of the diglycosylamine, a typical side-reaction during the amination procedure, was not observed by ^{1}H and ^{13}C NMR spectroscopy. The amination reaction yields stereospecifically the β-glycosylamines **8**–**14** as white, amorphous solids which could be stored at −20 °C under anhydrous conditions. Yields and analytical data were determined from ^{1}H NMR spectra in D_2O and are calculated on the integral ratio for the anomeric proton of the glycosyl amines. Owing to the instability of the glycosylamine in aqueous solutions the amination procedure was alternatively performed in DMSO. The oligosaccharide was dissolved in DMSO (20 mg/5 cm^3) and ammonium hydrogen carbonate was added until saturation. The mixture was kept for 24–28 h at 45 °C. Solid NH_4HCO_3 was added frequently to ensure saturation. The conversion was followed by ^{1}H NMR analysis in [$^2H_6$]DMSO. After complete conversion the solution was lyophilised. Utilizing DMSO prohibits the hydrolysis of the glycosylamines in aqueous solutions. In the case of very unstable glycosylamines the use of DMSO as a polar solvent system may prohibit the hydrolysis of already formed glycosylamines. Overall higher yields and more reliable NMR spectra could be obtained avoiding aqueous solutions for the preparation of glycosylamines.

General procedure for the syntheses of the N-linked building blocks 31–37

In a typical experiment, *N*,*N*′-diacetylchitobiosylamine **9** (200 mg, 472 μmol) was dissolved in DMSO (10 cm^3) and DIPEA (97 μl, 566 μmol, 1.2 mol equiv.) was added. The mixture was stirred at ambient temperature for 10 min and Fmoc-Asp(ODhbt)-OBu′ (262 mg, 472 μmol, 1 mol equiv.) was added subsequently. The solution turned immediately deep yellowish, indicating that the coupling reaction occurred instantaneously. The coupling reaction was monitored by RP-HPLC and TLC [chloroform–methanol–water (10:4:1)]. After complete disappearance of the starting glycosylamine **9** (30 min), the crude mixture was directly lyophilized to remove DMSO. The crude mixture was stirred for 6 h with an excess of acetic anhydride in pyridine (1:2) at ambient temp. to obtain the O-acetylated building block **25**. This was concentrated and co-concentrated with toluene and purified by column chromatography on silica gel 60 [VLC:$CHCl_3$–MeOH (30:1 ⟶ 10:1)]. The Bu′-ester was removed by treatment with neat TFA for 1 h at ambient temp. Finally, preparative RP-HPLC [gradient 70:30 (A:B) for 5 min, then to 0:100 (A:B) in 60 min] yielded the building block **32** in an overall yield of 65% (based on *N*,*N*′-diacetylchitobiose **2**).

Synthesis of the building blocks **31** and **33**–**37** was achieved similarly. In the case of the large oligosaccharide building blocks **35**–**37** O-acetylation with acetic anhydride and pyridine was performed at 50 °C for 12 h. Final purification of the building blocks was obtained by preparative RP-HPLC [gradient 70:30 (A:B) for 5 min, then to 0:100 (A:B) in 60 min]. The pure glycosylated N-linked building blocks **31**–**37** were fully characterised by electrospray mass spectroscopy (ES-MS) and ^{1}H and ^{13}C NMR spectroscopy. The retention times by analytical HPLC [gradient: 90:10 (A:B) for 5 min, then to 0:100 (A:B) in 30 min] were Fmoc-Asn(Ac_3GlcNAc)-OH **31**, t_R 21.33 min, Fmoc-Asn(Ac_3GlcNAcAc_2GlcNAc)-OH **32**, t_R 21.08 min, Fmoc-Asn(Ac_4Glc)-OH **33**, t_R 20.25 min, Fmoc-Asn($Ac_{10}Glc_3$)-OH **34**, t_R 26.25 min, Fmoc-Asn($Ac_{22}Glc_7$)-OH **35**, t_R 29.03 min. The purified N-linked building blocks **31**–**37** were fully characterized by ES-MS, and ^{1}H and ^{13}C NMR spectroscopy. ^{1}H and ^{13}C NMR data are presented in Tables 1 and 2, respectively.

Derivatisation of the mixture of the tri- and the di-antennary asialo-fetuin oligosaccharides with Fmoc-Asp(ODhbt)-OBu′ 16

The isolated and purified asialo-fetuin oligosaccharide fraction (20 mg, 10 μmol) was analyzed by Bio-Gel P-4 gel permeation chromatography and laser desorption mass spectrometry (LD-MS) using a Finnigan MAT laser desorption mass spectrometer. It was determined that the oligosaccharide fraction exclusively contained 20% of the dibranched complex structure **6** and 80% of the tribranched complex structure **7**. The mixture of the fetuin oligosaccharides **6** and **7** was dissolved in water (2 cm^3) and ammonium hydrogen carbonate (1.5 g) was added. The mixture was kept for 30 h at 45 °C. The formation of the oligosaccharide-glycosylamine was carefully monitored by ^{1}H NMR spectroscopy by the disappearance of the reducing α/β 1-H signals of the reducing oligosaccharides and the characteristic formation of the 1-H proton of the β-glycosylamine at δ_H

4.12 with the coupling constant of $J_{1,2}$ 8.3 Hz. The reaction was judged complete when the reducing oligosaccharide had been nearly consumed as determined by ^{1}H NMR analysis. The reaction mixture was then diluted with water (5 cm^3) and concentrated. This procedure was repeated five times and the formed oligosaccharide-glycosyl amine was re-dissolved in water (2 cm^3) and lyophilized several times. This procedure yielded quantitatively the glycosyl amines **13** and **14** (20 mg). The derivatization of the dibranched and the tribranched glycosylamines **13** and **14** was accomplished by dissolution of the oligosaccharide–glycosylamine mixture (20 mg) in DMSO (2 cm^3), adding of DIPEA (1.2 mol equiv.) and twice molar excess of Fmoc-Asp(ODhbt)-OBu^t **16**. The reaction was allowed to proceed at ambient temp. for 3 h. The formation of the oligosaccharide-amino acid building blocks **22** and **23** was monitored by analytical HPLC and TLC [ethyl acetate–methanol–water (2:3:3)]. When the reaction was completed, the mixture was lyophilized exhaustively. Separation of the derivatised oligosaccharides from the asparagine amino acid derivates was achieved by preparative HPLC [0→5 min 100:0 (A:B), then from 5→65 min to 50:50 (A:B) and from 65→85 min to 0:100 (A:B)] detected by absorption at 215 and 280 nm. The two N-linked oligosaccharides **22** and **23** eluted separately as single peaks and their purity was confirmed by analytical HPLC [0→5 min to 100:0 (A:B), then 5→35 min to 50:50 (A:B) and then 35→45 min to 0:100 (A:B). They were pooled separately and freeze dried]. Each of the major peaks were chromatographed again on analytical RP-HPLC and analyzed by ^{1}H NMR spectroscopy. Chromatographic data (HPLC retention time) t_R and yields after preparative HPLC. Analytical HPLC [gradient: 100:0 (A:B) for 5 min, 50 to 50 (A:B) in 35 min and then 0 to 100 in 10 min] Fmoc-Asn($Gal_2GlcNAc_2Man_3GlcNAc_2$)-OBu^t **22** t_R 16.06 min, Fmoc-Asn($Gal_3GlcNAc_3Man_3GlcNAc_2$)-OH **23** t_R 14.38 min. For the synthesis of the N-linked glycopeptides **45** and **46** containing the dibranched and the tribranched complex oligosaccharides, respectively, two different synthesis strategies were utilized.

The first strategy used the peracetylated building blocks **36** and **37**. The separated oligosaccharide-asparagine derivatives **22** (2 mg) and **23** (8 mg) were acetylated with pyridine–acetic anhydride (1:1; 2 cm^3) at 50 °C for 12 h. The mixtures were concentrated and co-concentrated with toluene and analyzed by analytical HPLC and ^{1}H NMR spectroscopy. Both techniques showed that complete O-acetylation of the building blocks **22** and **23** could not be achieved. The analytical HPLC showed a mixture of not-completely acetylated products **29** and **30**. However, the synthesis was continued by cleaving the *tert*-Bu ester of the oligosaccharide-asparagine derivatives **29** and **30** with treatment with neat TFA for 1 h. TFA was evaporated and it was co-evaporated with toluene and the compound was purified by semipreparative HPLC. The not completely acetylated building blocks **36** (2.6 mg) and **37** (12 mg) were, however, used in the synthesis of the glycopeptides **45** and **46**. Owing to the difficulties in peracetylating the oligosaccharide-asparagine building blocks **22** and **23** another strategy was followed utilizing building blocks with completely unprotected carbohydrate moieties.

In this second approach the unprotected oligosaccharide-asparagine derivatives **22** and **23** were purified and subsequent directly subjected to TFA treatment. Compounds **22** (2 mg) and **23** (8 mg) were kept in neat TFA for 1 h, respectively, in order to cleave the *tert*-Bu ester. Purification by semipreparative HPLC afforded the diantennary- and triantennary-complex building blocks **38** (1.2 mg) and **39** (5.2 mg), respectively. The pure oligosaccharide-asparagine building blocks **38** and **39** were characterized by ES-MS and ^{1}H NMR spectroscopy. HPLC retention times t_R were in analytical semipreparative HPLC [gradient: 100:0 (A:B) for 5 min, 50:50 (A:B) in 35 min and then 0:100 in 10 min]: Fmoc-Asn($Gal_2GlcNAc_2Man_3GlcNAc_2$)-OBu^t **38** t_R = 12.06 min, Fmoc-Asn($Gal_3GlcNAc_3Man_3$-$GlcNAc_2$)-OH **39** t_R 10.38 min. All four building blocks **36**–**39** have been used in two different approaches for the synthesis of the N-linked glycopeptides **45** and **46**.

Syntheses of the N-linked glycopeptides 40–46 (table 4): preparation of the macrosorb SPR 250 and PEGA resins

Syntheses of the glycopeptides **40**–**46** were performed in DMF on Macrosorb SPR 250 or alternatively PEGA-resin.[30] The resin (5 g, loading 0.2 mmol g^{-1}) was packed into a 50 cm^3 disposable syringe (Discardit II, Beckton Dickinson) fitted with a Teflon filter. The syringe was connected to a suction flask through a Teflon tube with a manual 2-way Teflon valve and the resin was swelled in DMF (20 cm^3) for 30 min. Excesses of reagents and DMF were removed by applying vacuum to the outlet of the syringe. The resin was derivatized with the HMPA-linker: The HMPA-linker (455 mg, 2.5 mmol), TBTU (750 mg, 2.37 mmol) and NEM (315 µl, 2.5 mmol) were dissolved in DMF (20 cm^3) and after 5 min added to the resin. After 2 h the resin was washed carefully with DMF (5 × 20 cm^3) and dichloromethane (5 × 10 cm^3). Fmoc-Lys(Boc)-OH (1.75 mg, 3.75 mmol) and *N*-methylimidazole (231 mg, 223 mm^3, 2.81 mmol) were dissolved in dichloromethane (20 cm^3) and MSNT (1.11 g, 3.71 mmol) was added. After 5 min the solution was added to the resin and the mixture was kept for 3 h. The resin was washed thoroughly with dichloromethane (5 × 10 cm^3) and then with DMF (5 × 10 cm^3), and unreactive amino groups of the resin were acetylated with a solution of 20% acetic anhydride in DMF (10 cm^3). After 20 min the resin was washed with DMF (5 × 10 cm^3) and diethyl ether (5 × 10 cm^3) and dried *in vacuo*.

General procedures for the solid-phase peptide synthesis (SPPS) of the N-linked glycopeptides 40–44

The derivatised resin was transferred into a 20-column custom-made Teflon reactor. (100 mg resin per column, loading 0.2 mmol g^{-1}). Throughout the synthesis N^{α}-Fmoc deprotection was effected by successive 2 min and 20 min treatments of the resin with 20% piperidine in DMF (1 cm^3/column). The washing procedure (10 vol of DMF) was repeated after each coupling/Fmoc deprotection step. Each batch of N^{α}-Fmoc-amino acid-OPfp ester (3 mol equiv.) and Dhbt-OH (1 mol equiv.), added as an auxiliary nucleophile, was dissolved in DMF (0.6 cm^3). In the case of the N-linked glycosylated asparagine building blocks just 1.1 mol equiv. were employed and coupled to the free amino group by activation with TBTU (1.5 mol equiv., this was found to be optimal) and DIPEA (1 mol equiv.). The solutions were added to the resins and then left for 4 h. In the case of the N-glycosylated building blocks **31**–**37** the reaction times were elongated to 12 h. After each coupling step the resin was washed, the Fmoc group removed, and the resin washed as described above. After coupling of the last amino acid, valine, the terminal Fmoc group was removed, the resin was washed successively with DMF (10 vol) and diethyl ether (10 vol), and dried *in vacuo*. Cleavage of the N-glycopeptides from the HMPA-linker and simultaneous amino acid deprotection were performed by treatment with 95% aq. TFA for 2 h. After cleavage, the soluble product filtered from the resin and the resin was washed three times with 95% aq. TFA. The combined filtrates were concentrated, then co-concentrated with toluene, and the glycopeptides were then precipitated by several triturations with diethyl ether. Residual solvent was removed under reduced pressure and the acetylated glycopeptides were dissolved in dry methanol (2 mg cm^{-3}) and O-deacetylated by addition of a catalytic amount of 1% sodium methoxide in methanol until pH paper indicated pH 8.5. The mixture was then stirred for 4–8 h at room temp., treated with solid CO_2, and concentrated. The crude glycopeptides were dissolved in water and purified by semipreparative HPLC, using a linear gradient 0–50% B in 50 min and then 50–100% in 10 min. The purified *N*-glycopeptides **40**–**44** were then

lyophilized from water and fully characterized by ES-MS and 1D and 2D ^{1}H NMR spectroscopy. Yields and ES-MS data are presented in Table 3. ^{1}H NMR data of the *N*-glycopeptides **40**–**44** are presented in Table 4.

Synthesis of the di- and tri-antennary N-linked glycopeptides 45 and 46

For the synthesis of the glycopeptides **45** and **46** the PEGA resin with the acid-labile HMPA linker was employed due to its better swelling and pore-size properties.[30] The PEGA resin (500 mg, 0.25 mmol g^{-1}) was placed in a syringe and allowed to swell in DMF (5 cm^3). After treatment of the resin with piperidine in DMF (20%) for 20 min, the resin was rinsed thoroughly with DMF (10 vol), dried *in vacuo*, and derivatized with the HMPA linker. HMPA (45.5 mg, 250 μmol), TBTU (75 mg, 237 μmol) and NEM (63 mm^3, 500 μmol) were dissolved in DMF (3 cm^3) and added to the resin after 5 min. After 2 h the resin was washed successively with DMF (20 vol) and dichloromethane (10 vol) and dried *in vacuo* for 24 h. Fmoc-Lys(Boc)-OH (175 mg, 375 μmol), *N*-methylimidazole (23.1 mg, 22.3 mm^3, 281 μmol) and MSNT (111 mg, 371 μmol) were dissolved in dichloromethane (3 cm^3) under argon. After 5 min the solution was added to the PEGA-resin and the whole was kept for 1 h. The resin was washed successively with dichloromethane (10 vol) and DMF (10 vol) and unchanged amino groups of the resin were acetylated with 20% acetic anhydride in DMF. The resin was washed successively with DMF (10 vol) and dichloromethane (10 vol) and dried *in vacuo*. The level of incorporation of the first amino acid lysine was determined by measuring the absorbance, at 290 nm, of the dibenzofullvene (= Fm)-piperidine adduct and comparing this with a standard curve. The subsequent amino acids were coupled as their Pfp esters (3 mol equiv.) with Dhbt-OH (1 mol equiv.) as auxiliary nucleophile. The resin was thoroughly dried *in vacuo* prior to the coupling of the di- and tri-antennary asparagine-building blocks **36**–**39**. The acetylated building blocks **36** and **37** were dissolved in 0.5 and 1 cm^3 of DMF, respectively. TBTU (1.5 mol equiv.) and DIPEA (1 mol equiv.) were added. The mixture was added to the resin and the whole was kept for 12 h. After rinsing with DMF (10 vol) the peptide synthesis was continued as described above. Cleavage of the glycopeptide from the resin was achieved with 95% aq. TFA for 2 h. The product solution was filtered from the resin, evaporated, co-evaporated with toluene, and the glycopeptide was precipitated by trituration with diethyl ether. The acetylated glycopeptides were dissolved in dry methanol (1 mg cm^{-3}) and O-deacetylated by addition of a catalytic amount of sodium methoxide (1%) in methanol at pH 9.0. The mixtures were stirred for 12 h at room temp., neutralized by addition of solid CO_2, and concentrated. The residue was dissolved in water and purified by semipreparative HPLC, using a linear gradient 0–50% B in 50 min and then 50–100% B in 10 min. The purified glycopeptides **45** and **46** were then characterized by MALDI-MS and ^{1}H NMR spectroscopy.

In the second approach the unprotected oligosaccharide-asparagine building blocks **38** and **39** were directly utilized in the MCPS. The building blocks **38** and **39** were dissolved in 0.5 cm^3 and 1 cm^3 of a DMF–DMSO (1 : 1) solution, respectively. DIPEA (1 mol equiv.) and TBTU (1.4 mol equiv.) were added and the solutions were added to the resin. This mixture was kept for 16 h and was then rinsed with DMF (10 vol). The peptide synthesis was continued as described above. Cleavage and deprotection of the glycopeptides **45** and **46** was achieved with 95% aq. TFA. The crude glycopeptides **45** and **46** were dissolved in water and directly purified by semipreparative HPLC using a linear gradient 0–50% B in 50 min and then 50–100% B in 10 min. The purified glycopeptides **45** and **46** were then characterized by MALDI-MS and ^{1}H NMR spectroscopy. The partial assignment of the ^{1}H NMR data for glycopeptides **45** and **46** is presented in Table 4.

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