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Silyl Protection in the Solid-Phase Synthesis Of *N*-Linked Glycopeptides. Preparation of Glycosylated Fluorogenic Substrates for Subtilisins

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Abstract—The trimethylsilyl (TMS) group was used for protection of the hydroxy groups of three disaccharide 1-amino-alditols and of the glycosylamines of glucose, maltotriose and maltoheptose. The per-*O*-trimethylsilylated derivatives were coupled with N^{α}-Fmoc-Asp(Cl)-OPfp **7** to give six glycosylated building blocks for the solid-phase synthesis of *N*-linked glycopeptides. Building block **8** was used in the synthesis of five internally quenched fluorescent substrates which were studied by enzymatic hydrolysis with savinase, a subtilisin-type enzyme.

Introduction

It is a general observation that proteolytic cleavage in the proximity of *O*- and *N*-linked carbohydrate moieties is slow or not possible.[1-6] The increased stability of glycoproteins towards proteases may be caused by the presence of spatially bulky carbohydrate moieties masking peptide fragments of the protein that would otherwise be accessible to degrading enzymes. Therefore, it would be of interest to evaluate the influence of a carbohydrate moiety close to the cleavage site.

Both *O*- and *N*-linked glycopeptides have been most effectively synthesized by a building block strategy, in which glycosylated amino acids are activated and coupled to a peptide linked to a solid support.[7] In solution synthesis of small glycopeptides, benzyl protection of the saccharide has often been employed, but since reductive cleavage is not compatible with all amino acids, the use of acyl protecting groups, which may be removed under mild basic conditions, has prevailed in the solid phase synthesis of glycopeptides. However, when using base treatment, low solubility of the intermediates and products is recurrently a crucial problem, in particular with multiple techniques, such as Multiple Column Peptide Synthesis (MCPS).[8] In the synthesis of *N*-linked glycopeptides, omission of the saccharide protection has been reported,[9-11] but this strategy is also not without problems.[7]

We have previously reported a general strategy employing acetyl or benzoyl protection of the saccharide in glycosylation of N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc) amino acid pentafluorophenyl (Pfp) esters[12,13] and direct incorporation of the well characterized products in complex glycopeptides.[8,14] Acetyl protection has also been used for the incorporation of mono- and disaccharide glycosylamines,[15] however, an attempt to apply this method to 1-amino-alditols[16] was not successful due to $O \rightarrow N$ acyl migration to the more basic primary amino group. In a previous communication[17] the strategy of using fully protected glycosylated amino acid building blocks for glycopeptide synthesis was extended by the use of easily removable trimethylsilyl (TMS) protection of a 1-amino-1-deoxy-D-glucitol moiety allowing a single acid cleavage and deprotection step in the synthesis of *N*-linked neoglycopeptides. The TMS groups were found to be stable in *N*,*N*-dimethylformamide (DMF), 20 % piperidine in DMF and 50 % morpholine in DMF, conditions which are often used in Fmoc-based solid-phase glycopeptide synthesis.

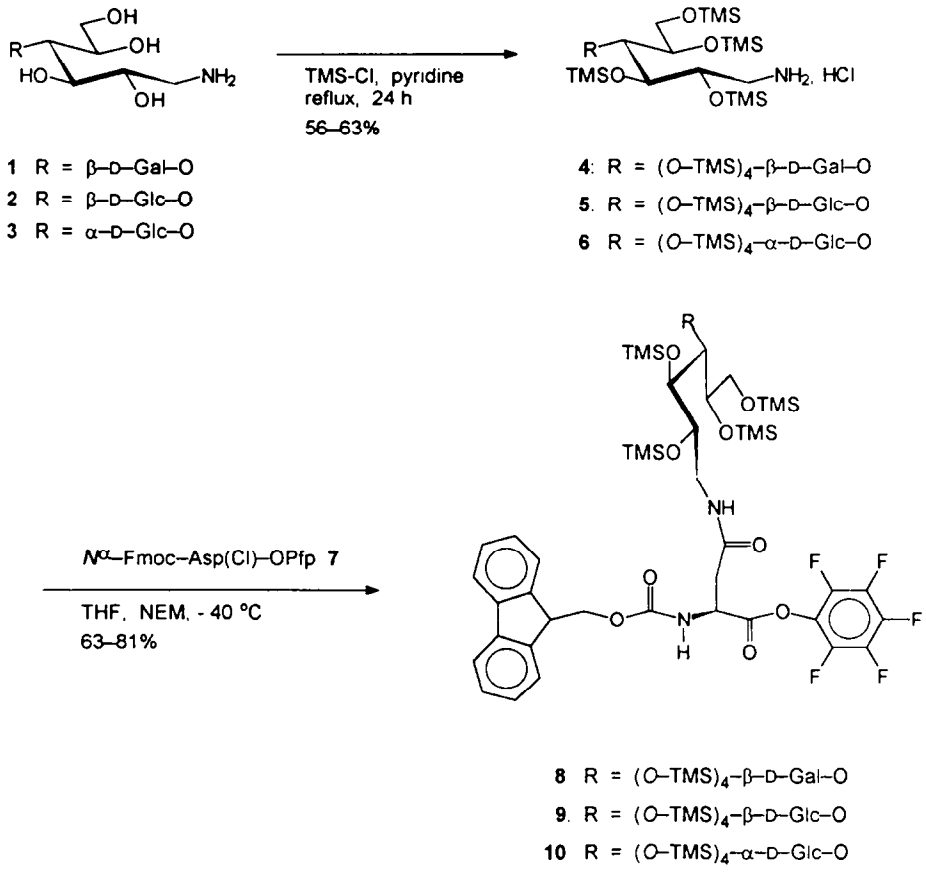
Silyl ethers, and TMS-ethers in particular, have been used extensively for the protection of hydroxy groups in organic synthesis,[18] and TMS-,[19] or more sterically crowded alkylsilyl-derivatives[20,21] of almost any substance, have been widely used in analysis by gas–liquid chromatography and mass spectrometry. In recent years several methods and reagents for silylation have been developed. The TMS group is easily removed by solvolysis in protic media, either in the presence of acid or base, whereas the more sterically crowded *tert*-butyldimethylsilyl group[22,23] offers stability under a wide range of conditions, yet may be removed easily by highly specific reagents, such as fluoride. For the TMS-protection of simple carbohydrate derivatives, a mixture of chlorotrimethylsilane (TMS-Cl) and hexamethyldisilazane in pyridine is generally used.[24] For less reactive compounds a recent procedure[25] describes the facile silylation of carbohydrates and sterically hindered alcohols using *N*,*O*-bis-trimethylsilylacetamide in pyridine with the addition of a catalytic amount of tetrabutylammonium fluoride (TBAF), a reagent which may be employed for the removal of TMS groups. For both of these methods high yields of the per-*O*-silylated products are reported and the work-up procedures are simple extraction and evaporation. The use of the TMS group for protection in glycopeptide synthesis has been further extended in the present work to disaccharide 1-

amino-alditols and, more importantly, to glycosylamines. The importance of few synthetic steps is crucial, especially in the case where only minute amounts of oligosaccharides are available for incorporation into glycopeptides. The versatility of using TMS groups as an easily introduced and easily removed protecting group also for larger oligosaccharide glycosylamines has therefore been examined and the results are reported here. We also report the synthesis of a series of internally quenched fluorescent protease substrates[26,27] carrying a disaccharide lactitol moiety. The glycosylated substrates as well as the unglycosylated control substrates were treated with savinase, a subtilisin-type protease, and the influence of the carbohydrate moiety near the cleavage site was evaluated.

Results and Discussion

Synthesis

It was previously reported that treatment of 1-amino-1-deoxy-D-glucitol with TMS-Cl in pyridine at room temperature gave penta-*O*-trimethylsilylated 1-amino-1-deoxy-D-glucitol hydrochloride in 94 % yield.[17] However, when the disaccharide 1-amino-alditols **1–3** were treated similarly, only partially silylated products were obtained. Instead, the crude alditols **1–3**, which are very hygroscopic compounds, were stirred with dry diethyl ether in order to extract most of the water that could be bound in the syrupy material. Upon decantation of the diethyl ether the alditols were treated with TMS-Cl in pyridine under reflux for 24 h and the octa-*O*-trimethylsilylated amino-alditol hydrochlorides **4–6** were isolated in ~60 % yield. The moderate yields may be due to the fact that the starting material contained more water than expected. It should be noted here, that since TMS groups are easily cleaved by acids, traces of hydrogen chloride (HCl) in the deuterated chloroform ($CDCl_3$) used for NMR spectroscopy lead to cleavage and to spectra, which may not reflect the actual distribution of products in the reaction mixture. As a general rule, $CDCl_3$ used in the present work was therefore filtered through activated alumina B immediately before use. Compounds **4–6** were used in a coupling reaction with N^{α}-Fmoc-Asp(Cl)-OPfp **7** to give the three building blocks **8–10**. The outcome of this coupling reaction was quite dependent on the experimental conditions. Thus, a solution of the amine in dry tetrahydrofuran (THF) containing two equiv. of *N*-ethyl-morpholine (NEM), to trap liberated HCl, was cooled to –40 °C and a solution of **7** in dry THF was added drop-wise from a separating funnel. The precipitation of *N*-ethyl-morpholine, hydrochloride (NEM•HCl) indicated an instantaneous reaction. The reaction mixture was allowed to warm to 20 °C and NEM•HCl was removed by filtration. The filtrate was concentrated and passed through a short column of dry silica gel to give the building blocks **8–10** in 56–81 % yield (1.5–2.5 g amounts) as syrupy compounds, the structures of which were confirmed by NMR spectroscopy and mass spectrometry. During the



1 R = β-D-Gal-O
2 R = β-D-Glc-O
3 R = α-D-Glc-O

4: R = (O-TMS)$_4$-β-D-Gal-O
5: R = (O-TMS)$_4$-β-D-Glc-O
6 R = (O-TMS)$_4$-α-D-Glc-O

8 R = (O-TMS)$_4$-β-D-Gal-O
9: R = (O-TMS)$_4$-β-D-Glc-O
10 R = (O-TMS)$_4$-α-D-Glc-O

Scheme I.

chromatographic purification partial hydrolysis of the TMS groups occasionally occurred resulting in only moderate yields. Compound **8**, carrying a lactitol moiety, was subsequently used in the synthesis of the glycosylated peptide substrates **25**, **27**, **29**, **31** and **33** (see Table 2).

Glycosylamines of mono- and disaccharides have been protected with acetyl groups and incorporated into glycopeptides,[15] however, extensive chromatographic purification was required after most of the synthetic steps. It would be of great advantage if the TMS group also could be employed for the protection of glycosylamines. Treatment of β-D-glycosylamines with TMS-Cl in pyridine always led to formation of several products and therefore another silylating agent *N*,*O*-bis-trimethylsilylacetamide, which recently was used for the preparation of trimethylsilyl ethers of carbohydrates,[25] was tested. Treatment of **11** in pyridine with 1.5 mol *N*,*O*-bis-trimethylsilylacetamide per mol hydroxy group at room temperature for 24 h gave quantitative conversion to the tetra-*O*-trimethylsilylated product **14**. The work-up procedure included quenching of excess *N*,*O*-bis-trimethylsilylacetamide with methanol followed by addition of hexane and extraction of the organic phase with water. Concentration of the organic phase gave **14** in 84 % yield and coupling with acid chloride **7** gave the TMS-protected glycosylated asparagine pentafluorophenyl ester **17** in 56 % yield. As for compounds **8**–**10** the final silica gel chromatography may have been the cause for the rather low yield, since loss of TMS groups led to retention on the silica gel. Compound **17** was characterized by mass spectrometry and NMR spectroscopy and the ^{13}C NMR spectrum is presented in Figure 1. In order to investigate the possibility of using TMS groups for the protection of larger oligosaccharide structures, maltotriose and maltoheptose were converted into their glycosylamines, **12** and **13** respectively, using ammoniumbicarbonate.[28,29] The efficiency of this reaction was approximately 80 % and since purification of the amine was not possible the crude mixtures were treated with *N*,*O*-bis-trimethylsilylacetamide in pyridine. The per-*O*-trimethylsilylated glycosylamines **15** and **16** accompanied by some trimethylsilylated starting material were isolated in 90 % estimated yields. The work-up procedure includes washing of the organic phase with water and excess ammonia, which otherwise gives problems in the following coupling reactions, was conveniently removed. Compounds **18** and **19** were

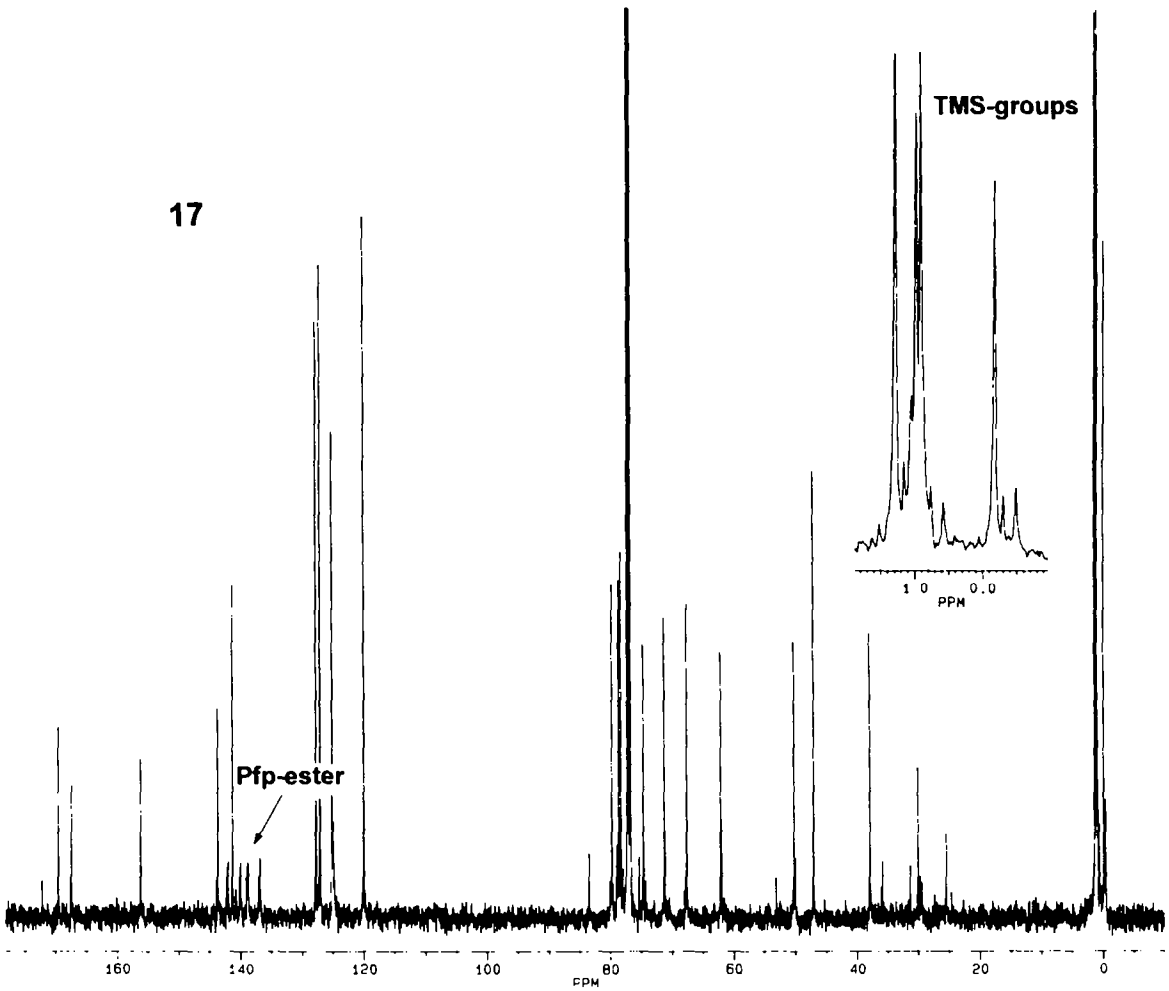


Figure 1. ^{13}C NMR Spectrum of building block **17** recorded at 125 MHz in $CDCl_3$ (300 K).

prepared and their structures confirmed by mass spectrometry but since trimethylsilylated maltotriose and maltoheptose, respectively, were present no further characterization was done at this stage. The three building blocks **17–19** were used in the synthesis of *N*-glycosylated tripeptides Leu-Asn-Glu-NH_2 **20–22** and as determined from HPLC traces of crude **20** and **21** the building blocks were quantitatively incorporated into the glycopeptides. In the case of **22** a peak with a shorter retention time contained the dipeptide Leu-Glu-NH_2, which may result from insufficient coupling time with the very large building block **19** (see Figure 2). The structure of all three glycopeptides was confirmed by mass spectrometry and NMR spectroscopy. The HPLC traces of **21** and **22** indicated only one peak containing the glycopeptide, yet the NMR spectra showed the presence of approximately 20 % of the unglycosylated tripeptide Leu-Asp-Glu-NH_2 (confirmed by mass spectrometry) clearly reflecting the efficiency of the formation of the glycosylamine. Despite the large differences in molecular weights (**21**; 859 Da, **22**; 1507 Da and Leu-Asp-Glu-NH_2; 374 Da) the glycopeptides and the unglycosylated tripeptide eluted with the same retention time in HPLC, for which there is no immediate explanation.

The 11 enzyme substrates, see Table 2, were synthesized in DMF on a custom-made, fully automated, continuous-flow peptide synthesizer or by MCPS[8,30,31] on PEGA[32] or Macrosorb SPR 250 resin, derivatized with a base-labile linker, 4-hydroxymethylbenzoic acid[33] (HMBA). In the automated synthesis, monitoring of the acylation steps[34] was not possible due to a persistent yellow colour of the resin after incorporation of 3-nitrotyrosine (Tyr(NO_2)), but cleavage of the Fmoc groups was followed by use of a UV-spectrophotometer at 320 nm during base treatment. In the present synthesis three different bases were used for cleavage of the Fmoc group (20 % piperidine in DMF, 50 % morpholine in DMF and 2 % piperidine and 2 % 1,8-diazabicyclo-[5,4,0]-undec-7-ene (DBU) in DMF). Preferably, 20 % piperidine in DMF should be used, since this reaction is fast (within 10 min) and piperidine is harmless to the protected carbohydrate moiety, whereas there is a risk of side reactions using DBU.[17] The acylation time for the glycosylated asparagine building block **8** was 7 h in the automated synthesis. According to analytical HPLC of the crude products, the building block was quantitatively incorporated into the glycopeptides. After the last acylation step the peptide was completely deprotected by treatment with 95 % aq. trifluoroacetic acid (TFA) followed by neutralization with 5 % ethyl-diisopropylamine in DMF or with 2 % piperidine/2 % DBU in DMF. The procedure with ethyl-diisopropyl-amine is to be recommended, since DBU is very

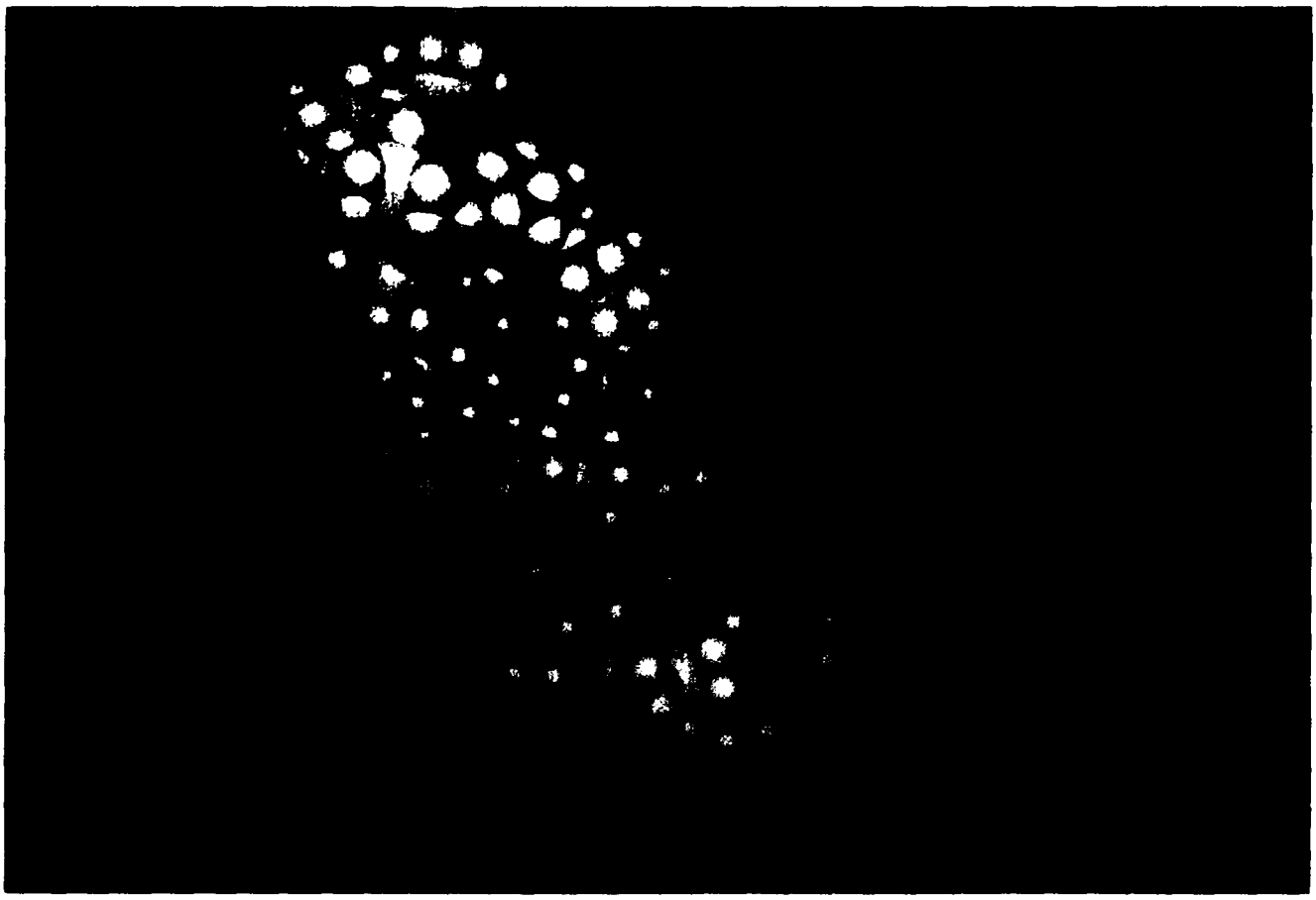
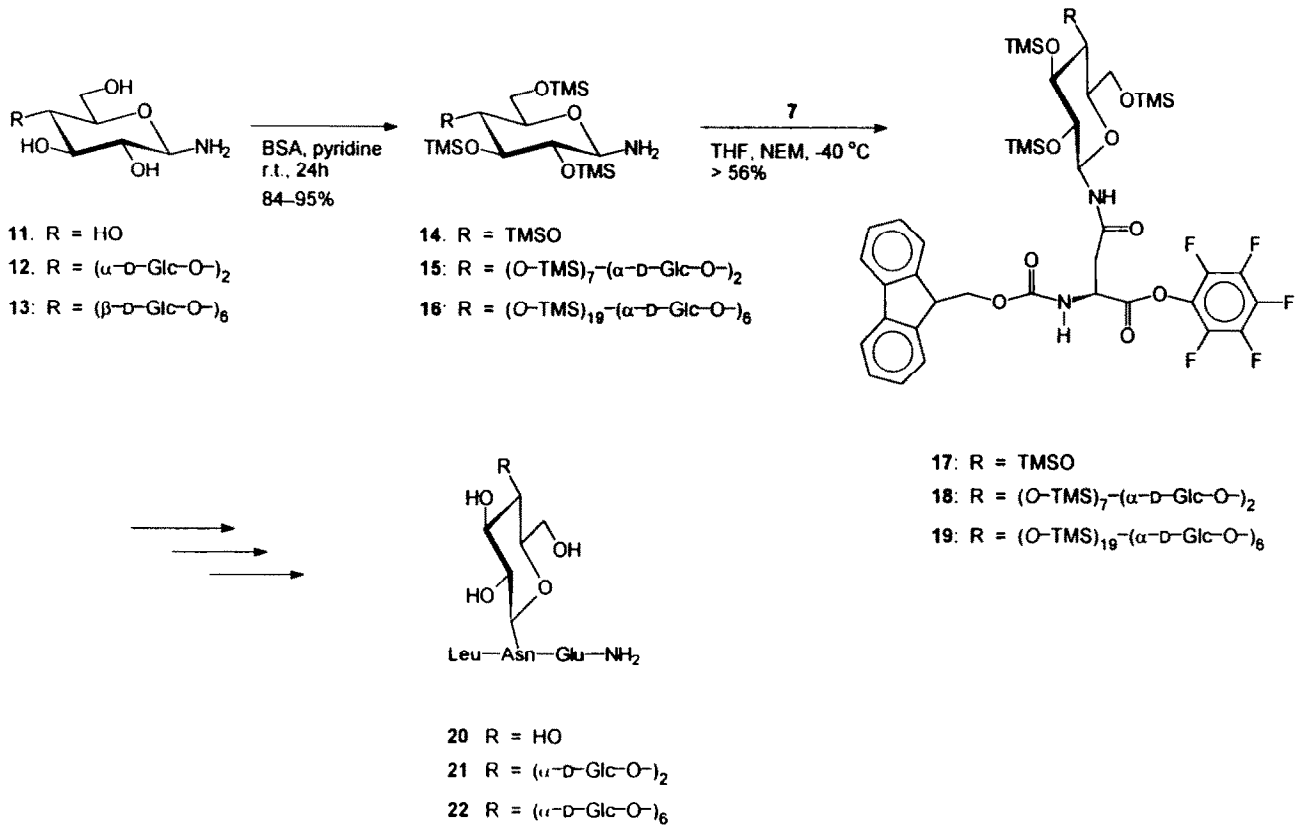


Figure 2. A space-filling model of building block **19** generated by combined molecular dynamics and energy minimization using the force-field Discover (Insight/Discover program package).

difficult to rinse out from the peptide and contaminates the crude product. After cleavage from the resin all the crude products were purified by reversed phase HPLC. The yields of isolated pure products varied from 25 to 71 %, except for compound **33**, which was isolated in a yield of only 12 % due to problems during the HPLC purification. All substrates were analyzed by analytical HPLC, amino acid analysis, and electrospray mass spectroscopy. The five neoglycopeptides were also analyzed by ^{1}H NMR spectroscopy (see Table 1) and in Figure 3 the ^{1}H NMR spectra of peptide **24** and the corresponding neoglycopeptide **25** are presented.



Scheme II.

Table 1. ^{1}H NMR chemical shifts (ppm) recorded at 500 or 600 MHz for compounds **25**, **27**, **29**, **31**, and **33** in 50 % CD_3COOD/H_2O (pH ~ 1.0) measured at 300 K[a]

		25	**27**	**29**	**31**	**33**
ABz	3-H	7.88	7.74	7.71	7.68 (8)	7.72
	4-H	7.52	7.47	7.48	7.41	7.48
	5-H	7.67	7.65	7.64	7.61 (1.5)	7.64
	6-H	7.49	7.45	7.46	7.40 (8.5)	7.46
Phe	$N^{\alpha}H$	-	8.55	8.56	8.50 (7)	8.56
	α	-	4.85	4.86	4.86	4.86
	β	-	3.20	3.26	3.24 (7;14)	3.24
	β'	-	3.17	3.13	3.12 (8.5)	3.12
	arom.	-	7.31-7.23	7.34-7.22	7.33-7.23	7.32-7.24
Gln	$N^{\alpha}H$	8.05	-	8.22	8.25 (7.5)	8.23
	α	4.75	-	4.71	4.73	4.73
	β	2.14	-	2.12	2.04	2.13
	β'	2.00	-	1.97	1.97	1.92
	γ,γ'	2.42	-	2.40	2.39 (7;13.5)	2.38
	CONH	7.44	-	7.45	7.45	7.45
	CONH'	6.84	-	6.85	6.85	6.85
Pro	α	4.45	4.37	4.40	4.44	4.42
	β	2.28	2.38	2.30	2.29 (8.5)	2.28

Table 1. *Continued.*

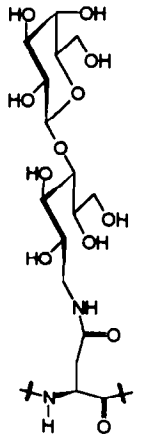
	β	1.98	2.33	1.97	1.99 (5)	1.96
	γ	2.03	2.01	2.06	2.08	2.6
	γ'	1.97	1.97	1.98	1.95	2.00
	δ	3.68	3.75	3.68	3.80	3.69
	δ	3.68	3.75	3.68	3.70	3.69
Leu	$N^{\alpha}H$	8.06	8.15	-	8.07 (7)	8.06
	α	4.35	4.32	-	4.36	4.37
	β	1.57	1.72	-	1.62	1.62
	β	1.62	1.59	-	1.57	1.58
	γ	1.66	1.70	-	1.66 (6.5;6.5)	1.64
	δCH_3	0.90	0.94	-	0.91	0.90
	$\delta' CH_3$	0.86	0.88	-	0.87	0.86
Asp	$N^{\alpha}H$	8.17	7.88	8.16	-	8.14
	α	4.71	4.70	4.71	-	4.68
	β	2.94	2.91	2.87	-	2.78
	β	2.85	2.81	2.86	-	2.70
Glu	$N^{\alpha}H$	7.98	7.82	8.12	8.05 (7)	-
	α	4.33	4.28	4.29	4.30	-
	β	1.97	1.96	1.93	1.97	-
	β'	1.84	1.86	1.90	1.85	-
	γ,γ'	2.26	2.25	2.24	2.23 (7.5)	-
Tyr(NO_2)	$N_{\alpha}H$	8.33	8.02	8.07	8.32 (8)	8.06
	α	4.75	4.77	4.75	4.72	4.68
	β	3.23	3.25	3.22	3.26 (5.5;14.5)	3.20
	β'	3.00	3.00	3.00	3.02 (9.5)	3.03
	2-H	7.97	7.97	7.96	7.99 (2)	7.97
	5-H	7.12	7.12	7.12	7.13	7.13
	6-H	7.56	7.57	7.56	7.58 (8.5)	7.55
Asp	$N^{\alpha}H$	8.10	8.10	8.11	8.10 (8)	8.19
	α	4.79	4.78	4.78	4.78	4.76
	β,β'	2.94	2.93	2.93	2.94 (6)	2.93
Asn	$N^{\alpha}H$	8.80	8.26	8.32	8.20 (7)	8.14
	α	5.01	5.02	4.70	4.72	4.68
	β	2.98	2.94	2.84	2.84 (6.5;15)	2.80
	β	2.90	2.73	2.90	2.78 (6.5)	2.87
	CONH	7.98	7.88	7.92	7.91 (5.5;5.5)	7.82
β-D-Gal(1-4)	1-H	4.52	4.55	4.53	4.54 (7.5)	4.54
	2-H	3.60	3.61	3.61	3.62 (9.5)	3.61
	3-H	3.68	3.68	3.68	3.68 (3.5)	3.68
	4-H	3.95	3.96	3.95	3.94	3.95
	5-H	3.92	3.93	3.94	3.95	3.94
	6-H	3.84-3.73	3.86-3.77	3.83-3.76	3.86 (3;11.5)	3.82-3.78
	6'-H	3.84-3.73	3.86-3.77	3.83-3.76	3.79	3.82-3.78
D-Glucitol	1-H	3.43	3.43	3.42	3.46 (5.5;13.5)	3.42
	1'H	3.43	3.36	3.42	3.41 (7)	3.35
	2-H	4.02	4.00	4.03	4.04 (4)	4.00
	3-H	3.79	3.78	3.82	3.81	3.80
	4-H	3.68	3.68	3.69	3.69	3.69
	5-H	3.92	3.93	3.94	3.95	3.94
	6-H	3.84-3.73	3.86-3.77	3.83-3.76	3.79 (3)	3.82-3.78
	6'-H	3.68	3.69	3.69	3.69	3.69

[a]First order coupling constants are given for compound **31** (in parenthesis, Hz). Corresponding coupling constants for compounds **25**, **27**, **29**, and **33** are identical within the range of ± 0.3 Hz.

Table 2. Kinetic data with substrates 23–33 at pH 8.5[a]

	scissile bond ↓ P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	k_{cat}/K_M (min^{-1} μM^{-1})
23	ABz—Phe—Gln—Pro—Leu—Asp—Glu—Tyr(NO_2)—Asp—OH	920
24[b]	ABz—**Asn**—Gln—Pro—Leu—Asp—Glu—Tyr(NO_2)—Asp—OH	2
25[b]	ABz—**Asn***—Gln—Pro—Leu—Asp—Glu—Tyr(NO_2)—Asp—OH	0.83
26	ABz—Phe—**Asn**—Pro—Leu—Asp—Glu—Tyr(NO_2)—Asp—OH	200
27	ABz—Phe—**Asn***—Pro—Leu—Asp—Glu—Tyr(NO_2)—Asp—OH	280
28	ABz—Phe—Gln—Pro—**Asn**—Asp—Glu—Tyr(NO_2)—Asp—OH	18
29	ABz—Phe—Gln—Pro—**Asn***—Asp—Glu—Tyr(NO_2)—Asp—OH	0.37
30[b]	ABz—Phe—Gln—Pro—Leu—**Asn**—Glu—Tyr(NO_2)—Asp—OH	4100
31[b]	ABz—Phe—Gln—Pro—Leu—**Asn***—Glu—Tyr(NO_2)—Asp—OH	960
32	ABz—Phe—Gln—Pro—Leu—Asp—**Asn**—Tyr(NO_2)—Asp—OH	72
33	ABz—Phe—Gln—Pro—Leu—Asp—**Asn***—Tyr(NO_2)—Asp—OH	1100

Asn* =



[a] P_5–P_4' Positions refer to the substrate and corresponding binding sites in the enzyme are denoted with S.[38]
[b] Substrates on which savinase exhibits carboxypeptidase activity, leading to release of the C-terminal Asp.

Enzymatic studies

Proteolytic enzymes usually exhibit a preference for certain peptide bonds in a polypeptide chain.[35,36] The rate of hydrolysis may be determined by the nature of the amino acid residues forming the scissile bond or by the nature of amino acids further away. The recent development of new techniques, such as MCPS[30,31] and the use of internally quenched fluorescent substrates,[26,27,37,38] has facilitated and accelerated the process of characterizing the substrate specificity of these enzymes. The internally quenched fluorescent substrates contain a fluorescent probe (*o*-aminobenzamide, ABz) and a quenching chromophore (3-nitrotyrosine, Tyr(NO_2)) such that cleavage of a peptide bond situated between these results in an increase in fluorescence. Complete quenching of fluorescence was observed over distances of more than 20 Å, corresponding to 6–7 amino acids, thereby allowing the use of long peptide substrates.

Subtilisins are endopeptidases with extensive binding sites, yet, are considered to be unspecific but with a

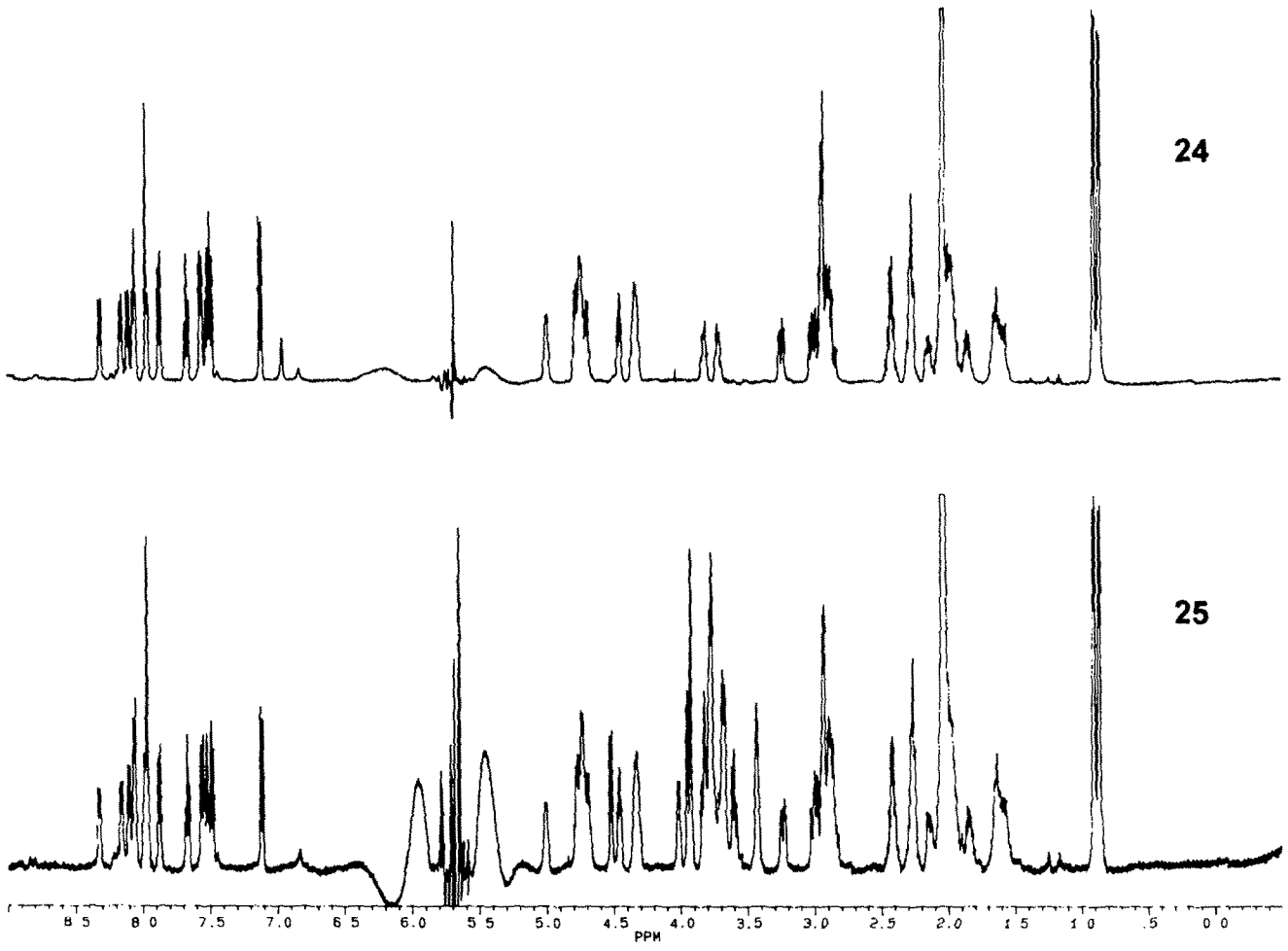


Figure 3. ^{1}H NMR Spectra of peptide substrate **24** and the corresponding neoglycopeptide **25** recorded at 500 MHz in 50 % CD_3COOD/H_2O.

well-defined substrate preference. A complete subsite mapping of savinase and subtilisin BPN', with respect to the eight subsites S_5 to S'_3*, has been performed using fluorogenic substrates, synthesized by MCPS.[27]

In the present work the substrate ABz-Phe-Gln-Pro-Leu-Asp-Glu-Tyr(NO_2)-Asp-OH, which is a substrate for savinase,[27] has been modified by sequential replacement of the amino acids between the donor–acceptor pair with a glycosylated asparagine residue carrying a disaccharide lactitol moiety. The activity and kinetic constants (k_{cat}/K_M) for substrates **23–33** were determined after treatment with savinase. Savinase has a broad specificity and consequently it is capable of cleaving a large variety of peptide bonds. However, by proper substrate design it is possible to direct the cleavage of a rather long peptide to one particular peptide bond. Thus it has been found that a proline is accepted by savinase in the P_2 position but only to a minor degree elsewhere.[27] The cleavage site between the ABz- and Tyr(NO_2) groups, responsible for the observed rate of cleavage, was checked for each substrate, using mass spectrometry. It was found that the cleavage site was as predicted (see Table 2). However, with substrates **24**, **25**, **30** and **31** release of the C-terminal Asp competed with this reaction. The reaction monitored in the fluorimeter is not due to this additional cleavage but only due to cleavage of the predicted scissile bond. It is estimated that the rate is not influenced by the presence or absence of an Asp in the P_4' position since this residue does not seem to interact with the enzyme.[40]

The results show that k_{cat}/K_M for the hydrolysis of a substrate with a lactitol moiety in the P_1 position is 50-fold lower than that for the hydrolysis of the corresponding substrate without this carbohydrate group. A lactitol group in the P_1' position is less detrimental to hydrolysis (five-fold reduction). This effect of a bulky carbohydrate group is consistent with the P_1'–S_1' and in particular the P_1–S_1 interactions being important for the rate of hydrolysis.[27] From this point of view it is interesting that the lactitol group at the P_4 position only reduces k_{cat}/K_M three-fold since the P_4–S_4 interactions are considered as important for hydrolysis as the P_1–S_1 interactions. The lack of a more pronounced effect of

*The enzyme binding sites are denoted S_1, S_2, ..., S_i and S'_1, S'_2, ...,S'_i away from the scissile bond toward the N- and the C-terminus, respectively.[39] Amino acid residues in the substrates are referred to as P_1, P_2, ..., P_i and P'_1, P'_2, ..., P'_i in correspondence with the binding site.

the bulky carbohydrate group could be due to the fact that hydrophilic side chains in the P_4 position do not bind within the S_4 pocket in the way characteristic of hydrophobic groups.[41] Thus, it must be expected that the lactitol group binds to the surface of the enzyme and with only minor steric limitations.

The k_{cat}/K_M for the hydrolysis of a substrate with the carbohydrate group at the P_3 position is slightly larger than that of the corresponding unglycosylated substrate. This is consistent with the X-ray studies [40,42–44] showing that the P_3 side chain protrudes into the solvent, and as a consequence, its effect on hydrolysis is limited. The 15-fold increase in k_{cat}/K_M due to glycosylation of the P_2' side-chain could be due to additional interactions with the enzyme, since the S_2' site is poorly defined and its contribution to catalysis is limited.[27]

Conclusion

The present study shows that TMS groups may conveniently be used as carbohydrate protecting groups in the synthesis of *N*-linked glycopeptides and the incorporation of TMS-protected glycosylated amino acids into glycopeptides was not affected by the size of the carbohydrate moiety.

The limitation in the procedure presented is the formation of the glycosylamine, since the TMS groups were easily introduced and the coupling reaction to form the building blocks proceeded instantaneously, despite the size of the TMS-derivative.

In addition, the enzymatic studies show that a subtilisin-type protease like savinase has the capacity to cleave in the proximity of a carbohydrate moiety, but preferably it should be located away from the P_1 position of the substrate. Other proteases may share this ability but in each case it must be verified experimentally, in order to draw general conclusions.

Experimental

Merck Silica Gel 60 H was dried at 120 °C for > 24 h prior to use. DMF was freshly distilled by fractional distillation at reduced pressure and kept over 3 Å molecular sieves. Thionyl chloride, pyridine and THF were distilled prior to use. Concentrations were performed under reduced pressure at temperatures < 30 °C (bath). *p*-((α-Fmoc Amino)-2,4-dimethoxybenzyl)-phenoxyacetic acid (Rink-amide-linker),[45] HMBA and suitably protected N^{α}-Fmoc amino acids were purchased from NovaBiochem (Switzerland), Macrosorb SPR 250 resin from Sterling Organics (England), TMS-Cl, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH), NEM and *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU) from Fluka (Switzerland) and *N*,*O*-bis-trimethylsilyl-acetamide, *N*-methyl imidazole (MeIm), 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazolide[46] (MSNT) and TFA from Merck (Germany). N^{α}-Fmoc-Tyr(NO_2)-OH and *o*-*tert*-butyloxycarbonyl-aminobenzoyl Dhbt-ester (Boc-ABz-ODhbt) were prepared as previously described.[26] The peptides and the glycopeptides were hydrolyzed with 6 M HCl at 110 °C for 24 h and the amino acid composition was determined on a Pharmacia LKB Alpha Plus amino acid analyser, Asn and Gln were determined as Asp and Glu, respectively. Electrospray mass spectra (ESMS) were recorded in the positive mode on a VG Quattro Mass Spectrometer, with 50 % aqueous acetonitrile as the liquid phase. NMR Spectra were recorded on a Bruker AM-500 or a Bruker AMX-600 MHz spectrometer. The ^1H and ^{13}C resonances were assigned by ^1H, ^{13}C, ^1H–^1H COSY, ^{13}C–^1H correlation, ROESY and TOCSY experiments. The 2-D NMR spectroscopy was performed as previously described.[8] NMR Spectra were recorded in D_2O or 50 % deuterated acetic acid/H_2O (CD_3COOD/H_2O) (reference: acetic acid at 2.03 ppm (^1H)) or in $CDCl_3$ (reference: chloroform at 7.3 ppm (^1H) and 77.0 ppm (^{13}C)), which was filtered through basic alumina. Preparative HPLC was performed on a Waters system with a 600 controller, a 991 photodiode array detector, equipped with a preparative flow cell, and a model 600 pump with modified 80 mL/min pump heads. The system was fitted with a switchable Delta Pak (25 × 200 mm, 10 mL/min) and a preparative radial pack column (50 × 300 mm, 20 mL/min), both packed with reversed phase C_{18} or a Shodex DS-2013 column (20 × 300 mm, 4 mL/min). Analytical HPLC was performed using a Waters RCM 8 × 10 module with a Waters 8 NV C_{18} (4 μm) column (1 mL/min). The solvent system was buffer A; 0.1 % TFA and buffer B; 0.1 % TFA in 90 % acetonitrile–10 % water and detection was at 215 or 320 nm. Savinase from *Bacillus lentus* was prepared and purified as previously described.[27] The enzymatic hydrolysis of peptide and glycopeptide substrates was performed as described[27] and followed on a Perkin Elmer luminescence spectrometer LS 50.

1-Amino-alditols of lactose **1**, cellobiose **2** and maltose **3** as well as β-D-glucopyranosylamine **11** were prepared as previously described.[16,47] The β-D-glycosylamines of maltotriose **12** and maltoheptose **13** were prepared essentially as previously described,[28,29] except for the fact that the ion-exchange step was omitted and the crude products, which according to NMR were between 80 and 90 % pure, were used directly in the next step. N^{α}-Fmoc-Asp(Cl)-OPfp **7** was prepared from N^{α}-Fmoc-Asp(OBu^t)-OPfp as previously described.[15]

*1-Amino-1-deoxy-2,3,5,6-tetra-*O*-trimethylsilyl-4-*O*-(2,3,4,6-tetra-*O*-trimethylsilyl-β-D-galactopyranosyl)-D-glucitol hydrochloride (4)*

1-Amino-1-deoxy-4-*O*-β-D-galactopyranosyl-D-glucitol **1** (1.5 g, 4.4 mmol) was stirred with dry diethylether (50 mL) for one week, then the diethylether was decanted leaving amorphous **1**. Pyridine (80 mL, 992 mmol) and TMS-Cl (55 mL, 435 mmol) was added and the reaction mixture was heated to reflux (oil bath) for 24 h. The reaction mixture was concentrated, suspended in dry pentane, filtered through dry Celite and the filtrate

concentrated to leave crude title compound **4** (2.33 g, 56 %, syrup), which was more than 90 % pure according to ^{1}H NMR. The ^{1}H NMR spectrum showed eight trimethylsilyl resonances between 0.1 and 0.3 ppm and a broad peak at 7.8 ppm which integrated to three protons ($NH_3^+Cl^-$).

1-Amino-1-deoxy-2,3,5,6-tetra-O-trimethylsilyl-4-O-(2,3,4,6-tetra-O-trimethylsilyl-β-D-glucopyranosyl)-D-glucitol hydrochloride (5)

Compound **5** was prepared in 63 % yield (1.58 g) from 1-amino-1-deoxy-4-*O*-β-D-glucopyranosyl-D-glucitol **2** as described for compound **4**.

1-Amino-1-deoxy-2,3,5,6-tetra-O-trimethylsilyl-4-O-(2,3,4,6-tetra-O-trimethylsilyl-α-D-glucopyranosyl)-D-glucitol hydrochloride (6)

Compound **6** was prepared in 60 % yield (2.0 g) from 1-amino-1-deoxy-4-*O*-α-D-glucopyranosyl-D-glucitol **3** as described for compound **4**.

N^α-(Fluoren-9-ylmethoxycarbonyl)-N^γ-[2,3,5,6-tetra-O-trimethylsilyl-4-O-(2,3,4,6-tetra-O-trimethylsilyl-β-D-galactopyranosyl)-1-deoxy-D-glucitol-1-yl]-L-asparagine pentafluorophenyl ester (8)

Compound **4** (1.60 g, 1.67 mmol) was dissolved in THF (15 mL) and NEM (442 μL, 3.5 mmol) was added. The mixture was added dropwise to a solution of **7** (1.73 mmol) in THF (20 mL) at –40 °C. *N*-Ethyl-morpholine hydrochloride precipitated instantaneously and the reaction mixture was allowed to warm to 20 °C. The hydrochloride was removed by filtration, the filtrate concentrated, redissolved in dry dichloromethane (DCM) (2 mL) and passed through a short column of dry silica gel [ethyl-acetate–light petroleum (1:12)]. Title compound **8** was isolated as a syrup (1.51 g, 68 %). δ_H(500 MHz; $CDCl_3$): 4.59 (J_{1a2a} = 7.5 Hz, 1a-H), 3.61 (J_{2a3a} = 9.5 Hz, 2a-H), 3.41 (J_{3a4a} = 3 Hz, 3a-H), 3.90 (4a-H), 3.93 (J_{5a6a} = 2.5 Hz, 5a-H), 3.85 (J_{6a6a} = 11 Hz, 6a-H), 3.80 (6a'-H), 3.70 (J_{1b2b} = 9 Hz, J_{1b1b} = 13.5 Hz, 1b-H), 3.48 ($J_{1b'2b}$ = 3 Hz, 1b'-H), 3.64 (J_{2b3b} = 5.5 Hz, 2b-H), 3.42 (J_{3b4b} = 2.5 Hz, 3b-H), 3.95 (J_{4b5b} = 3 Hz, 4b-H), 4.05 (J_{5b6b} = 6 Hz, $J_{5b6b'}$ = 7 Hz, 5b-H), 3.85 (6b-H and 6b'-H), Asn 6.41 ($J_{\alpha,H}$ = 9.5 Hz, N^αH), 5.03 (H^α), 3.21 ($J_{\alpha,\beta}$ = 4.3 Hz, $J_{\beta,\beta'}$ = 16.5 Hz, H^β), 2.81 ($J_{\alpha,\beta'}$ 4, $H^{\beta'}$), 6.43 (N^γH), Fmoc 4.30 (H^9), 4.54 ($J_{9,H}$ = 6 Hz, $J_{H,H'}$ = 9.5 Hz, CH_2), 4.30 ppm (CH_2'); δ_C(125.77 MHz; $CDCl_3$): 103.3 (C-1a), 71.7 (C-2a), 75.3 (C-3a), 71.2 (C-4a), 74.4 (C-5a), 60.5 (C-6a), 40.9 (C-1b), 71.7 (C-2b), 75.2 (C-3b), 76.7 (C-4b), 70.2 (C-5b), 63.6 (C-6b), Asn 50.6 (C^α), 37.6 (C^β), Fmoc 47.1 (C^9), 67.5 (CH_2), Pfp 136.8–142 ppm. ESMS 1461.8 $[M + K]^+$, $C_{61}H_{103}F_5N_2O_{15}Si_8$ requires *M*, 1422.5.

N^α-(Fluoren-9-ylmethoxycarbonyl)-N^γ-[2,3,5,6-tetra-O-trimethylsilyl-4-O-(2,3,4,6-tetra-O-trimethylsilyl-β-D-glucopyranosyl)-1-deoxy-D-glucitol-1-yl]-L-asparagine pentafluorophenyl ester (9)

Compound **9** was prepared in 63 % yield (1.58 g, syrup) from **5** and **7** as described for compound **8**. δ_H(500 MHz; $CDCl_3$): 4.85 (J_{1a2a} = 7.5 Hz, 1a-H), 3.20 (J_{2a3a} = 6.5 Hz, 2a-H), 3.41 (J_{3a4a} = 2 Hz, 3a-H), 3.41 (4a-H), 3.22 (J_{5a6a} = 5.5 Hz, 5a-H), 3.87 ($J_{6a6a'}$ = 11 Hz, 6a-H), 3.67 (6a'-H), 3.64 (1b-H and 1b'-H), 3.87 (2b-H), 3.87 (J_{3b4b} = 6 Hz, 3b-H), 3.80 (4b-H), 4.21 (5b-H), 3.87 (6b-H and 6b'-H), Asn 6.45 ($J_{\alpha,H}$ = 9.5 Hz, N^αH), 5.01 (H^α), 3.13 ($J_{\alpha,\beta}$ = 4.5 Hz, $J_{\beta,\beta'}$ = 16.5 Hz, H^β), 2.85 ($J_{\alpha,\beta'}$ = 4 Hz, $H^{\beta'}$), 6.49 (N^γH), Fmoc 4.29 (H^9), 4.51 (CH_2, $J_{9,H}$ = 7 Hz, $J_{H,H'}$ = 10.5 Hz), 4.36 ppm (CH_2', $J_{9,H'}$ = 7.5 Hz); δ_C(125.77 MHz; $CDCl_3$): 101.7 (C-1a), 74.9 (C-2a), 77.8 (C-3a), 71.9 (C-4a), 77.6 (C-5a), 62.5 (C-6a), 41.2 (C-1b), 70.9 (C-2b), 78.2 (C-3b), 75.7 (C-4b), 76.7 (C-5b), 63.8 (C-6b), Asn 50.6 (C^α), 37.5 (C^β), Fmoc 47.0 (C^9), 67.6 (CH_2), Pfp 136.8–142 ppm. ESMS 1445.3 $[M + Na]^+$, $C_{61}H_{103}F_5N_2O_{15}Si_8$ requires *M*, 1422.5.

N^α-(Fluoren-9-ylmethoxycarbonyl)-N^γ-[2,3,5,6-tetra-O-trimethylsilyl-4-O-(2,3,4,6-tetra-O-trimethylsilyl-α-D-glucopyranosyl)-1-deoxy-D-glucitol-1-yl]-L-asparagine pentafluorophenyl ester (10)

Compound **10** was prepared in 81 % yield (2.39 g, syrup) from **6** and **7** as described for compound **8**. δ_H(500 MHz; $CDCl_3$): 4.88 (J_{1a2a} = 3.5 Hz, 1a-H), 3.43 (J_{2a3a} = 9.5 Hz, 2a-H), 3.77 (J_{3a4a} = 9 Hz, 3a-H), 3.53 (J_{4a5a} = 9 Hz, 4a-H), 3.70 (5a-H), 3.77 (6a-H and 6a'-H), 4.01 (J_{1b2b} = 7.5 Hz, $J_{1b1b'}$ = 12.5 Hz, 1b-H), 3.43 ($J_{1b'2b}$ = 3 Hz, 1b'-H), 4.07 (2b-H), 3.70 (3b-H), 3.77 (4b-H), 4.08 (5b-H), 3.77 (6b-H), 3.70 (6b'-H), Asn 6.30 ($J_{\alpha,H}$ = 9 Hz, N^αH), 5.01 (H^α), 3.18 ($J_{\alpha,\beta}$ = 4 Hz, $J_{\beta,\beta'}$ = 16 Hz, H^β), 2.81 ($J_{\alpha,\beta'}$ = 4 Hz, $H^{\beta'}$), 6.08 (N^γH), Fmoc 4.28 (H^9), 4.52 ($J_{9,H}$ = 6.8 Hz, $J_{H,H'}$ = 10 Hz, CH_2), 4.32 ppm ($J_{9,H'}$ = 7.5 Hz, CH_2'); δ_C(125.77 MHz; $CDCl_3$): 100.3 (C-1a), 73.7 (C-2a), 74.0 (C-3a), 71.9 (C-4a), 73.2 (C-5a), 61.8 (C-6a), 42.3 (C-1b), 71.8 (C-2b), 74.9 (C-3b), 80.6 (C-4b), 77.3 (C-5b), 64.5 (C-6b), Asn 50.6 (C^α), 37.8 (C^β), Fmoc 47.1 (C^9), 67.5 (CH_2), Pfp 136.8–142 ppm. ESMS 1423.7 $[M + H]^+$, $C_{61}H_{103}F_5N_2O_{15}Si_8$ requires *M*, 1422.5.

2,3,4,6-Tetra-O-trimethylsilyl β-D-glucosylamine (14)

β-D-Glucosylamine **11** (1.0 g, 5.6 mmol) was suspended in pyridine (54 mL, 670 mmol) and *N,O*-bis-trimethylsilylacetamide (8.2 mL, 33.5 mmol) was added. After 24 h at room temperature, methanol (2 mL) was added and the reaction mixture was extracted by addition of hexane. The organic phase was washed with water, dried ($MgSO_4$) and concentrated to give title compound **14** (1.84 g, 84 %, syrup). ESMS 468.7 $[M + H]^+$, $C_{18}H_{45}NO_5Si_4$ requires *M*, 467.2.

Deca-O-trimethylsilylated β-D-maltotriosylamine (15)

Crude β-D-maltotriosylamine **12** was prepared from 0.5 g maltotriose and converted into the TMS-derivative in 95 % yield (1.16 g, syrup) as described for compound **14**. ESMS 1224.8 $[M + H]^+$, $C_{48}H_{113}NO_{15}Si_{10}$ requires *M*, 1223.6.

Docosa-O-trimethylsilylated β-D-maltoheptosylamine (16)

Crude β-D-maltoheptosylamine **13** was prepared from 200 mg maltoheptose and converted into the TMS-derivative in 86 % yield (410 mg, syrup) as described for compound **14**. ESMS 2741.3 $[M + H]^+$, $C_{108}H_{249}NO_{35}Si_{22}$ requires *M*, 2740.0.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{γ}-(2,3,4,6-tetra-O-trimethylsilyl-β-D-glucopyranosyl)-L-asparagine pentafluoro-phenyl ester (17)

Compound **14** (1.84 g, 3.94 mmol) was dissolved in THF (25 mL) and NEM (510 μL, 4.0 mmol) was added. The mixture was added dropwise to a solution of **7** (4.0 mmol) in THF (25 mL) at –40 °C. *N*-Ethyl-morpholine hydrochloride precipitated instantaneously and the reaction mixture was allowed to warm to 20 °C. The hydrochloride was removed by filtration, the resulting filtrate concentrated, redissolved in dry DCM (2 mL) and passed through a short column of dry silica gel [ethyl acetate–light petroleum (1:9)]. The title compound **17** was isolated as a syrup (2.1 g, 56 %). δ_H(500 MHz; $CDCl_3$): 5.13 (J_{12} = 9 Hz, H-1), 3.55, 3.46 and 3.38 (H-2, H-3 and H-4), 3.27 (H-5), 3.82 (J_{56} = 1.5 Hz, $J_{66'}$ = 11.5 Hz, H-6), 3.66 (H-6'), Asn 6.23 and 5.80 ($N^{\alpha}H$ and $N^{\gamma}H$), 5.05 (H^{α}), 3.23 ($J_{\alpha,\beta}$ = 4 Hz, $J_{\beta,\beta'}$ = 17 Hz, H^{β}), 2.93 ($J_{\alpha,\beta'}$ 4, $H^{\beta'}$), Fmoc 4.28 (H^9), 4.51 ($J_{9,H}$ = 7.5 Hz, $J_{H,H'}$ = 10.5 Hz, CH_2), 4.38 ppm ($J_{9,H'}$ = 7.5 Hz, $CH_{2'}$); δ_C(125.77 MHz; $CDCl_3$): 79.8, 78.7, 78.3, 74.6 and 71.3 (C-1, C-2, C-3, C-4 and C-5), 62.1 (C-6), Asn 50.2 (C^{α}), 37.9 (C^{β}), Fmoc 47.0 (C^9), 67.6 (CH_2), Pfp 136.8–142 ppm. ESMS 971.6 $[M + H]^+$, $C_{43}H_{59}F_5N_2O_{10}Si_4$ requires *M*, 970.3.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{γ}-(deca-O-trimethylsilyl-β-D-maltotriosyl)-L-asparagine pentafluoro-phenyl ester (18)

Compound **18** was prepared from **15** and **7** as described for compound **17** except for the fact that the crude product was not filtered through silica gel, but used directly in the synthesis of compound **21**. The title compound **18** was confirmed by ESMS 1772.4 $[M + HCOOH]^+$, $C_{73}H_{127}F_5N_2O_{20}Si_{10}$ requires *M*, 1726.7.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{γ}-(docosa-O-trimethylsilyl-β-D-maltoheptosyl)-L-asparagine pentafluoro-phenyl ester (19)

Compound **19** was prepared from **16** and **7** as described for compound **17** except for the fact that the crude product was not filtered through silica gel, but used directly in the synthesis of compound **22**. The title compound **19** was confirmed by ESMS 3289.4 $[M + HCOOH]^+$, $C_{133}H_{263}F_5N_2O_{40}Si_{22}$ requires *M*, 3243.4.

Leu-Asn(β-D-glucosyl)-Glu-NH_2 (20)

Compound **20** was synthesized by the syringe method,[15] on PEGA-resin[32] (0.36 g, 0.24 mmol/g) using the Rink-amide linker and N^{α}-Fmoc-Glu(OBu^t)-OPfp, N^{α}-Fmoc-Leu-OPfp and compound **17** with the addition of Dhbt-OH. Two equiv. of compound **17** were used and acylation was done for 36 h. Peptide cleavage and complete deprotection was done with 95 % aq. TFA (25 mL, 2 h) and the peptide was purified by semipreparative HPLC to give title compound **20** (40 mg, 87 %). δ_H(500 MHz; 50 % CD_3COOD/H_2O, pH 0.53): Leu 4.07 (H^{α}), 1.74 (H^{β} and $H^{\beta'}$), 1.78 (H^{γ}), 0.94 (2 δCH_3), Asn 8.70 ($J_{NH\alpha}$ = 7.5 Hz, $N^{\alpha}H$), 4.88 (H^{α}), 2.94 ($J_{\alpha\beta}$ = 6 Hz, $J_{\beta\beta'}$ = 16.5 Hz, H^{β}), 8.65 (J_{NH1} = 8.5 Hz, $N^{\gamma}H$), Glu 8.27 ($J_{NH\alpha}$ = 7.5 Hz, $N^{\alpha}H$), 4.42 (H^{α}), 2.17 (H^{β}), 1.97 ($H^{\beta'}$), 2.45 (H^{γ} and $H^{\gamma'}$), 7.57 (CONH), 7.08 (CONH'), glucosyl 4.97 (J_{12} = 8.5 Hz, H-1), 3.43 (J_{23} = 9 Hz, H-2), 3.59 (J_{34} = 9 Hz, H-3), 3.43 (H-4), 3.51 (H-5), 3.89 (J_{56} = 1 Hz, $J_{66'}$ = 12 Hz, H-6), 3.72 ppm ($J_{56'}$ = 5 Hz, H-6'). ESMS 536.5 $[M + H]^+$, $C_{21}H_{37}N_5O_{11}$ requires *M*, 535.2.

Leu-Asn(β-D-maltotriosyl)-Glu-NH_2 (21)

Compound **21** was synthesized using compound **18** as described for compound **20**. Semipreparative HPLC afforded one major peak (50 mg, 56 %) which contained the title compound **21** with approximately 20 % of the unglycosylated parent peptide Leu-Asp-Glu-NH_2. δ_H(500 MHz; 50 % CD_3COOD/H_2O, pH 0.50): chemical shifts for Leu, Asn and Glu are identical to those of compound **20**, 5.3 ppm (2 anomeric protons). ESMS 860.6 $[M + H]^+$, $C_{33}H_{57}N_5O_{21}$ requires *M*, 859.4.

Leu-Asn(β-D-maltoheptosyl)-Glu-NH_2 (22)

Compound **22** was synthesized using compound **19** as described for compound **20**. Semipreparative HPLC afforded two peaks, one major peak (23 mg, 61 %) which contained the title compound **22** with approximately 20 % of the unglycosylated parent peptide Leu-Asp-Glu-NH_2 and one minor peak (8 mg) which contained the dipeptide Leu-Glu-NH_2. δ_H(500 MHz; 50 % CD_3COOD/H_2O, pH 0.37): chemical shifts for Leu, Asn and Glu are identical to those of compound **20**, 5.3 ppm (6 anomeric protons). ESMS 1509.2 $[M + H]^+$ and 755.1 $[M + 2H]^{2+}$, $C_{57}H_{97}N_5O_{41}$ requires *M*, 1507.6.

Peptide synthesis, general

Synthesis of the peptides and glycopeptides was carried out on a custom-made, fully automated, continuous-flow peptide synthesizer or by MCPS and were performed in DMF. N^{α}-Fmoc-Protected Pfp esters (3 equiv.) of aspartic acid (OBu^t), glutamic acid (OBu^t), leucine, phenylalanine and proline were coupled with the addition of Dhbt-OH (1 equiv.). In the multiple-column synthesis N^{α}-Fmoc-Asn-OPfp and N^{α}-Fmoc-Gln-OPfp (3 equiv.) were used and coupled with the addition of Dhbt-OH (1 equiv.), and in the automated synthesis protected N^{α}-Fmoc-Gln(Trt)-OH and N^{α}-Fmoc-Asn(Trt)-OH (3 equiv.) were used and coupled with TBTU (2.7 equiv.) and NEM (6 equiv.).[48] The TMS-protected glycosylated asparagine building block **8** (2

equiv.) was coupled with addition of Dhbt-OH (1 equiv.), N^{α}-Fmoc-Tyr(NO_2)-OH (3 equiv.) was incorporated by the TBTU procedure, and the anthranilic acid was coupled using Boc-ABz-ODhbt[26] (3 equiv.).

Anchoring of the linker and the first amino acid

HMBA (87 mg, 0.57 mmol) was dissolved in DMF (2 mL) and cooled to 0 °C before addition of TBTU (180 mg, 0.56 mmol) and NEM (0.15 mL, 1.16 mmol). After 10 min this mixture was added to PEGA-resin (2 g, 0.185 mmol/g) which had been swelling in DMF (40 mL, 40 min). The flask was gently shaken and after 35 min the resin was filtered, rinsed with DMF (6 × 20 mL), DCM (10 × 20 mL) and lyophilized for 2 days. MeIm (36 µL, 0.44 mmol) was added under argon to a solution of N^{α}-Fmoc-Asp(OBu^t)-OH (236 mg, 0.58 mmol) in dry DCM (24 mL, distilled from diphosphorous pentoxide) and this solution was added with pressure via a double ended needle to a flask containing MSNT[46] (172 mg, 0.58 mmol). The resulting solution was then transferred to a flask containing the dried resin. After 30 min the resin was filtered and rinsed with DCM (3 × 20 mL), DMF (3 × 20 mL) and DCM (3 × 20 mL). The procedure was repeated and the derivatized resin was lyophilized. The degree of incorporation of aspartic acid was 79 % as determined by quantitative amino acid analysis. Macrosorb SPR 250 resin (0.25 mmol/g) was derivatized with HMBA and aspartic acid by the same procedure as described above and the incorporation of aspartic acid was 84 % as determined by amino acid analysis.

Multiple-column synthesis of compounds 23, 26–30, 32 and 33

The derivatized resin was distributed into the wells of a 20-column teflon block (60 mg in each column, 0.0087 mmol) and swelled in DMF for 1 h. Capping of free amino groups with 10 % acetic anhydride in DMF for 10 min was followed by washing each column with DMF (0.75 mL) 10 times. Synthesis cycle: removal of Fmoc groups was performed with 2 % 1,8-diazabicyclo-[5,4,0]-undec-7-ene (DBU) / 2 % piperidine in DMF (1 min + 30 min) for the peptides and 50 % morpholine in DMF (1 min + 30 min)for the glycopeptides after coupling of the glycosylated amino acid. This was followed by washing with DMF (0.5 min × 5, 2 min × 5 and 0.5 min × 5) and addition of a solution of the protected amino acid in DMF. The synthesis block was gently shaken overnight (acylation times 17–20 h) and the columns were washed with DMF (0.5 min × 5, 2 min × 5 and 0.5 min × 5). After the last acylation the resins were washed successively with DMF (× 10) and DCM (× 10) and dried by air-flow for 1.5 h. The resins were then treated, in the columns, with 95 % aq. TFA for 3 h, rinsed with 95 % aq. acetic acid (5 min × 4), neutralized with 5 % ethyl-diisopropylamine in DMF (2 min x 2), rinsed with DMF (× 10) and DCM (× 10) and dried by air-flow for 1.5 h. The synthesis block was taken apart and each column was connected with a 5 mL glass tube. Cleavage from the resin was performed by treatment with 3 × 0.3 mL 0.10 M NaOH (the resin was stirred between each addition to be completely wetted) for a total of 2 h. The resins turned red and the basic solutions (yellow) were eluted from the columns by supplying pressure to the column inlet with a rubber ball. The resins were rinsed with water (7 × 0.3 mL). The solutions were neutralized with 0.10 M HCl (0.9 mL) and lyophilized. All the crude peptides (including 5 mg NaCl) were purified by reversed-phase HPLC and analyzed by HPLC, amino acid analysis (see Table 3) and mass spectrometry. The glycopeptides **27**, **29** and **33** were also analyzed by ^{1}H NMR spectroscopy (see Table 1).

Automated synthesis of compounds 24, 25 and 31

Derivatized PEGA-resin (0.25 g, 0.036 mmol), see above, was used in the synthesis of compounds **6** and **11** and derivatized Macrosorb SPR 250 resin (0.45 g, 0.094 mmol), see above, was used in the synthesis of compound **7**. Free amino groups were capped with 10 % acetic anhydride (40 µL, 10 min) followed by washing with DMF. Removal of Fmoc groups was performed with 20 % piperidine in DMF for 10 min, and the cleavage was followed by a UV-spectrophotometer at 320 nm. Acylation times were 2 h for TBTU couplings, 7 h for the glycosylated asparagine residue **8** and 5 h for the Pfp- and Dhbt-esters. After completed synthesis the PEGA-resin was rinsed with DMF and DCM and lyophilized. The Macrosorb SPR 250 resin was rinsed with DMF and diethylether and dried by

Table 3. Amino acid analysis of compounds **23–33**. (Theoretical values in parenthesis.)

Compound	Asp	Glu	Leu	Phe	Pro	Tyr(NO_2)
23	2.13 (2)	2.16 (2)	0.88 (1)	0.92 (1)	0.99 (1)	0.91 (1)
24	2.87 (3)	2.12 (2)	1.01 (1)	-	0.99 (1)	1.00 (1)
25	2.92 (3)	2.12 (2)	1.01 (1)	-	0.99 (1)	0.96 (1)
26	2.85 (3)	1.15 (1)	0.97 (1)	0.96 (1)	1.09 (1)	0.97 (1)
27	2.84 (3)	1.11 (1)	1.01 (1)	0.99 (1)	1.08 (1)	0.96 (1)
28	2.86 (3)	2.14 (2)	-	0.98 (1)	1.01 (1)	1.01 (1)
29	2.91 (3)	2.15 (2)	-	0.99 (1)	1.00 (1)	0.95 (1)
30	2.00 (2)	2.18 (2)	0.90 (1)	0.94 (1)	1.02 (1)	0.95 (1)
31	2.05 (2)	2.05 (2)	0.94 (1)	0.95 (1)	1.01 (1)	0.92 (1)
32	2.91 (3)	1.08 (1)	1.07 (1)	0.99 (1)	0.96 (1)	0.99 (1)
33	2.92 (3)	1.10 (1)	1.03 (1)	1.03 (1)	1.00 (1)	0.92 (1)

passing nitrogen through the column. Protecting groups were removed by treatment with 95 % aq. TFA (10 mL) for 2 h and the resin was then rinsed with 95 % aq. acetic acid (10 mL), DMF (5 × 10 mL), neutralized with 2 % piperidine/2 % DBU in DMF (10 mL) and rinsed with DMF (5 × 10 mL) and DCM (5 × 10 mL). The resins were lyophilized and the peptides were cleaved from the solid support by treatment with 0.1 M NaOH (10 mL) for 2 h and extracted from the resins with 0.1 M NaOH (5 mL) and water (3 × 10 mL). The extracts were pooled and neutralized with 0.1 M HCl. Lyophilization afforded the crude peptides (including 87 mg NaCl) and they were all analyzed by analytical HPLC and purified by preparative HPLC. The purified products were analyzed by HPLC, amino acid analysis (see Table 3), mass spectrometry and ^{1}H NMR spectroscopy (compounds **25** and **31**, see Table 1).

ABz-Phe-Gln-Pro-Leu-Asp-Glu-Tyr(NO$_2$)-Asp-OH (23)

The crude product (12.2 mg, 118 %) was purified by semipreparative HPLC (Delta Pak column; 10 min 25 % B, then a linear gradient of 25–70 % B in 90 min; retention time 39 min) to give pure **23** (4 mg, 39 %). Amino acid analyses are presented in Table 3. ESMS 1191.1 $[M + H]^+$, $C_{54}H_{67}N_{11}O_{20}$ requires *M*, 1189.5.

ABz-Asn-Gln-Pro-Leu-Asp-Glu-Tyr(NO$_2$)-Asp-OH (24)

The crude product (123 mg, 293 %) was purified by semipreparative HPLC (Shodex column; 10 min 12 % B, then a linear gradient of 12–60 % B in 50 min; retention time 59 min) to give pure 24 (13.5 mg, 33 %). Amino acid analyses are presented in Table 3. ESMS 1157.7 $[M + H]^+$ $C_{49}H_{64}N_{12}O_{21}$ requires *M*, 1156.4.

ABz-Asn[4-O-(β-D-galactopyranosyl)-1-deoxy-D-glucitol-1-yl]-Gln-Pro-Leu-Asp-Glu-Tyr(NO$_2$)-Asp-OH (25)

The crude product (135 mg, 255 %) was purified by semipreparative HPLC (Shodex column; 10 min 12 % B, then a linear gradient of 12–60 % B in 50 min; retention time 54 min) to give pure **25** (28 mg (53 %). Amino acid analyses and selected ^{1}H NMR data are presented in Tables 3 and 1, respectively. ESMS 1484.1 $[M + H]^+$ $C_{61}H_{86}N_{12}O_{31}$ requires *M*, 1482.6.

ABz-Phe-Asn-Pro-Leu-Asp-Glu-Tyr(NO$_2$)-Asp-OH (26)

The crude product (14.7 mg, 144 %) was purified by semipreparative HPLC (Delta Pak column; 10 min 25 % B, then a linear gradient of 25–65 % B in 40 min; retention time 37 min) to give pure **26** (3 mg, 30 %). Amino acid analyses are presented in Table 3. ESMS 1177.0 $[M + H]^+$, $C_{53}H_{65}N_{11}O_{20}$ requires *M*, 1175.4.

ABz-Phe-Asn[4-O-(β-D-galactopyranosyl)-1-deoxy-D-glucitol-1-yl]-Pro-Leu-Asp-Glu-Tyr(NO$_2$)-Asp-OH (27)

The crude product (14.7 mg, 112 %) was purified by semipreparative HPLC (Delta Pak column; 10 min 10 % B, then a linear gradient of 25–65 % B in 40 min; retention time 30 min) to give pure **27** (4.7 mg, 36 %). Amino acid analyses and selected ^{1}H NMR data are presented in Tables 3 and 1, respectively. ESMS 1503.1 $[M + H]^+$ $C_{65}H_{87}N_{11}O_{30}$ requires *M*, 1501.6.

ABz-Phe-Gln-Pro-Asn-Asp-Glu-Tyr(NO$_2$)-Asp-OH (28)

The crude product (13.6 mg, 131 %) was purified by semipreparative HPLC (Delta Pak column; 10 min 25 % B, then a linear gradient of 25–65 % B in 40 min; retention time 26 min) to give pure **28** (3 mg, 30 %). Amino acid analyses are presented in Table 3. ESMS 1192.0 $[M + H]^+$ $C_{52}H_{62}N_{12}O_{21}$ requires *M*, 1190.4.

ABz-Phe-Gln-Pro-Asn[4-O-(β-D-galactopyranosyl)-1-deoxy-D-glucitol-1-yl]-Asp-Glu-Tyr(NO$_2$)-Asp-OH (29)

The crude product (15.7 mg, 119 %) was purified by semipreparative HPLC (Delta Pak column; 10 min 25 % B, then a linear gradient of 25–65 % B in 40 min; retention time 21 min) to give pure **29** (8.5 mg, 64 %). Amino acid analyses and selected ^{1}H NMR data are presented in Tables 3 and 1, respectively. ESMS 1518.0 $[M + H]^+$ $C_{64}H_{84}N_{12}O_{31}$ requires *M*, 1516.5.

ABz-Phe-Gln-Pro-Leu-Asn-Glu-Tyr(NO$_2$)-Asp-OH (30)

The crude product (12.4 mg, 120 %) was purified by semipreparative HPLC (Delta Pak column; 10 min 25 % B, then a linear gradient of 25–70 % B in 90 min; retention time 32 min) to give pure 30 (5 mg, 49 %). Amino acid analyses are presented in Table 3. ESMS 1190.2 $[M + H]^+$ $C_{54}H_{68}N_{12}O_{19}$ requires *M*, 1188.5.

ABz-Phe-Gln-Pro-Leu-Asn-[4-O-(β-D-galactopyranosyl)-1-deoxy-D-glucitol-1-yl]-Glu-Tyr(NO$_2$)-Asp-OH (31)

The crude product (255 mg, 177 %) was purified by semipreparative HPLC (Delta Pak column; linear gradient of 20–60 % B in 120 min; retention time 45 min) to give pure **31** (101 mg, 71 %). Amino acid analyses and selected ^{1}H NMR data are presented in Tables 3 and 1, respectively. ESMS 1516.2 $[M + H]^+$, $C_{66}H_{90}N_{12}O_{29}$ requires *M*, 1514.6.

ABz-Phe-Gln-Pro-Leu-Asp-Asn-Tyr(NO$_2$)-Asp-OH (32)

The crude product (12.5 mg, 120 %) was purified by semipreparative HPLC (Delta Pak column; 10 min 25 % B, then a linear gradient of 25–60 % B in 40 min; retention time 32 min) to give pure **32** (2.5 mg, 25 %). Amino acid analyses are presented in Table 3. ESMS 1176.0 $[M + H]^+$, $C_{53}H_{68}N_{12}O_{19}$ requires *M*, 1174.5.

ABz-Phe-Gln-Pro-Leu-Asp-Asn[4-O-(β-D-galactopyranosyl)-1-deoxy-D-glucitol-1-yl]-Tyr(NO$_2$)-Asp-OH (33)

In the purification of the crude product (13.6 mg, 104 %) by semipreparative HPLC, the compound was eluted within the front fractions on both Delta Pak and Shodex columns. Therefore it was necessary to make the

purification by preparative analytical HPLC (linear gradient of 25–40 % B in 50 min; retention time 23 min). This afforded 1.5 mg of pure **33** (12 %). Amino acid analyses and selected ^{1}H NMR data are presented in Tables 3 and 1, respectively. ESMS 1501.6 $[M + H]^+$ $C_{65}H_{88}N_{12}O_{29}$ requires M, 1500.6.

Determination of enzyme activity and kinetic constants for compounds 23–33

The substrates were dissolved in DMF at concentrations of 3–15 μM. A total of 50 μL of substrate solution was added to 2450 μL of assay buffer (50 mM Bicine, 2 mM $CaCl_2$, 0.1 M KCl, pH 8.5) to give initial substrate concentrations in the cuvette, s_0, of 0.06–0.3 μM. The initial fluorescence, $E_{initial}$, was determined, and 50 μL of an enzyme solution, appropriately diluted into the assay buffer, was added (final enzyme concentrations, e_0, of 0.1–100 nM). To prevent loss of enzyme due to adsorption on surfaces, dilution of enzyme was carried out in polypropylene micro test tubes which had been filled with 0.1 % poly(ethylene glycol) 20.000, drained and oven dried to coat the surface. The cleavage of substrate was followed with time by monitoring the emission at 420 nm (10 nm slit) upon excitation at 320 nm (10 nm slit), at 25 °C. At each substrate concentration, s_0, the initial velocity for the cleavage of substrate, v_0, was determined from the slope of the initial part of the progress curve (emission versus time). The k_{cat}/K_M values were determined from initial velocities using the relation $v_0 = (k_{cat}/K_M)e_0s_0$ which is valid at low substrate concentrations ($s_0 << K_M$) for systems that obey Michaelis–Menten kinetics. For each substrate, the validity of this equation was ascertained by using a minimum of three substrate concentrations. The determination of k_{cat}/K_M values was ± 5 % and the values are presented in Table 2.

Determination of cleavage sites in compounds 23–33

To ascertain that the glycopeptides were cleaved at the predicted site, the scissile peptide bond was determined. Lyophilized substrate (70–300 nmol) was dissolved in 5 μL DMF and 195 μL assay buffer was added. Enzyme was added to the substrate medium and the degree of hydrolysis was followed by analytical HPLC (5–100 % B in 15 min). The reaction was quenched with addition of 20 % TFA (10 μL) and the reaction products were separated by analytical preparative HPLC (5–100 % B in 30 min). The peptide products were collected, lyophilized and analyzed using electrospray mass spectrometry.

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