Evaluation of the effect of glycosylation on the enzymic hydrolysis of peptides

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The glycosylated building block N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{γ}-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosyl]-L-asparagine pentafluorophenyl ester **7** has been synthesized and incorporated into the solid-phase synthesis of a panel of peptides and N-linked glycopeptides. These have been synthesized as internally quenched fluorogenic compounds where the position of the glycosylated asparagine residue is sequentially varied. Enzymic hydrolysis of these substrates with Savinase has been followed by fluorescence and the k_{cat}/K_M-values have been obtained. It is observed that the glycopeptides are less susceptible to proteolytic degradation than are the corresponding peptides.

The importance of carbohydrates for the biological activity and the structure of proteins is well recognized.[1] In addition, oligosaccharides have been shown to enhance the thermal stability of proteins[1,2] and to protect them against proteolytic degradation.[1,3] Detailed investigation of the influence of glycosylation on the enzymic hydrolysis of peptides is therefore of interest. The enzyme Savinase was studied in a model reaction on the hydrolysis of glycosylated compared with non-glycosylated peptides.

Savinase is a subtilisin-type enzyme. Subtilisins are serine endopeptidases and comprise an extended binding cleft with up to eight binding subsites (denoted S_5 to S_3').[4] Previous subsite-mapping studies[5,6] and X-ray crystal structure studies[7] have revealed that the subsites S_1 and S_4 are important for substrate specificity. The peptide ABz-Phe-Gln-Pro-Leu-Asp-Glu-Tyr-(NO_2)-Asp-OH (Table 1) was shown to be a suitable substrate for this enzyme. An aromatic residue was desirable at the P_4 position. A proline residue was accommodated preferentially at the P_2 position relative to the other sites. This directed the site of bond cleavage to the bond between the P_1 and the P_1' residues of the substrate (shown in Table 1).

We have previously reported a study on the effect of attachment of lactitol by reductive amination and acylation of lactose on the enzymic hydrolysis of a series of peptides that are substrates of Savinase.[8] A lactitol moiety, N-linked to an asparagine residue, was introduced at various positions along the parent peptide (Table 1). The results of this investigation revealed that the most pronounced effect of the sugar residue was observed at positions adjacent to the site of bond cleavage. Modification in P3 increased the rate of hydrolysis while the lactitol moiety in all other positions gave substrates with increased stability. Since the opened ring on the lactitol moiety introduces additional interactions between the peptide and the enzyme, we here propose to perform a comparative study introducing the much less flexible disaccharide lactose.

The present paper describes the synthesis of a series of peptides and the corresponding N-linked glycopeptides (Table 1) in order to examine the effect of the introduction of a lactose residue on the enzymic hydrolysis of these substrates. A series of substrates have been designed where each amino acid in the parent peptide has been sequentially replaced by an unglycosylated and glycosylated asparagine residue to afford the corresponding peptides and glycopeptides. The proline residue has been kept constant for all the substrates since it is important for directing the site of bond cleavage to the bond between the P_1 and P_1' residues. These compounds are substrates for the enzyme Savinase. They have been synthesized as internally quenched fluorogenic compounds.[5] The 2-aminobenzoyl (ABz)[5] group is employed as the fluorogenic probe and is introduced at the N-terminus of the peptides. The 3-nitrotyrosine [Tyr(NO_2)][5] residue is employed as the quenching chromophore and is located towards the C-terminus. The fluorescence of the ABz group is intramolecularly quenched by Tyr(NO_2) by long-range resonance-energy transfer.[5] Enzymic cleavage of the substrates results in an increase in fluorescence and enables the calculation of the enzyme activity and the kinetic constant (k_{cat}/K_M) for each substrate.

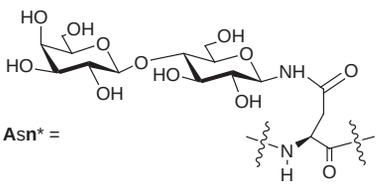
Results and discussion

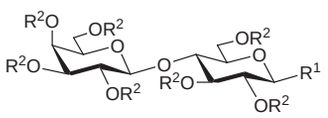
The strategy chosen for the synthesis of the peptides and glycopeptides shown in Table 1, was the building-block strategy,[9] where a glycosylated building block is first synthesized and subsequently incorporated into solid-phase peptide synthesis. The primary step was the synthesis of a glycosylated building block, where a protected β-D-lactose unit was N-linked to a protected asparagine residue. Initial attempts to procure such a building block involved the synthesis of β-D-lactosylamine as its per-*O*-trimethylsilylated derivative. Hence, β-D-lactosylamine **1** was prepared by the reaction of lactose with aqueous ammonium bicarbonate (ammonium hydrogen carbonate).[10] Subsequent attempts to protect the hydroxy groups of β-D-lactosylamine **1** with trimethylsilyl groups were unsuccessful. Treatment of lactosylamine **1** with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) and a catalytic amount of tetrabutylammonium fluoride (TBAF)[11] did not afford the desired product **2**. Per(trimethylsilylation) was also attempted with a mixture of hexamethyldisilazane and trimethylsilyl chloride;[12] however, a mixture of compounds resulted, possibly from incomplete silylation.

At this point, another approach to the glycosylated building block was contemplated, where the trimethylsilyl protecting groups were replaced with acetyl groups. The following route was followed to procure the desired block. The reaction of per-*O*-acetylated α/β-D-lactopyranoside **3** with trimethylsilyl azide in the presence of stannic chloride ($SnCl_4$)[13] afforded the glycosyl azide **4** in 91% yield. Reduction of the azide **4** in the presence of Pd/C in methanol–triethylamine afforded five other products in addition to the desired amine, as observed by TLC.

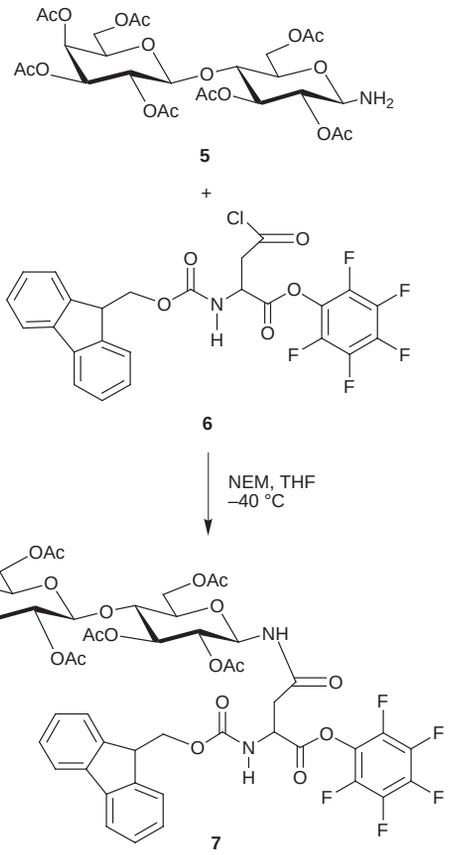
Table 1 Kinetic data for the substrates **8**–**18** at pH 8.5

	Scissile bond ↓								k_{cat}/K_M (min^{-1} μM^{-1})
	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3' P_4'	
8	ABz-Phe-	Gln-	Pro-	Leu-	Asp-	Glu-	Tyr(NO_2)-	Asp-OH	1125
9	ABz-Asn-	Gln-	Pro-	Leu-	Asp-	Glu-	Tyr(NO_2)-	Asp-OH	1.76
10	ABz-Asn*-	Gln-	Pro-	Leu-	Asp-	Glu-	Tyr(NO_2)-	Asp-OH	0.39
11	ABz-Phe-	Asn-	Pro-	Leu-	Asp-	Glu-	Tyr(NO_2)-	Asp-OH	296
12	ABz-Phe-	Asn*-	Pro-	Leu-	Asp-	Glu-	Tyr(NO_2)-	Asp-OH	204
13	ABz-Phe-	Gln-	Pro-	Asn-	Asp-	Glu-	Tyr(NO_2)-	Asp-OH	11
14	ABz-Phe-	Gln-	Pro-	Asn*-	Asp-	Glu-	Tyr(NO_2)-	Asp-OH	0.30
15	ABz-Phe-	Gln-	Pro-	Leu-	Asn-	Glu-	Tyr(NO_2)-	Asp-OH	4749
16	ABz-Phe-	Gln-	Pro-	Leu-	Asn*-	Glu-	Tyr(NO_2)-	Asp-OH	1407
17	ABz-Phe-	Gln-	Pro-	Leu-	Asp-	Asn-	Tyr(NO_2)-	Asp-OH	2260
18	ABz-Phe-	Gln-	Pro-	Leu-	Asp-	Asn*-	Tyr(NO_2)-	Asp-OH	852





		R^1	R^2
1:	β	NH_2	H
2:	β	NH_2	TMS
3:	α/β	OAc	Ac
4:	β	N_3	Ac
5:	β	NH_2	Ac



Scheme 1

However, hydrogenation in the presence of Lindlar's catalyst in THF–methanol–triethylamine[14] for 16 h afforded an almost quantitative amount of the glycosylamine **5**.

Condensation of the glycosylamine **5** with N^{α}-Fmoc-Asp(Cl)-OPfp **6** [obtained by the treatment of N^{α}-Fmoc-Asp(OBu^t)-OPfp with trifluoroacetic acid (TFA), followed by thionyl dichloride][15] was performed in the presence of *N*-ethylmorpholine (NEM) at −40 °C. Compound **7** was isolated in 31% yield after chromatographic purification (Scheme 1).

The peptides **8**, **11**, **13**, **15**, **17** and the glycopeptides **10**, **12**, **14**, **16**, **18** were synthesized by solid-phase peptide synthesis on PEGA resin.[16] Derivatization of the resin with a base-labile linker, 4-(hydroxymethyl)benzoic acid (HMBA), was performed by the *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) procedure.[17] The anchoring of the first amino acid residue was effected by the method of Blankemeyer-Menge *et al.*[18] Hence, esterification of N^{α}-Fmoc-Asp(OBu^t)-OH with the hydroxy function of the HMBA linker was performed with 2,4,6-mesitylenesulfonyl 3-nitro-1,2,4-triazolide (MSNT) in the presence of 1-methylimidazole (MeIm) under dry conditions and an inert atmosphere. Subsequent amino acids were added as their N^{α}-Fmoc-protected Pfp-esters. One mol equiv. of 3,4-dihydo-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) was added as the auxiliary nucleophile to enhance the reactivity of the pentafluorophenyl esters. The cleavage of the N^{α}-Fmoc protecting group was accomplished by the use of 20% piperidine in DMF. The use of 50% morpholine in DMF to affect the deprotection of the Fmoc group as reported previously[8] had resulted in the isomerization of the α-aspartimide to the β-aspartimide for compound **17**. Similar reports in the literature[19] indicate that this isomerization is particularly favoured when an Asp residue is succeeded by an Asn followed by a Tyr, which is the case for the peptide **17**. This isomerization was avoided by the use of 20% piperidine in DMF for Fmoc removal for this study. Amino acid sequencing was performed for compound **17** in order to confirm the absence of isomerization. The final ABz group was introduced with 3,4-dihydro-4-oxo-1,2,3-benzotriazol-3-yl 2-(*tert*-butyloxycarbonylamino)benzoate (Boc-ABz-ODhbt)[5] and the coupling was allowed to proceed for 16 h. After completion of the synthesis, the peptides and glycopeptides were deprotected by treatment with 95% aqueous trifluoroacetic acid (TFA) followed by neutralization with 5% ethyldiisopropylamine in DMF. The substrates were cleaved from the resin with aqueous sodium hydroxide (0.1 M) with concurrent removal of the acetyl protecting groups on the lactose moiety in the glycopeptides. Cleavage of the substrates from the resin, was followed by observing the colour of the resin, which lost its yellow colour after the substrates had been cleaved. Although the peptides required a single treatment with sodium hydroxide for cleavage, the glycopeptides required two to three. All compounds were purified by reversed-phase HPLC and analysed by amino acid analysis (see Table 3) and electrospray mass spectroscopy. The glycopeptides **10**, **12**, **14**, **16**, **18** were characterised by ^{1}H NMR spectroscopy (Table 2).

Enzymic study

The peptides and the glycopeptides **8**–**18** have been synthesized as internally quenched fluorogenic compounds. The fluorogenic ABz group and the quenching Tyr(NO_2) residue were placed at opposite sides of the site of bond cleavage. Determination of the enzyme activity was performed on a luminescence spectrometer. The ABz group was excited at 320 nm and its emission was monitored at 420 nm. In the absence of the enzyme the fluorescence was quenched intramolecularly by Tyr(NO_2). Upon addition of the enzyme Savinase an increase in the fluorescence was observed, which stabilized after some time. The activity and kinetic constants (k_{cat}/K_M) obtained for each substrate are presented in Table 1.

The general trend observed was that the introduction of the

Table 2 ^{1}H NMR chemical shifts (ppm) recorded at 500 or 600 MHz for compounds **10**, **12**, **14**, **16**, **18** in 50% CD_3CO_2D–H_2O (pH 1.4) measured at 300 K

		10	**12**	**14**	**16**	**18**
Abz	3-H	7.88	7.72	7.71	7.71	7.75
	4-H	7.50	7.45	7.49	7.46	7.51
	5-H	7.66	7.63	7.65	7.63	7.67
	6-H	7.47	7.44	7.46	7.44	7.49
Phe	$N^{\alpha}H$		8.52	8.57	8.54	8.58
	α		4.82	4.85	4.85	4.87
	β		3.16	3.24	3.24	3.26
	β′		3.13	3.12	3.12	3.14
	arom.	—	7.22–7.28	7.22–7.28	7.22–7.28	7.22–7.28
Gln	$N^{\alpha}H$	8.34		8.32	8.25	8.24
	α	4.72		4.72	4.72	4.74
	β	2.13		2.12	2.08	2.10
	β′	1.98		1.97	1.95	1.96
	γ,γ′	2.41		2.40	2.37	2.40
Pro	α	4.46	4.34	4.34	4.42	4.43
	β	2.28	2.35	2.28	2.28	2.29
	β′	1.94	1.99	1.99	2.01	1.97
	γ	2.02	1.99	2.02	2.01	1.99
	γ′	2.02	1.99	2.02	2.01	1.99
	δ	3.70	3.75	3.71	3.72	3.71
	δ′	3.81	3.75	3.71	3.67	3.71
Leu	$N^{\alpha}H$	8.07	7.95		8.07	8.06
	α	4.34	4.30		4.34	4.38
	β	1.62	1.68		1.62	1.63
	β′	1.57	1.58		1.56	1.59
	γ	1.64	1.67		1.64	1.63
	δCH_3	0.90	0.90		0.90	0.91
	$\delta' CH_3$	0.85	0.87		0.86	0.87
Asp	$N^{\alpha}H$	8.16	7.75	8.19		8.14
	α	4.69	4.72	4.72		4.67
	β	2.90	2.90	2.90		2.86
	β′	2.84	2.81	2.90		2.86
Glu	$N^{\alpha}H$	7.97	7.77	8.07	8.08	
	α	4.31	4.28	4.28	4.28	
	β	1.96	1.94	1.97	1.89	
	β′	1.83	1.84	1.91	1.84	
	γ,γ′	2.25	2.25	2.26	2.21	
Tyr(NO_2)	$N^{\alpha}H$	8.07	7.99	8.07	8.27	8.06
	α	4.73	4.74	4.73	4.71	4.70
	β	3.24	3.25	3.23	3.26	3.23
	β′	3.00	2.98	3.00	3.00	3.04
	H-2	7.97	7.97	7.97	8.01	7.98
	H-5	7.11	7.12	7.12	7.13	7.15
	H-6	7.56	7.56	7.55	7.59	7.55
Asp	$N^{\alpha}H$	8.11	8.09	8.11	8.08	8.19
	α	4.77	4.77	4.76	4.78	4.78
	β,β′	2.93	2.92	2.93	2.94	2.94
Asn	$N^{\alpha}H$	8.78	8.25	8.34	8.19	8.17
	α	5.02	5.03	4.73	4.72	4.72
	β	3.02	3.04	3.01	2.91	3.00
	β′	2.97	2.79	2.99	2.86	2.82
	CONH	8.68	8.56	8.62	8.60	8.50
β-D-Gal(1→4)	H-1	4.44	4.45	4.44	4.44	4.45
	H-2	3.60	3.61	3.60	3.63	3.62
	H-3	3.68	3.68	3.68	3.68	3.68
	H-4	3.95	3.95	3.95	3.95	3.97
	H-5	3.72	3.70	3.66	3.72	3.74
	H-6	3.82	3.82	3.83	3.83	3.85
	H′-6	3.77	3.75	3.78	3.77	3.74
D-Glc	H-1	5.00	4.94	5.00	5.04	4.96
	H-2	3.47	3.46	3.48	3.49	3.47
	H-3	3.70	3.66	3.71	3.71	3.73
	H-4	3.64	3.70	3.63	3.64	3.63
	H-5	3.63	3.57	3.62	3.59	3.63
	H-6	3.91	3.91	3.91	3.91	3.95
	H′-6	3.81	3.86	3.82	3.82	3.83

lactose unit into the peptide at various positions along the chain protected the peptide from proteolytic degradation. The effect of the disaccharide was dependent upon its position along the chain. Although the P_4 position is important for substrate–enzyme binding, only a 4-fold loss in activity was observed upon glycosylation at this position as compared with Asn. This is not surprising since the S4 of the protein is a deep hydrophobic pocket unable to interact with any polar groups. The lack of interaction of the two substrates with this subsite is reflected in a low rate of hydrolysis for both **9** and **10**. Glycosylation of P3 (**11**→**12**) did not influence the rate of hydrolysis and this is in agreement with the known lack of specificity of this subsite and with molecular dynamics modelling of the enzyme–substrate complex showing the P3 side chain to extend

Table 3 Amino acid analysis for substrates **8**, **10**–**18**

Compound	Asp	Glu	Leu	Phe	Pro	Tyr(NO_2)
8	2.09 (2)	2.10 (2)	0.98 (1)	0.97 (1)	0.95 (1)	0.99 (1)
10	3.10 (3)	2.06 (2)	0.96 (1)		1.00 (1)	0.88 (1)
11	3.03 (3)	1.01 (1)	0.99 (1)	1.00 (1)	0.97 (1)	1.01 (1)
12	3.05 (3)	1.06 (1)	1.02 (1)	0.99 (1)	0.98 (1)	0.90 (1)
13	2.98 (3)	2.03 (2)		0.98 (1)	1.03 (1)	0.98 (1)
14	2.97 (3)	2.16 (2)		0.98 (1)	0.98 (1)	0.91 (1)
15	1.98 (2)	2.09 (2)	1.00 (1)	0.98 (1)	0.96 (1)	0.98 (1)
16	2.01 (2)	2.09 (2)	0.99 (1)	0.98 (1)	0.99 (1)	0.94 (1)
17	3.02 (3)	1.03 (1)	0.99 (1)	1.00 (1)	0.95 (1)	1.00 (1)
18	3.03 (3)	1.05 (1)	1.01 (1)	1.00 (1)	0.98 (1)	0.91 (1)

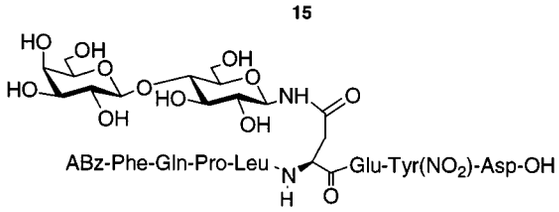
away from the binding site (data not shown). The P2 subsite was maintained as Pro throughout this study to effectively direct the cleavage to the intended scissile bond. The maximum effect of the sugar residue was observed when the site of glycosylation was in P1 adjacent to the site of bond cleavage (**13**→**14**). Thus, glycosylation at the P_1 position results in a 40-fold loss in enzyme activity and the overall activity was very low for both substrates, indicating the requirement for a hydrophobic side chain in this subsite.

The substitution effect at the P_1' position was not large (**15**→**16**); nevertheless, a decrease in activity of 3.5-fold was observed upon glycosylation and, furthermore, substrates with overall increased activity were obtained. Glycosylation at the P_2' position gave similar results and the rate of hydrolytic cleavage decreased by a factor of 3 upon glycosylation (**17**→**18**). Inspection of the crystal structure shows a shallow patch on the enzyme suited for interaction with the sugar moiety close to the P′ subsites. The trends were similar and more pronounced with the lactose substitution compared with those observed upon substitution with lactitol.[8]

The retardation of the rate of hydrolysis by the introduction of the disaccharide could be due to steric or conformational effects. In order to further investigate the effect of the sugar, time-resolved fluorescence studies were performed on three substrates. Time-resolved fluorescence spectroscopy allows the calculation of the distance between a fluorophore and a chromophore that quenches the fluorescence by resonance-energy transfer.[20] Since it operates on a nanosecond time scale, it allows the observation of even subtle conformational changes.[21] The peptide **15**, and the glycopeptides **16** and **19**,[8] where the carbohydrate was a lactose and a lactitol residue respectively, were investigated.[22] However, the same fluorescence decays were observed for each of the three substrates (Fig. 1). A full analysis of the decay curves has not been attempted here; however, the data clearly show a fast decay compared with the single exponential decay of the unquenched ABz (τ_D 7.6 ns). This confirms that the donor–quencher pair are at distances shorter than the Förster distance of 31 Å, giving rise to strong quenching. It is to be expected that a large change in conformational populations will affect the fluorescence decay dramatically. Therefore our results suggest that glycosylation of the peptide does not effect the conformation of the peptide backbone. The differences in the rates of enzymic hydrolysis that are observed are probably due to a steric effect of the carbohydrate moiety.

ABz-Phe-Gln-Pro-Leu-Asn-Glu-Tyr(NO_2)-Asp-OH

15



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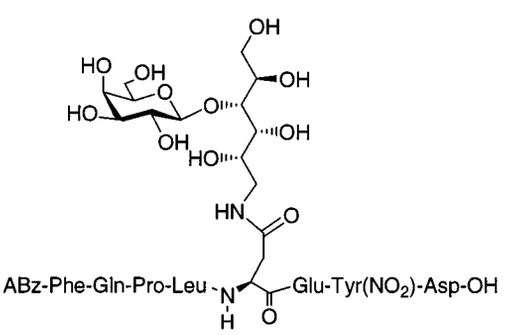

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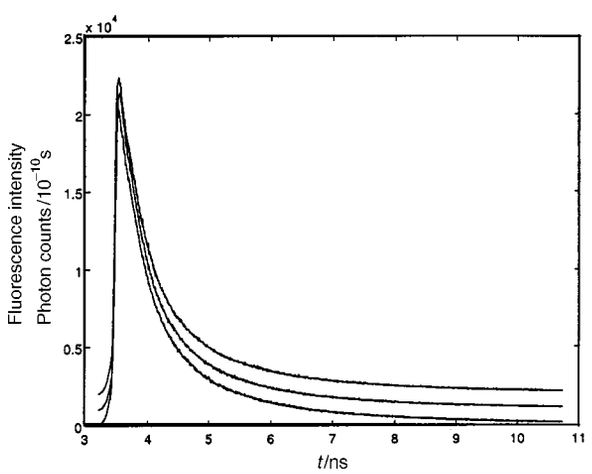

Fig. 1 Fluorescence decay for the peptides **15**, **16** and **19** measured by TCSPC[22] with the timescale in nanoseconds given on the *x*-axis. The decay curves completely overlapped, so in order to show the three curves these are shifted axially.

Conclusions

In conclusion, glycosylation stabilizes the peptide substrates to various degrees depending on the site of glycosylation relative to the scissile bond. The most dramatic effect was observed in P1 and the stabilising effect was similar to that observed with a more flexible and polar lactitol residue. The substrate side chains, which according to modelling studies point away from the active site, also tolerate the substitution without significant loss of specificity whereas those buried in the hydrophobic pockets of the enzyme do not. As seen by fluorescence decay these effects do not result from differences in conformation of the substrates due to glycosylation.

Experimental

General experimental

Analytical TLC was performed on Merck Silica Gel 60 F_{254}

aluminium sheets with detection by UV light and by charring with sulfuric acid. Compounds were purified by medium-pressure chromatography on Kieselgel 60 (230–400 mesh). For chromatography under anhydrous conditions, Kieselgel 60 (230–400 mesh) was dried at 120 °C for >24 h prior to use. Solvents were purchased from Labscan Ltd. (Dublin, Ireland). Light petroleum was the 60–80 °C fractions. Dichloromethane was distilled over P_4O_{10}. DMF was freshly distilled by fractional distillation at reduced pressure and kept over 3 Å molecular sieves. Thionyl dichloride, pyridine and THF were distilled prior to use. Concentrations were performed under reduced pressure at temperatures <30 °C (bath). HMBA and suitably protected N^{α}-Fmoc amino acids were purchased from NovaBiochem (Switzerland), Macrosorb SPR 250 resin from Sterling Organics (England), trimethylsilyl chloride (TMS-Cl), Dhbt-OH, NEM and TBTU from Fluka (Switzerland) and BSA, MeIm, MSNT and TFA from Merck (Germany). N^{α}-Fmoc-Tyr(NO_2)-OH and Boc-ABz-ODhbt were prepared as previously described.[5] The peptides and the glycopeptides were hydrolysed 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 (ES-MS) were recorded in the positive mode on a VG Quattro Mass Spectrometer from Fisons, with 50% aqueous acetonitrile as the liquid phase. NMR spectra were recorded on a Bruker AM-500 or a Bruker AMX-600 MHz spectrometer. For all compounds, the assignment of ^{1}H and ^{13}C spectra was based on 2D proton–proton shift-correlation spectra. The assignment of ^{13}C NMR spectra was based on carbon–proton shift correlation. NMR spectra were recorded in $CDCl_3$, D_2O or 50% deuterated acetic acid–water (CD_3CO_2D–H_2O) [reference: acetic acid at 2.03 ppm (^{1}H)] or in $CDCl_3$ [reference: chloroform at 7.3 ppm (^{1}H) and 77.0 ppm (^{13}C)]. 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^{-1} pump heads. The system was fitted with a switchable Delta Pak (25 × 200 mm, 10 ml min^{-1}) and a preparative radial pack column (50 × 300 mm, 20 ml min^{-1}), both packed with reversed-phase C_{18} or a Shodex DS-2013 column (20 × 300 mm, 4 ml min^{-1}). Analytical HPLC was performed using a Waters RCM 8 × 10 module with a Waters 8 NV C_{18} (4 μm) column (1 ml min^{-1}). 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. Mps were measured on a Büchi melting-point apparatus and are uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter, and $[a]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. Microanalysis was provided by Leo Pharmaceutical Products (Ballerup, Denmark) or Department of Chemistry, Copenhagen University (Denmark). Savinase from *Bacillus lentus* was prepared and purified as previously described.[6] The enzymic hydrolysis of peptide and glycopeptide substrates was performed as described[6] and followed on a Perkin-Elmer luminescence spectrometer LS 50.

Solid-phase peptide synthesis. General procedure

Synthesis of the peptides was performed on the PEGA resin in DMF. The resin (2 g, 2.0 mmol) was allowed to swell in DMF (25 ml) for 2 h and was treated with 20% piperidine in DMF (20 ml) for 10 min to remove any chloride ions and expose the free amino groups on the resin. For the substrates **8**–**18** the resin was washed with DMF (5 × 20 ml) and was derivatized with the HMBA linker in the presence of TBTU (185 mg, 0.58 mmol) and NEM (0.15 ml, 1.18 mmol) as previously described.[8] The anchoring of the first amino acid was performed with N^{α}-Fmoc-Asp(OBu^t)-OH (284 mg, 0.69 mmol), MeIm (40 ml, 0.50 mmol) and MSNT (204 mg, 0.69 mmol) in anhydrous CH_2Cl_2 (25 ml) essentially as previously described.[8] The degree of incorporation of the aspartic acid was determined by quantitative amino acid analysis and was found to be 60–65% in both cases. The synthesis was performed simultaneously, in a 20-well Teflon block, each peptide being synthesized in two adjacent wells. Each well contained 50–60 mg of derivatised resin. Free amino groups were capped with 10% acetic anhydride in DMF for 10 min. The cleavage of the N^{α}-Fmoc protecting group was accomplished by treatment with 20% piperidine in DMF for 10 min. This was followed by washing with DMF (1.5 min × 6). The coupling of subsequent amino acids was performed with the N^{α}-Fmoc-protected Pfp esters (3 equiv.). The side chains on the aspartic acid and glutamic acid were protected with *tert*-butyl groups. Glutamine was used as its triphenylmethyl (trityl) derivative, lysine as its *tert*-butoxycarbonyl (Boc), and threonine as its *tert*-butyl derivative. Dhbt-OH (1 equiv.) was added for each coupling as the auxiliary nucleophile to catalyse the acylation. Couplings were allowed to proceed for 4–6 h; 2 equiv. of the glycosylated asparagine building block **7** were employed, the final aminobenzoyl group was introduced with Boc-ABz-ODhbt[5] and the coupling was allowed to proceed for 16 h. Following the final coupling, the resin was washed with piperidine (30 min), followed by DMF (×10) and CH_2Cl_2 (×10) and dried for 2 h. For the substrates **8**–**18** treatment of the resin with 95% aqueous TFA for 3 h effected side-chain deprotection. The wells were washed with 95% aqueous acetic acid (4 × 5 min) followed by neutralization with 5% ethyldiisopropylamine in DMF (2 × 2 min). Final washes were performed with DMF (×10) and CH_2Cl_2 (×10) and the resin was air dried for 2 h. The cleavage of the substrates from the resin was carried out with aqueous sodium hydroxide (0.1 M) for 3 h, which also effected the removal of the acetyl protecting groups on the lactosylamine moiety in the glycopeptides. Cleavage of the substrates from the resin was followed by observing the colour of the resin, which lost its yellow colour after the substrates had been cleaved. Although the peptides required a single treatment with sodium hydroxide for cleavage, the glycopeptides required two to three. The solutions were concentrated and lyophilised. All compounds were purified by reversed-phase HPLC and analysed by amino acid analysis and electrospray mass spectroscopy.

2,3,6-Tri-*O*-acetyl-4-*O*-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosyl azide 4. Stannic [tin(IV)] chloride (0.8 ml, 6.8 mmol) in anhydrous CH_2Cl_2 (26 ml) was added to a solution of 1,2,3,6-tetra-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α/β-D-glucopyranose **3**[23] (3.4 g, 5.0 mmol) and trimethylsilyl azide (0.8 ml, 6.0 mmol) in CH_2Cl_2 (26 ml). The reaction mixture was stirred at room temperature, under an inert atmosphere, for 4 h at which point TLC indicated completion of reaction. The reaction mixture was washed successively with water and saturated aqueous sodium hydrogen carbonate (×2). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was purified by column chromatography with hexane–ethyl acetate as eluant (R_f = 0.36) to afford the *title compound* **4** (3.0 g, 91%) as a foam, $[a]_D^{20}$ −12.1 (*c* 13.7 in CH_2Cl_2); δ_C($CDCl_3$) 20.48, 20.62, 20.72, 20.79 (7 × $COCH_3$), 60.77 (C-6′), 61.72 (C-6), 66.57 (C-4′), 69.06 (C-2′), 70.77, 70.93, 70.99 (C-2, -3′, -5), 72.53 (C-3), 74.81 (C-5), 75.78 (C-4), 87.71 (C-1), 101.12 (C-1′), 169.04, 169.46, 169.60, 170.02, 170.08, 170.28 (7 × $COCH_3$); δ_H($CDCl_3$) 1.90, 1.98, 1.99, 2.00, 2.07, 2.08 (21H, 6 × s, 7 × $COCH_3$), 3.74 (1H, ddd, $J_{4,5}$ 9.8, $J_{5,6a}$ 5.1, $J_{5,6b}$ 2.2 Hz, H-5), 3.86 (1H, t, $J_{3,4+4,5}$ 19.0 Hz, H-4), 3.91 (1H, m, H-5′), 4.1–4.2 (3H, m, H-6a, -6a′, -6b′), 4.51 (1H, d, $J_{1',2'}$ 7.9 Hz, H-1′), 4.54 (1H, dd, $J_{5,6b}$ 2.2, $J_{6a,6b}$ 12.7 Hz, H-6b), 4.66 (1H, d, $J_{1,2}$ 8.5 Hz, H-1), 4.66 (1H, t, $J_{1,2+2,3}$ 18.4 Hz, H-2), 5.23 (1H, dd, $J_{2',3'}$ 10.4, $J_{3',4'}$ 3.6 Hz, H-3′), 5.14 (1H, t, $J_{1',2'}$ 7.9, $J_{2',3'}$ 10.4 Hz, H-2′), 5.25 (1H, t, $J_{2,3+3,4}$ 18.5 Hz, H-3), 5.39 (1H, dd, $J_{3',4'}$ 3.5, $J_{4',5'}$ 0.9

Hz, H-4′) (Calc. for $C_{26}H_{35}N_3O_{17}$: C, 47.20; H, 5.33; N, 6.35. Found: C, 47.08; H, 5.35; N, 6.63%).

2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosylamine 5. The lactopyranosyl azide **4** (0.5 g, 0.75 mmol) was dissolved in a mixture of methanol (11 ml) and THF (11 ml). Triethylamine (0.21 ml, 1.5 mmol) and Lindlar's catalyst (0.7 g) were added and the reaction mixture was stirred under a positive pressure of hydrogen for 16 h. The reaction mixture was filtered through Celite, concentrated *in vacuo* below 15 °C and column chromatographed with ethyl acetate–methanol (5 : 0.25) as eluant ($R_f = 0.38$). The title compound **5** was obtained (0.45 g, 96%). ^{1}H and ^{13}C NMR data were the same as previously published.[15] ES-MS 636.12 $[M + H]^+$. $C_{26}H_{37}NO_{17}$ requires *M*, 635.2.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{γ}-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosyl]-L-asparagine pentafluorophenyl ester 7. A solution of 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosylamine **5** (0.45 g, 0.75 mmol) and NEM (0.1 ml, 0.8 mmol) in anhydrous THF (5 ml) was added dropwise to N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-aspartic acid 4-chloride 1-pentafluorophenyl ester **6** in anhydrous THF (5 ml) at −40 °C under an inert atmosphere. The reaction mixture was warmed to room temperature, filtered, concentrated and column chromatographed on a short column of pre-dried silica gel with hexane–ethyl acetate (1 : 1.8) as eluant ($R_f = 0.4$). The title compound **7** was obtained as a powder (0.26 g, 31%). ^{1}H and ^{13}C NMR data were in agreement with published data.[15] ES-MS 1139.2 $[M + H]^+$. $C_{51}H_{51}F_5N_2O_{22}$ requires *M*, 1138.9.

ABz-Phe-Gln-Pro-Leu-Asp-Glu-Tyr(NO_2)-Asp-OH 8. The crude product 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 t_R 45.3 min) to give pure **8** (13 mg, 55%). Amino acid analysis is presented in Table 3. ES-MS 1190.7 $[M + H]^+$. $C_{54}H_{67}N_{11}O_{20}$ requires *M*, 1189.5.

ABz-Asn-Gln-Pro-Leu-Asp-Glu-Tyr(NO_2)-Asp-OH 9. This compound was available from previous studies.[8]

ABz-Asn-{N^{γ}-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]}-Gln-Pro-Leu-Asp-Glu-Tyr(NO_2)-Asp-OH 10. The crude product was purified by semipreparative HPLC (Delta Pak column; 0 min 10% B, then a linear gradient of 0–45% B in 90 min; t_R 69 min) to give pure **10** (6.0 mg, 41%). NMR data and amino acid analysis are presented in Tables 2 and 3. ES-MS 1481.8 $[M + H]^+$. $C_{61}H_{84}N_{12}O_{31}$ requires *M*, 1480.6.

ABz-Phe-Asn-Pro-Leu-Asp-Glu-Tyr(NO_2)-Asp-OH 11. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 0% B, then a linear gradient of 0–75% B in 65 min; t_R 57 min) to give pure **11** (14 mg, 61%). Amino acid analysis is presented in Table 3. ES-MS 1176.6 $[M + H]^+$. $C_{53}H_{65}N_{11}O_{20}$ requires *M*, 1175.4.

ABz-Phe-Asn-{N^{γ}-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]}-Pro-Leu-Asp-Glu-Tyr(NO_2)-Asp-OH 12. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 0% B, then a linear gradient of 0–70% B in 80 min; t_R 59 min) to give pure **12** (19.0 mg, 63%). NMR data and amino acid analysis are presented in Tables 2 and 3. ES-MS 1500.8 $[M + H]^+$. $C_{65}H_{85}N_{11}O_{30}$ requires *M*, 1499.6.

ABz-Phe-Gln-Pro-Asn-Asp-Glu-Tyr(NO_2)-Asp-OH 13. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 25% B, then a linear gradient of 25–75% B in 90 min; t_R 33 min) to give pure **13** (11 mg, 48%). Amino acid analysis is presented in Table 3. ES-MS 1191.5 $[M + H]^+$. $C_{52}H_{62}N_{12}O_{21}$ requires *M*, 1190.5.

ABz-Phe-Gln-Pro-Asn-{N^{γ}-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]}-Asp-Glu-Tyr(NO_2)-Asp-OH 14. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 10% B, then a linear gradient of 10–70% B in 80 min; t_R 49 min) to give pure **14** (14.2 mg, 46%). NMR data and amino acid analysis are presented in Tables 2 and 3. ES-MS 1515.8 $[M + H]^+$. $C_{64}H_{82}N_{12}O_{31}$ requires *M*, 1514.5.

ABz-Phe-Gln-Pro-Leu-Asn-Glu-Tyr(NO_2)-Asp-OH 15. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 25% B, then a linear gradient of 25–70% B in 90 min; t_R 46 min) to give pure **15** (14.3 mg, 61%). Amino acid analysis is presented in Table 3. ES-MS 1189.6 $[M + H]^+$. $C_{54}H_{68}N_{12}O_{19}$ requires *M*, 1188.5.

ABz-Phe-Gln-Pro-Leu-Asn-{N^{γ}-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]}-Glu-Tyr(NO_2)-Asp-OH 16. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 25% B, then a linear gradient of 25–70% B in 90 min; t_R 36 min) to give pure **16** (15 mg, 50%). NMR data and amino acid analysis are presented in Tables 2 and 3. ES-MS 1514.0 $[M + H]^+$. $C_{66}H_{88}N_{12}O_{29}$ requires *M*, 1512.7.

ABz-Phe-Gln-Pro-Leu-Asp-Asn-Tyr(NO_2)-Asp-OH 17. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 25% B, then a linear gradient of 25–70% B in 90 min; t_R 41 min) to give pure **17** (10.0 mg, 43%). Amino acid analysis is presented in Table 3. ES-MS 1175.9 $[M + H]^+$. $C_{53}H_{68}N_{12}O_{19}$ requires *M*, 1174.5.

ABz-Phe-Gln-Pro-Leu-Asp-Asn-{N^{γ}-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]}-Tyr(NO_2)-Asp-OH 18. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 25% B, then a linear gradient of 25–50% B in 50 min; t_R 39 min) to give pure **18** (12.4 mg, 41%). NMR data and amino acid analysis are presented in Tables 2 and 3. ES-MS 1199.7 $[M + H]^+$. $C_{65}H_{86}N_{12}O_{29}$ requires *M*, 1498.6.

*Conformational assessment for compounds **15**, **16** and **19** by fluorescence decay measurement.* The fluorescence decay for the ABz moiety in peptides **15**, **16** and **19** were measured by Time-Correlated Single-Photon Counting as described previously.[22] All samples were prepared in tris buffer (pH 8.5) and the concentrations were in the region of 10 μmol, where the intermolecular energy transfer is negligible. Data were acquired to approximately 2×10^4 counts in the maximum channel and for comparing the decay curves these were scaled to the same maximum count.

*Determination of enzyme activity and kinetic constants for compounds **8**–**18**.* The substrates were dissolved in DMF at concentrations of 3–19 μM. 50 μL of substrate solution was added to 2450 μL of assay buffer [50 mM *N*,*N*-bis-(2-hydroxyethyl)glycine (bicine), 2 mM $CaCl_2$, 0.1 M KCl (pH = 8.5)] to give initial substrate concentrations in the cuvette, s_o, of 0.06–0.38 μM. The initial fluorescence was determined and 10 μL of an appropriately diluted enzyme solution was added to the assay buffer, final enzyme concentration e_o being 0.1–100 nM. Cleavage of the substrate was followed with time by monitoring the emission at 420 nm (10 nm slit) upon excitation at 320 nm (10 nm slit). At each substrate concentration, s_o, the initial velocity of cleavage was determined from the slope of the initial part of the progress curve (emission *vs.* time). The k_{cat}/K_M-values were determined from the initial velocities with the relation $v_o = (k_{cat}/K_M)e_o s_o$ which is valid at low substrate concentrations ($s_o \ll K_M$) 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 devi-

ations observed during repeated determination of k_{cat}/K_M were ~5% and the k_{cat}/K_M-values are presented in Table 3.

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