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Synthesis and Conformational Analysis of N-glycopeptides that Contain Extended Sugar Chains

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Abstract

Maltooligosaccharides with 2-7 sugar moieties were converted into β-1-amino-1-deoxy derivatives and were coupled to N^{α}-fluorenylmethoxycarbonyl-L-aspartic acid α-*tert*-butyl β-pentafluorophenyl ester. After trifluoroacetic acid deprotection, the resulting glycosylated asparagines were used as building blocks for the solid-phase synthesis of T-cell epitopic glycopeptide analogues. The coupling efficiencies of the glycoamino acid synthons and the acid and base stability of the resulting glycopeptides indicate the applicability of this solid-phase synthetic protocol for the incorporation of sugars that are comparable in size with that of the natural carbohydrate antennae of N-glycoproteins. The sugars placed into N-terminal position did not affect the strong α-helical structure of the peptides, but inhibited the disulfide-bridge formation of proximal cysteine residues in a carbohydrate length-dependent manner.

Introduction

Although many eukaryotic proteins carry covalently linked oligosaccharides, at present little is known about the role(s) of the sugar side chains in biological processes.[1] Since synthetic glycopeptide models are considered to be effective tools for investigating the impact of glycosylation on the biological, physical, and chemical properties of protein fragments, the development of widely applicable techniques for solid phase synthesis of glycopeptides is of considerable interest. The preparation of short and medium-sized (5-20 amino acid residues) peptides carrying mono- and disaccharide moieties has been reported for both the O-glycosylated[2] and the N-glycosylated series.[3-5] Numerous serine- and threonine-bound saccharides contain carbohydrate chains consisting of one or two sugar moieties, and appropriate glycopeptides can be made without considerable difficulty, provided that α-anomeric connection is achievable.[6] The extended sugar chain of asparagine bound glycopeptides[1] makes their synthesis more complicated. Since the side-chain carboxyl group of the aspartic acid is prone to succinimide formation with the amide group of its C-terminal amino acid when activation of that particular carboxyl is attempted in peptides,[7] the use of N-terminally protected glycosylated asparagine synthons is the method of choice for N-glycopeptide synthesis.[8] Application

of the methodologies of classical sugar chemistry is less suitable for the custom synthesis of longer sugar-conjugted N-glycopeptides because the conventionally used bulky sugar-hydroxyl protecting groups slow down the coupling reactions to such an extent that the coupling efficiencies are not compatible with the accepted rates for automated solid-phase synthesis.[3] N^{α}-protected N^{β}-glycosylated asparagines that lack sugar side chain protection, however, couple much faster[3] and their use can be justified by two observations. First, similar to the unprotected serine and threonine hydroxyls,[9] the activated amino acids that are currently commonly used in peptide synthesis do not acylate the free hydroxyl groups of the sugars regardless of the identity of the commonly used activating agents.[3,10] Second, most of the glycosidic bonds survive the trifluoroacetic acid (TFA) treatment used for the final cleavage and deprotection of the peptides.[11] Although this methodology was successfully applied for N-glycopeptides containing carbohydrate chains of 1-3 sugar moieties,[12,13] the natural antennae in N-glycoproteins consist of at least 6-10 sugar residues. Due to the unavailability of the required amount of the sugar systems found in glycoproteins, simplifications are needed in the design of the chemical synthesis of the glycopeptides. A continuously growing number of reports suggests that one of the major functions of both N- and O-linked glycosylation is to form or maintain the secondary-tertiary structure of the proteins rather than to bind to the appropriate biological counterparts.[14] It was demonstrated by spectroscopic evidence that glycosylation affects the secondary structure of synthetic peptides[15-17] but this effect does not require the presence of the natural acetamido group at C2, and is independent of the anomeric configuration of the interconnected sugars.[15] Based on these observations, we selected α-anomeric oligomers of D-glucose to develop methodology for the synthesis of glycopeptides with extended sugar chains. The maltobiose, -tetraose, -hexaose, and -heptaose are easily available and inexpensive sugars (an attractive feature for later peptide-based drug design[18]), and the upper range of this scale is now comparable in size with the natural carbohydrate antennae.

Recent reports of increased solubility and, consequently, oral availability of peptide drug leads upon glycosylation[19] have indicated the applicability of glycopeptides in rational drug design. In our experience, glycosylated versions of peptide T, octa-, and pentapeptide fragments of the glycoprotein gp120 of the California HIV isolate showed a dramatically increased resistance to serum proteases, especially when the sugar was positioned at the N-termini,[20] the usual site of peptidase cleavage in this peptide family.[21] From this perspective it is tempting to speculate that terminal glycosylation will provide peptide drug candidates with favorable physical and biological properties.

Incorporation of mono- and disaccharides into the mid-chain position of dodeca- and tridecapeptides breaks the dominant α-helical conformation as demonstrated by CD measurements in water-trifluoroethanol (TFE) mixtures.[15] The conservation of α-helical structure in the non-bonded state (as opposed to the MHC-bound extended conformation[22]) is thought to be important in maintaining the biological functions of T-cell epitopes,[23] and perhaps of applied pharmaceuticals.

This paper reports the synthesis of new glycosylated asparagine synthons with extended sugar length and their applicability in solid phase peptide synthesis. Because the size of the sugar may mimic the natural antennae in glycoproteins if function such as T-cell activity is not specific to the structure of the sugar,[24] these models may be useful for the design of syntheses of glycopeptides with natural antennae. The synthons used here were built onto N-terminal positions of T-cell determinant peptides which correspond to a sugar-dependent and a sugar-independent epitope of the hemagglutinin of influenza virus.[25] The effect of the sugar side chain on the conformation of the peptide was also scrutinized by CD and nuclear magnetic resonance (NMR)

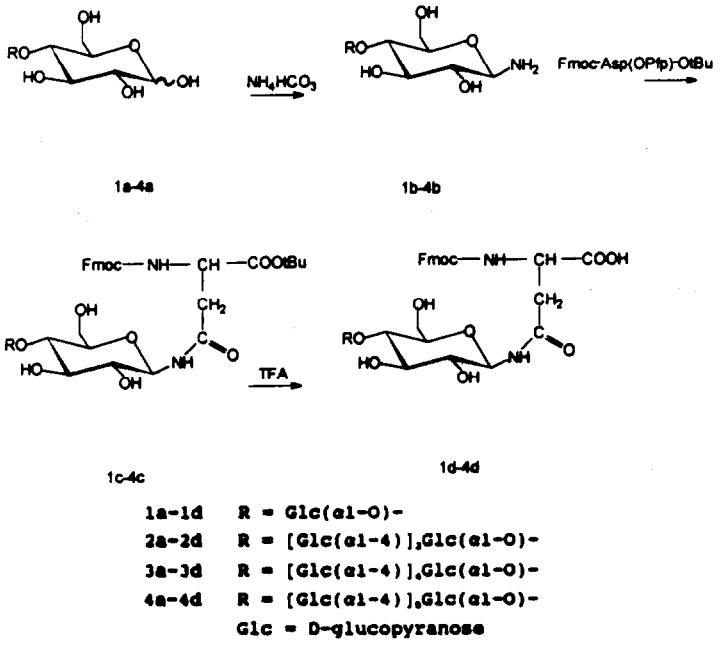
spectroscopy. Since post-translationally modified peptides are believed to bind to the major histocompatibility complex but fail to be further recognized,[24,26] this model is appropriate for determining if glycosylation of T-cell epitopes of autoantigens is suitable for down-regulating unwanted immune responses.[25]

Results and Discussion

Glycopeptide Synthesis.

Synthesis of glycosylated asparagines. Maltobiose (**1a**), maltotetraose (**2a**), maltohexaose (**3a**), and maltoheptaose (**4a**) were converted into 1-amino-1-deoxy derivatives (**1b-4b**) by using saturated ammonium bicarbonate.[27] (**Scheme I.**)

Scheme I.



1a-1d R = Glc(α1-O)-
2a-2d R = [Glc(α1-4)]$_2$Glc(α1-O)-
3a-3d R = [Glc(α1-4)]$_3$Glc(α1-O)-
4a-4d R = [Glc(α1-4)]$_4$Glc(α1-O)-
Glc = D-glucopyranose

The crude 1-amino-1-deoxy sugars were selectively N-acylated in a mixture of dimethyl formamide/water[11] with N$^{\alpha}$-fluorenylmethoxycarbonyl-L-aspartic acid α-*tert*-butyl β-pentafluorophenyl ester (Fmoc-Asp(OPfp)-O^{t}Bu) yielding the asparagine conjugates (**1c-4c**). The crude disaccharide-coupled asparagine derivative (**1c**) was the only one that could be purified by trituration with ether and water,[11,13] because as the length of the sugar increased the asparagine conjugates (**2c-4c**) became increasingly water soluble. In order to remove the unreacted 1-amino sugars, **2c-4c** were subjected to reverse phase high performance liquid chromatography (RP-HPLC) purification; it was found that the longer the sugar, the lower the yield (Table 1). Because of the expense of long sugars, we collected the HPLC flow-through containing

Table 1. Characterization of the Glycosylated Asparagine Conjugates

Compound #	RP-HPLC ret. time (min)	LSIMS ($M+H^+$)	Yield (percent)
1c	36.46	735	35
2c	35.02	1060	23
3c	34.14	1383	14
4c	33.72	1546,1564 ($M+Na^+$)	15
1d	29.92	679	34
2d	28.73	1003	22
3d	27.82	1327,1349 ($M+Na^+$)	13
4d	27.57	1512($M+Na^+$)	13

the nonreacted oligosaccharides and after removal of the solvent by lyophilization, **4a** was subjected to a second round of amination[27] and treatment as described above. By doing this, **4c** was obtained in comparable yield and purity as in the first synthesis. By using this method any reducing sugar can be converted into its asparagine conjugate with high efficiency.

The *tert*-butyl group of **1c-4c** was removed by treatment with 100% TFA for 20 min yielding the synthons **1d-4d** that were subsequently used as building blocks for the solid-phase synthesis of glycopeptides. The analytical data of these Fmoc-Asn(sugar)-OH derivatives are listed in (Table 1). Analytical RP-HPLC and ^{1}H-NMR did not indicate any significant cleavage at either the N- or O-glycosidic bonds after the 20 min acid treatment indicating that oligosaccharide side chains of any length survive the conventional acidic conditions by which glycopeptides are cleaved off the polystyrene-polyepoxy copolymer resin. The stability of the majority of the glycosidic bonds of mono- and disaccharides during TFA treatment has been reported earlier.[10,11,13,28] In order to determine the acid stability of the longest sugar and to find optimal conditions for the final cleavage of the glycopeptides from the acid-sensitive resin, **4d** was kept in TFA at room temperature and the decomposition was monitored by analytical HPLC (Figure 1). The rate of decomposition increased by adding

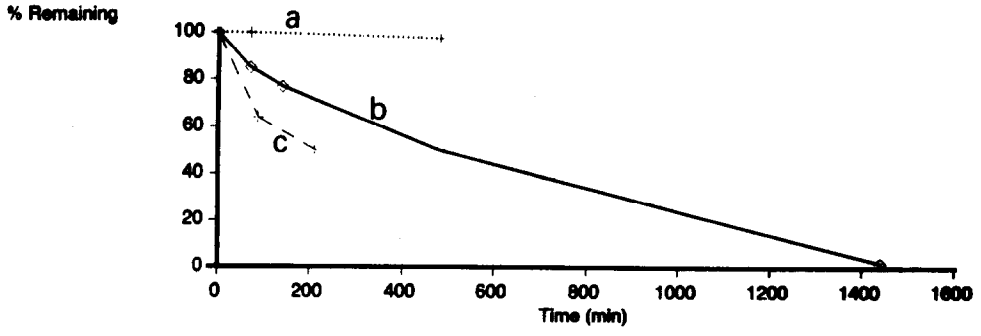


Figure 1. Decomposition of the glycosidic bonds in different solvents used during the conditions of solid-phase peptide synthesis; a: N(maltohexaose) KYVKQNTLKLA (**7**) in 20% piperidine/DMF, b: Fmoc-Asn (Glc_7)-OH (**4d**) in 100% TFA, c: Fmoc-Asn (Glc_7)-OH (**4d**) in 95% TFA and 5% water.

5% water to the TFA. For this reason, we recommend using a 1-h cleavage time and avoiding the addition of water (Figure 1). It has been suggested that water be added as part of a scavenger mixture for peptides that contain certain guanidino-protecting groups of arginine residues.[29]

Synthesis of glycopeptides. The suitability of synthons **1d-4d** for solid phase peptide synthesis was tested by coupling their pentafluorophenyl ester derivatives to resin bound T-cell epitopic peptides CTLIDALLGDPH (coupled with **1d, 2d, 4d**) and KYVKQNTLKLA (coupled with **1d, 3d**). Pentafluorophenyl esters of **1d-4d** were formed in situ in dimethylformamide (DMF) (in which **1d** and **2d** are very soluble while **3d** and **4d** are moderately soluble). Compounds **1d** and **2d** were allowed to acylate the free N-terminal amino group of the resin-bound peptide using a 4 molar excess in a 0.2 molar solution overnight. The coupling efficiencies were 78% and 53%, respectively, indicating increased steric hindrance as the length of the sugar side chain increased. Because of the cost and lower solubility in DMF of the six and seven sugar-coupled asparagine derivatives, **3d** and **4d** were coupled using a 10% excess of the peptide in a 0.05 molar solution. On this occasion, the coupling efficiencies were 24% and 30%, respectively. In contrast, higher coupling efficiencies were reported during the synthesis of O-glycopeptides, using a trisaccharide-conjugated threonine residue.[30] In order to compare the applicability of the 2-(^{1}H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate activation method,[31] **3d** was also activated and coupled using this reagent. No significant difference in the coupling efficiencies of this longer sugar conjugate was found. After removal of the Fmoc protecting group with 20% piperidine for 20 min, the glycopeptides **2-4, 6-7** were cleaved off the resin with 95% TFA and 5% thioanisole (v/v) for 60 min. RP-HPLC profiles of the crude products showed an additional, more hydrophilic peak eluting earlier than the asparagine-deleted peptide.[32] No significant amount of other side products were detected. Table 2 summarizes the properties of the glycopeptides purified by preparative HPLC. Due to the increased hydrophilicity, the retention times of the peptides conjugated with longer carbohydrate side chains are lower; the decrease of the retention times, however, is not linear. Similar to our earlier experiments with glycopeptides bearing mono- and disaccharides,[3,32] the difference in the retention times decreases as the size of the sugar homologs increases. Because this nonlinear decrease in retention time is observed for both sets of glycopeptides as well as for the Fmoc-protected glycosylated asparagine derivatives,

Table 2. Characterization of the Peptides and Glycopeptides Corresponding to the Influenza Virus Hemagglutinin

Peptide #	Sequence[a]	RP-HPLC ret time	LSIMS ($M+H^+$)
1	NCTLIDALLGDPH	35.8 (41.3)	1382
2	N(maltobiose)CTLIDALLGDPH	34.4 (39.2)	1705
3	N(maltotetraose)CTLIDALLGDPH	33.8 (38.4)	2029
4	N(maltoheptaose)CTLIDALLGDPH	33.3 (38.0)	2516
5	NKYVKQNTLKLA	23.6	1420
6	N(maltobiose)KYVKQNTLKLA	22.4	1766
7	N(maltohexaose)KYVKQNTLKLA	21.9	2392

[a] All peptides were amide at the C-termini and free at the N-termini.

the possibility of conformational orientation on the surface of the bonded phase[32,33] is not likely and may indicate that no change in the conformation of the parent peptides occurs after glycosylation.

Currently, 20% piperidine in DMF is the reagent of choice for the repetitive removal of the N-terminal Fmoc protecting group during automated solid-phase peptide synthesis.[34] We investigated the stability of glycopeptide **7** in the above solvent mixture to obtain insights into the general applicability of our procedure. RP-HPLC did not show any decomposition of the O- and N-glycosydic bonds (Figure 1), indicating that further elongation of the peptide backbone after incorporating synthons **1d-4d** in the mid-chain position is predicted to be trouble-free.

Secondary Structural Analysis of the Peptides and Glycopeptides.

CD studies. CD spectra of the peptides and glycopeptides (**1-7**) were measured in TFE/water mixtures. TFE is frequently used to decrease the dielectric constant of water[35] and to stabilize incipient peptide conformations.[36] It is reported that TFE does not induce ordered structures in protein fragments if the same sequence of the native protein lacks ordered structures.[37] Because of this and also because of their excellent solubilizing properties, TFE-water mixtures are favored solvents for chiroptical studies of small- and medium-sized peptides.

The CD spectra of the peptides below 250 nm are dominated by the most abundant amide chromophore of the chiral peptide backbone.[38] However, in the case of glycopeptides, especially N-glycopeptides, the amide bonds of the asparagine-sugar linkage (and the extra amide bonds of the acetamido-sugars in natural antennae) need to be considered as it was shown earlier for short- and medium-sized peptides that carried N-acetyl-glucosamine moieties.[15,39] The current study aims to get insights into the effect of the elongation of the carbohydrate chain on the spectral contribution of the N-glycosidic bond. Due to its proximity to the rigid hexopyranose ring, this sugar-amide bond is located in a well-defined, strongly chiral environment. The larger the ratio of the number of sugar amides: peptide backbone amides, the more pronounced CD contribution of the

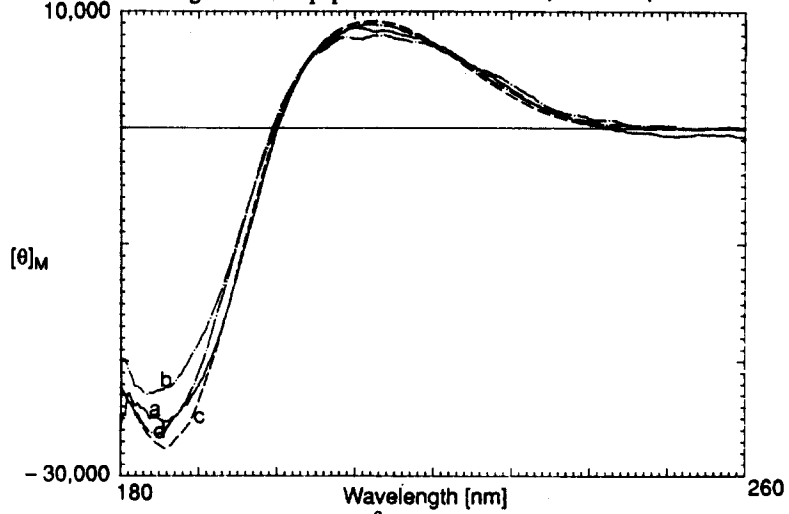


Figure 2. CD spectra of the sugar standards Nβ-[O-α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-L-asparagine (**1e**); a: in water (—); b: in 50% TFE (—.—.), and 1-acetamido-1-deoxy-β-D-glucose; c: in water (— — —); d: in 75% TFE (—.—.—).

sugar side chain can be predicted. In order to estimate this CD contribution, the CD spectra of the asparagine and various glycosylated asparagine residues were examined. While the asparagine has practically no CD effect in the 180-260 nm region, the N^{β}-[O-α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-L-asparagine (**1e**) shows a strong negative band at 187 nm and a weaker positive band at 209 nm in water and in different water/TFE mixtures (Figure 2), suggesting that the CD contribution of the amide of the glycosylated asparagine arises mostly from the chirality of the sugar. This was further verified by the observation that the CD spectra of 1-acetamido-1-deoxy-β-D-glucose are identical with the CD spectra of **1e** at each of the solvent compositions examined (Figure 2) as the CD spectra of 1,2-diacetamido-1,2-dideoxy-β-D-glucopyranose[15,39] and of N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine are. We have also examined the effect of elongation of the sugar chain on the CD of the glycosylated asparagine derivatives. The CD spectra of **1d-4d** were almost identical in any solvent studied (Figure 3). Comparison of the CD spectra of **1e** (Figure 2), **1d**, and Fmoc-Asn-OH (Figure 3) in 75% TFE demonstrates that the spectral contribution of the Fmoc group is negligible to the chiral perturbing effect of the sugar moieties. Although the increased solvent dependence of the CD spectra after incorporation of the Fmoc group suggests some influence of the fluorene chromophore, the increase of the

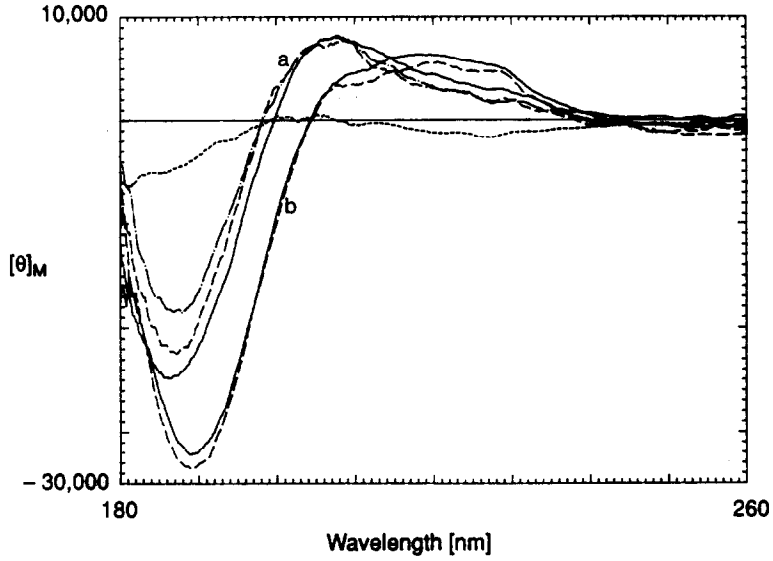


Figure 3. CD spectra of the Fmoc protected glycosylated asparagine conjugates. Curves a in 75% TFE: Fmoc-Asn(Glc_2)-OH (**1d**) (—), Fmoc-Asn(Glc_4)-OH (**2d**) (— — —), Fmoc-Asn(Glc_7)-OH (**4d**) (—.—.—), Fmoc-Asn-OH (--------). Curves b in water: Fmoc-Asn(Glc_2)-OH (**1d**) (—), Fmoc-Asn(Glc_4)-OH (**2d**) (— — —).

length of the sugar chain does not effect the CD spectra in any solvent systems studied. This indicates that neither the second ring, nor the more remote pyranose rings that lack the amide group, nor the (presumably) helical conformation of the malto-oligosaccharide chain[41] has a significant effect on the CD contribution of the N-glycosidic bond. Consequently, the CD spectra of any of the glycosylated asparagine standards of the maltooligosaccharide series can be used for estimating the chiral side chain contribution of the N-glycosidic

bond. We have subtracted the CD spectra of **1e** from the CD spectra of all of the glycopeptides **2-4** and **6-7** in order to obtain the true CD profile of the peptide backbone. Since the CD spectra of the glycosylated asparagine (**1e**) itself do not show any solvent dependence (Figure 2), any change in the CD spectra of the glycopeptides upon changes in the composition of the solvents can be attributed to the conformational differences of the peptide backbone.

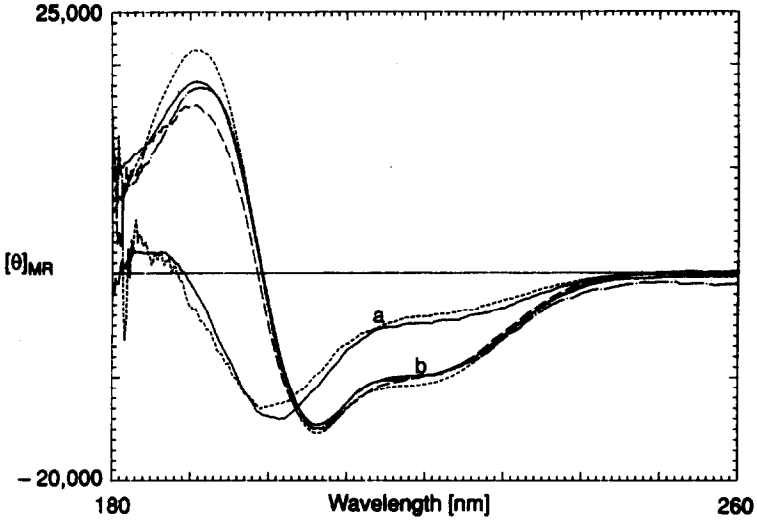


Figure 4. CD spectra of peptides **1-4**. Curves a in water: NCTLIDALLGDPH (—), N(maltoheptaose)CTLIDALLGDPH (--------). Curves b: NCTLIDALLGDPH in 25% TFE (—) and in 100% TFE (------), N(maltoheptaose)CTLIDALLGDPH in 25% TFE (— — —) and in 90% TFE (—.—.—).

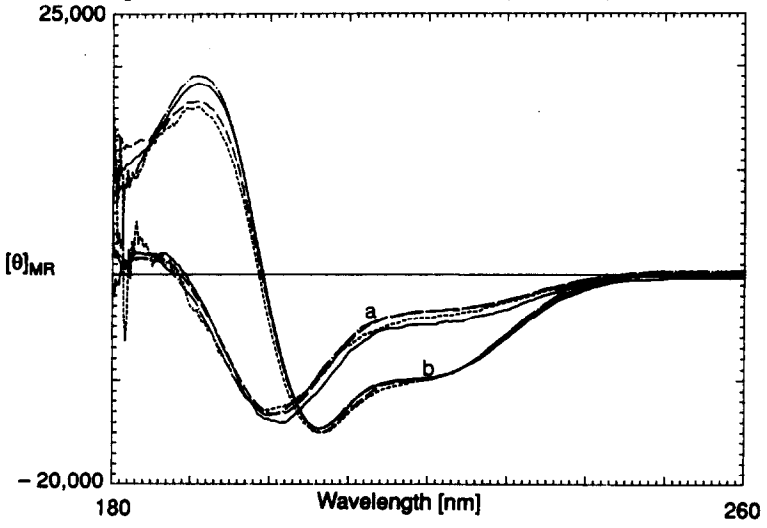


Figure 5. CD spectra of peptides **1-4**. Curves a in water: NCTLIDALLGDPH (—), N(maltobiose)CTLIDALLGDPH (.—.—.—), N(maltotetraose)CTLIDALLGDPH (— — —), N(maltoheptaose)CTLIDALLGDPH (-----). Curves b in 25% TFE: NCTLIDALLGDPH (—), N(maltobiose)CTLIDALLGDPH (.—.—.—), N(maltotetraose)CTLIDALLGDPH (— — —), N(maltoheptaose)CTLIDALLGDPH (-----).

The CD spectra of parent, nonglycosylated peptide **1** in water shows a strong, wide, negative band at 200 nm and a strong shoulder at 225 nm (Figure 4) as an indication of the predominance of a random conformer population.[38] Nevertheless, the widening of this band and the strong shoulder indicates the presence of conformers with more or less ordered structures. A continuous spectral change is observed between 0% and 25% TFE but no further change in the CD spectra is detected in the 25-100% TFE range. In 25-100% TFE the CD spectra of the peptide exhibits a positive band at 191 nm, a negative band at 207 nm and a negative band at 221 nm indicating the dominance of α-helix conformation (Figure 4). After subtracting the sugar amide contribution the CD spectra of glycopeptides **2-4** and their nonglycosylated parent peptide exhibited very similar characteristic features at any given solvent composition (Figures 4 and 5). Glycopeptides carrying smaller sugars are more soluble in TFE than their analogues carrying longer sugars. This limited solubility of the glycopeptides is noticeable at the 4-sugar derivative and is fully manifested at the 7-sugar-conjugated peptide **4**, which is not at all soluble in TFE and could only be studied at 90% TFE (Figure 4).

The CD spectrum of peptide **5** in water is dominated by a strong negative band at 197 nm indicating the presence of mostly unordered conformer population (Figure 6). When the TFE composition is increased from 0 to 90%, the spectra show a continuous transition between the two extrema with an isodichroic point at 203 nm. The presence of the isodichroic point indicates that only two conformers are present in the conformational equilibrium. Due to their insolubility in TFE, the CD spectra of peptides **5-6** were examined in 90% TFE/water; in 90% TFE, the spectrum of the nonglycosylated peptide exhibits the same characteristic features as **1** showing a high population of α-helical structure (Figure 6). The greater α-helix forming capability of **1**

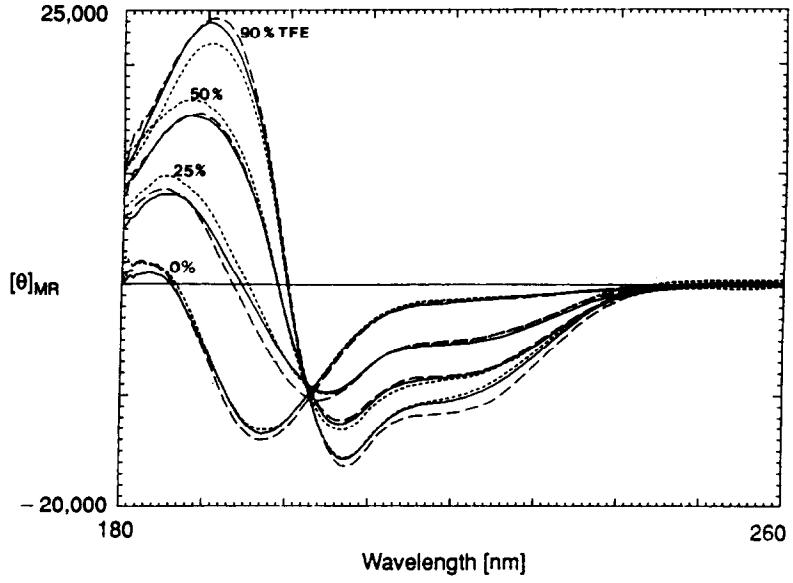


Figure 6. CD titration spectra of peptides **5-7** in 0-90% TFE. NKYVKQNTLKLA (—), N(maltobiose)KYVKQNTLKLA (-------), N(maltohexaose)KYVKQNTLKLA (— — —).

compared to **5** is apparent from the redshift of the band exhibited by **1** when measured in water and the unchanged spectrum obtained in 25-100% TFE when compared with the continuous spectral change of **5** in this region (Figures 4-6). After subtracting the sugar amide contribution the resulting CD spectra of glycopeptides **6-7** were again almost identical with the CD spectra of their nonglycosylated analogue **5** in all solvents (Figure 6). These results indicate that the secondary structure of the α-helical T-cell epitopic peptides **1** and **5** could be conserved after incorporation of the studied linear oligosaccharide chains into N-terminal positions.

Table 3. ^1H NMR Characterization Data of the Glycosylated Asparagine Derivatives in DMSO-D_6

Hydrogen atom		Chemical shift (ppm) and coupling constants (Hz)		
		2d	**4d**	**1e**
Fmoc group	H-1	7.71	7.71	
	H-2	7.33	7.33	
	H-3	7.42	7.42	
	H-4	7.89	7.89	
	CH,CH_2	4.15-4.30	4.20-4.30	
Asn residue	αNH	7.54	7.54	5.58
	αCH	4.37	4.37	3.66
	βCHa	2.68	2.70	2.76
	βCHb	2.54	2.55	2.56
	βNH	8.54	8.54	8.84
Sugar residues	H-1	4.75	4.75	4.71
	H-2	3.14	3.15	3.13
	H-3	3.50	3.50	3.43
1-	H-4	3.46	3.46	3.30
	H-5	3.25	3.25	3.20
	H-6a	3.67	3.67	3.52
	H-6b	3.56	3.56	3.62
	$J_{1,NH}$	9	9	8.4
	$J_{1,2}$	9	9	8.9
2-	H-1	5.00-5.05	5.00-5.05	5.01
	H-2	3.23		
	H-3	3.36		
	H-4	3.05-3.7	3.05-3.7	3.04
	H-5	3.48		
	H-6a	3.42		
	H-6b	3.58		
	$J_{1,2}$	3.5		

Table 4. ^{1}H NMR Assignment of N(maltobiose)CTLIDALLGDPH in DMSO-D_6 and the NOESY and ROESY Crosspeaks Found in CD_3CN-D_2O Mixture

Amino acid	NH	α	β	δ	γ	$d_{\alpha N}(i, i+3)$	$d_{\alpha\alpha}(i, i+3)$
Asn	8.28	4.38	2.76				
			2.48				
Cys	7.88	4.38	3.31			+	+
			2.99				
Thr	8.08	4.18	3.95	1.03			+
Leu	8.02	3.67	1.82	1.57			+
			1.68				
Ile	7.86	4.09	1.68	1.05		+	
				1.38			
Asp	8.30	4.47	2.68				+
			2.45				
Ala	7.85	4.17	1.16			+	+
Leu	7.79	4.19	1.65	2.02			
			1.48				
Leu	7.83	4.22	1.70	2.04			
			1.42				
Gly	7.80	4.17					
		2.65					
Asp	8.28	4.55	2.78				
			2.70				
Pro	8.3	4.18	1.98				
			1.65				
His	8.87	4.72	2.68	7.22	8.53		
			2.39				

Sugar part						
Sugar	H-1	H-2	H-3	H-4	H-5	H-6
Glc1	4.73	3.12	3.44	3.32	3.24	3.53
						3.63
Glc2	5.01	5.23	3.36	3.04	3.48	3.42
						3.60

NMR studies. Table 4 lists the ^{1}H-NMR assignment of peptide **2** by using 2D DQFCOSY and TOCSY experiments in phase-sensitive mode in dimethyl sulfoxide-d_6. Because dimethyl sulfoxide usually destroys ordered secondary structure due to its strong hydrogen bond-forming capacity,[42] through space interactions of peptide **2** were investigated in aqueous CD_3CN solution in which the peptide shows α-helical CD spectra. The crosspeak sequence (Table 4) that was found by 2D NOESY and ROESY experiments[43] and the lack of crosspeaks between more distant amino acids suggest a tightly packed α-helical structure in the region of Cys^2-Gly^{10}, which confirms the data obtained from CD. No crosspeaks indicating any interaction between the sugar side chain and the peptide backbone were found. This proves why the sugar had no impact on the structure of the peptide backbone found by CD.

Because of the through space distance of the peptide backbone amides and the sugar amide of the asparagine side chain, no significant secondary interactions and/or exciton coupling between the side chain sugar amide and the backbone can occur.[38] As a consequence of this, the CD spectra of the glycopeptides are a mathematical sum of the CD of the peptide backbone and the sugar amide.[39,40]

Dimerization studies. Peptides **1-4** contain a cysteine residue proximal to the asparagine to be glycosylated. Dimerization by disulfide bond formation may play a role in the T-cell stimulatory activity of the peptides as demonstrated earlier.[44] The disulfide bridge is formed spontaneously when the peptides are kept in solution for an extended time and during the workup and purification procedures. We have measured the rate of the dimerization of peptides **1-4** in 80% TFE in which the conformation and solubility of the peptides are similar. It was found that increasing the length of the oligosaccharide side chain on the neighboring amino acid of the cysteine residue gradually delays the process of dimerization, probably due to increasing steric hindrance (Figure 7). This finding indicates that glycosylation can affect the physical properties of glycoproteins and may

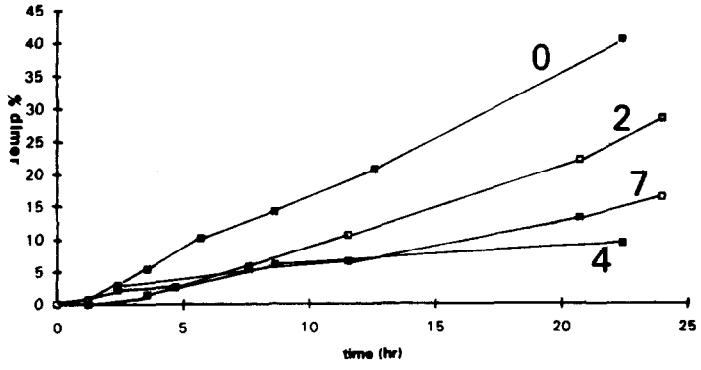


Figure 7. Dimerization studies of peptides **1-4** in 80% TFE. The numbers correspond to the number of sugars incorporated.

explain why cysteine residues are not preferred between the glycosylated asparagines and the hydroxy amino acids two residues downstream in the natural N-glycosylation sites (Asn-X-Ser/Thr) occurring in glycoproteins.[45]

Experimental Section

Liquid secondary ion mass spectroscopy (LSIMS) experiments were carried out in the positive mode using a VG Analytical ZAB-E instrument. Thioglycerol was used as liquid matrix. ^{1}H-NMR studies were performed on a Bruker 500 spectrometer at 313 K. The HPLC system consisted of two Beckman 110A pumps controlled by AI 406 interface and System Gold software. A Beckman 160 UV detector was set to 214 nm. Solvent A was 0.1% aqueous TFA, solvent B was 0.1% TFA in acetonitrile. Initial solvent composition was 95% A. Chromatographic conditions were I: column C_{18}, 4.6 x 250 mm, linear gradient of 1.33%/min solvent B starting at 5 min after injection. II: preparative column C_{18}, 21.2 x 150 mm, linear gradient of 0.1%/min solvent B.

Peptide Synthesis. Nonglycosylated peptides were synthesized on a Milligen 9050 automated synthesizer. Fmoc-protected amino acid pentafluorophenyl esters were used for peptide chain assembly on a polystyrene-polyethylene-glycol graft copolymer resin using standard continuous flow protocol. Completion of coupling and deprotection cycles were monitored by online ultraviolet absorbance at 365 nm. A mixture of 95% TFA and 5% thioanisole (v/v) was used for cleavage of the peptides from the solid support. The cleavage mixture was filtered into dry ether, the precipitated crude peptides were then washed with ether and purified by RP-HPLC. Peptides containing free cysteine side chains were treated with dithiothreitol for 30 min before HPLC purification. The final peptides had free amino groups at the N-termini and an amide group at the C-termini.

Preparation of Glycosylamines. All of the starting oligosaccharides were purchased from Sigma. The glycosylamines (1-amino-1-deoxy-sugars) were synthesized using the same protocol as described earlier.[11]

N$^{\alpha}$-fluorenylmethoxycarbonyl-N$^{\beta}$-maltotetraosyl-L-asparagine (2d). An ice cold solution of 1.5 mmol Fmoc-Asp-O^{t}Bu, 1.5 mmol pentafluorophenol and 1.5 mmol diisopropyl-carbodiimide in 1.5 ml DMF was stirred for 90 min and 500 mg (0.75 mmol) crude **2b** in 1.5 ml DMF/water (2/1; v/v) was added. Stirring was continued at room temperature for 48 h, after which the solvent was evaporated in vacuo and the solid residue was triturated with ether several times. The product was purified by preparative HPLC (LSIMS and HPLC data are listed in Table 1).

For the cleavage of the *tert*-butyl group, the **1c-4c** derivatives were dissolved in TFA and after 20 min the TFA was evaporated in vacuo and the final free acids were triturated with ether.

Nα-fluorenylmethoxycarbonyl-Nβ-maltohexaosyl-L-asparagine (3d). To a 1 ml solution of 1 mmol Fmoc-Asp(OPfp)-O^{t}Bu in DMF (prepared by the above method) 500 mg (0.5 mmol) **3b** was added in 1.5 ml DMF/water (2/1). The procedure then followed as described above.

Nα-fluorenylmethoxycarbonyl-Nβ-maltoheptaosyl-L-asparagine (4d). To a 1 ml solution of 1 mmol Fmoc-Asp(OPfp)-O^{t}Bu (prepared by the above method) 500 mg of **4b** was added (0.43 mmol) in 1.5 ml DMF/water (2/1, v/v). The procedure was then the same as above.

Nβ-maltobiosyl-L-asparagine. Fifteen mg of HPLC purified **1d** was dissolved in 20% piperidine/DMF for 10 min and the solvent was removed in vacuo. The solid residue was triturated with ether and the solvent was filtered off. The crude solid product was dissolved in water, then injected onto a preparative HPLC column and the flow was collected and lyophilized. (LSIMS: 457M+H$^+$, 479 M+Na$^+$.)

Coupling of the glycosylated asparagines to peptides. The glycosylated asparagine derivatives **1d-4d** and 1.1 equivalent pentafluorophenol were dissolved in DMF, and mixed with the resin-coupled peptides that had free N-termini. One and a tenth equivalent 1,3-diisopropyl-carbodiimide was added and the mixture was shaken for 24 h. The resin was sequentially washed with DMF, 20% piperidine/DMF for 20 min (to remove the Fmoc group) and finally with DMF. The glycopeptides were cleaved off the resin and worked up as described above. Coupling efficiencies were determined from the crude cleaved peptides comparing the peak areas of the glycosylated and the Asn-deleted peptides.

1d and **2d** were coupled in 4 molar excess in 0.2 molar solution.
3d and **4d** were coupled to 1.1 equivalent peptide resin in 0.05 molar solution.
Properties of the final glycopeptides are summarized in Table 2.

CD Measurements. CD spectra were taken on a Jasco J720 dichrograph at room temperature in a 0.2 mm pathlength quartz cell. Double distilled water and NMR grade TFE (Aldrich) were used as solvents. The spectra are the average of 4-6 scans at 0.2 nm steps. In spite of the presumably high UV absorption of the Fmoc group, the PMT voltage was still in the acceptable 0-1000 V range in the full 180-260 nm scale. All spectra that are shown are unsmoothed and baseline-corrected. The spectral contribution of the chirally perturbated side chain amide of the glycosylated asparagine was estimated by subtracting the CD spectrum of **1e** from the CD spectra (expressed in molar ellipticity) of the glycopeptides. The sugar-corrected spectra were divided by the number of amino acid residues to calculate the mean residue ellipticity (expressed in deg cm^2 dmol^{-1}). The concentration of the peptides was around 0.5 mg/ml except when the concentration dependence of the CD spectra was checked (between 0.1-2 mg/ml). The corrected glycopeptide concentration was calculated by comparing the HPLC peak area of the parent peptide to the glycosylated peptides after injection of the solution measured in CD.

Dimerization Studies. Deionized water was left to stand in an open vial for 2 days at room temperature. It was then used to prepare an 80% TFE/water mixture. This mixture was left to stand in an open vial for one day. The peptides were dissolved in this equilibrated solution in approximately 0.5 molar concentration, and were immediately co-injected onto the HPLC column. The initial peak areas were used to adjust each of the peptides to the same concentration. The peptide solutions were left to stand at room temperature in sealed vials and were injected at times indicated in Figure 5. The quantities of the dimerized products were calculated by

comparing the peak areas of the dimer and the monomer peptides. The quantities of the dimer forms that were present at the first run (at zero time) were subtracted after each calculation.

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