Internally quenched fluorogenic, α-helical dimeric peptides and glycopeptides for the evaluation of the effect of glycosylation on the conformation of peptides

Seema Mehta, Morten Meldal, Vito Ferro, Jens Ø. Duus and Klaus Bock

Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Copenhagen, Denmark

A panel of α-helical, dimeric coiled-coil peptides has been designed and synthesized for the evaluation of the effect of glycosylation on the conformation of these coiled-coil peptides. Two glycosylated building blocks, N^{α}-(fluoren-9-ylmethoxycarbonyl)-*O*-(2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-β-D-glucopyranosyl)-L-threonine pentafluorophenyl ester 8 and N^{α}-(fluoren-9-ylmethoxycarbonyl)-*O*-{2,3,4-tri-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)benzoylamino]-6-deoxy-β-D-glucopyranosyl}-L-threonine pentafluorophenyl ester 9 containing the fluorogenic 2-aminobenzamide (Abz) group, have been synthesized. These compounds have been obtained by the glycosylation of N^{α}-Fmoc-Thr-OPfp with the corresponding glycosyl trichloroacetimidate donors and have been incorporated into the solid-phase synthesis of the peptides 1–3 and 7 and glycopeptides 4–6. Compounds 1 and 4–7 have been synthesized as internally quenched fluorogenic compounds where the Abz group has been employed as the fluorogenic probe and 3-nitrotyrosine Tyr(NO_2) as the quenching chromophore. Steady-state fluorescence studies have provided evidence to support the dimerization of the α-helical peptides. Denaturation studies, by fluorescence as well as CD spectroscopy, indicate that the introduction of a carbohydrate moiety into the coiled-coil peptides has a significant destabilizing effect on the α-helicity.

Introduction

During the last decade research has emphasized the important role played by carbohydrates in both the structure and biological functions of proteins.[1] Glycosylation has been shown to be important for cell surface expression,[1] modulation of intermolecular interactions,[1] efficient secretion of proteins,[1,2] the thermal stability of proteins,[1,2] and protein solubility[1,3] to name but a few. Glycosylation is essential for the proper folding and transport of certain proteins.[1,4] In order for us to have a better understanding of the mechanisms involved in these processes it is important to have an insight into the conformational flexibilities of the glycosylated peptide chain. One of the points that needs to be addressed concerns the role of carbohydrates in imparting particular conformations to the glycopeptides. We are interested in studying the effect of protein glycosylation on the secondary structure of peptides.

The coiled-coil structural motif is frequently found in proteins. It has been known for its structural role in fibrous proteins such as myosin, keratin and fibrinogen.[5] It is encountered in a number of DNA-binding proteins and is a convenient model system with which to study the interaction of peptide chains during protein folding.

The study of the influence of glycosylation on the conformation of peptides which form well defined parallel α-helical dimers is of considerable interest. In aqueous solution, such peptides exist in an equilibrium between the α-helical dimeric state and the random coil monomeric state.[6] The α-helical dimeric state may be visualized as a pair of α-helices wrapped around each other to form a superhelix. In order to assess the effect of glycosylation on the conformation of such peptides a shift in the monomer–dimer equilibrium upon glycosylation of the parent peptide could be measured. Earlier studies aimed at investigating the conformational effects of glycosylation in glycopeptides have employed a number of techniques, such as nuclear magnetic resonance (NMR) spectroscopy[7] and circular dichroism (CD) spectroscopy.[8] However, solution conformations derived from NMR spectroscopy are results of conformational averaging over millisecond time-scales and hence are somewhat limited. Fluorescence energy transfer (FET) has frequently been used to study the structure and dynamics of various biomolecules. More recently, this technique has been adopted for the examination of glycopeptide conformation.[9] The technique complements the established methods by operating on a more rapid nanosecond time-scale, and by providing long-range, intramolecular distances between the fluorogenic probes. This enables the detection of even subtle conformational changes. Moreover, FET is a highly sensitive technique which requires substrates in only micromolar concentrations.

The present paper describes the synthesis of fluorescent peptides and glycopeptides and the initial investigations of the effect of glycosylation on the secondary structure of α-helical coiled-coils.

Results and discussion

Design of α-helical dimeric peptides and glycopeptides

Previous work[10] has provided a design for double-stranded, coiled-coil peptides. These peptides form parallel α-helical homodimers. The design consists of a heptapeptide repeating unit denoted as 'abcdefg' (Fig. 1 and structures **1**–**7**). The choice and position of the amino acid residues in each heptad, as well as the length of the peptide chain, are important for the stability of the coiled-coil. Leucine residues are placed at the 'a' and 'd' positions of the heptapeptide. They form the internal core of the coiled-coil and provide stability through van der Waalls and hydrophobic interactions [Fig. 1(*a*)]. Positioning of oppositely charged amino acids, specifically, glutamic acid at the 'e' position and lysine at the 'g' position of the heptapeptide provide additional stability to the dimer by interhelical electrostatic interaction. Due to the high intrinsic α-helical propensity of alanine and its small steric requirements,[6,10] it has been incorporated into the peptide at a number of positions. The heptapeptide is repeated four times to provide a peptide with 28 residues, which is the minimum length required to form stable

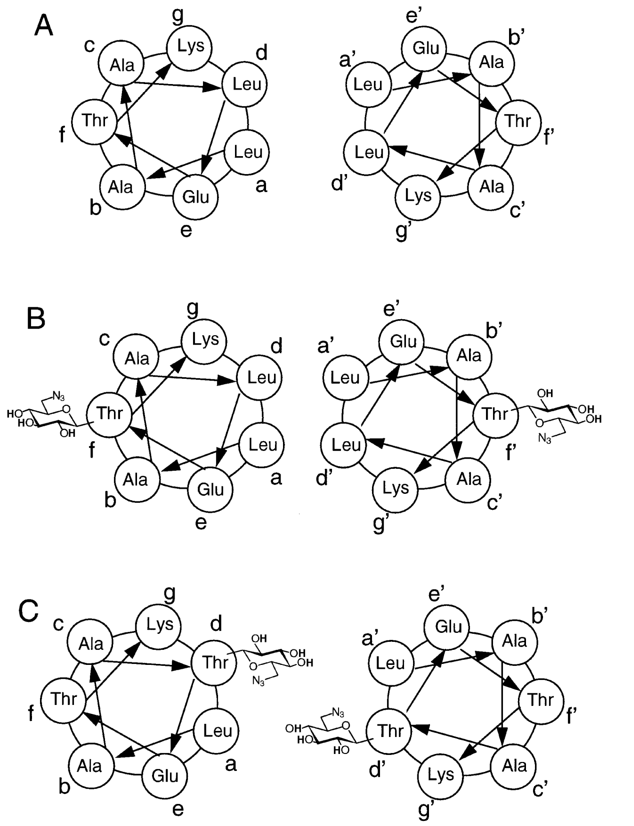


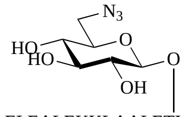
Fig. 1

abcdefg

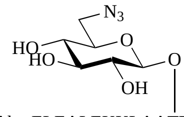
1 Abz-ELEALEKKLAALETKLQALEKKLEALEY(NO_2)G-NH_2

2 Ac-Y(NO_2)ELEALEKKLAALETKLQALEKKLEALEYG-NH_2

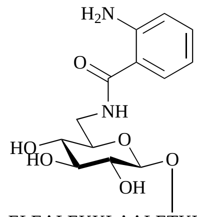
3 Abz-ELEALEKKLAALETKLQALEKKLEALEYG-NH_2



4 Abz-ELEALEKKLAALETKLQALEKKLEALEY(NO_2)G-NH_2



5 Abz-ELEALEKKLAATETKLQALEKKLEALEY(NO_2)G-NH_2



6 Ac-ELEALEKKLAALETKLQALEKKLEALEY(NO_2)G-NH_2

7 Abz-ELEALEKKLAALETKLQY(NO_2)LEKKLEALEYG-NH_2

coiled-coils.[11] The terminal carboxy group is protected as the carboxamide and the terminal amino group as its acetyl derivative, to avoid helix-destabilizing interactions with the helix dipole. We maintained this basic design of O'Neill and DeGrado[6] and performed certain modifications. The peptides and glycopeptides described in this paper were synthesized as internally quenched fluorescent compounds in which a fluorescent group was placed at one end of the substrate and a quenching group at the other end. In order to incorporate fluorescent probes into this design, the modifications presented in Fig. 1 and structures **1**–**7** were performed.

FET between the fluorogenic chromophore (energy donor) and the quenching chromophore (energy acceptor) occurs *via* resonance (or long-range) energy transfer.[12–14] To ensure efficient FET it is important to have a high quantum yield of the fluorescence donor and a good spectral overlap between the emission band of the fluorogenic chromophore and the absorption band of the quenching chromophore.[13] One donor–acceptor pair that meets these criteria very well is Abz and Tyr(NO_2).[14] The Abz group exhibits an emission maximum at 420 nm upon excitation at 320 nm. This coincides with the absorption maxima of Tyr(NO_2). Moreover, both these groups are easily incorporated by solid-phase synthesis and they have minimal influence on the conformational and amphipathic properties of the peptide products. Thus, the fluorescent probe Abz was introduced at the amino terminus of the peptides and glycopeptides and the quenching chromophore, Tyr(NO_2), was positioned towards the carboxy terminus (structures **1**–**7**). A threonine residue was introduced at position 15 in the peptides, which corresponded to the 'f' position of the second heptad. This facilitated the introduction of the carbohydrate moiety, in the case of glycopeptides.

It was expected that peptides of this design would dimerize in a parallel fashion.[6,10] In order to provide further support for this by fluorescence the peptides **2** and **3** were designed as control compounds. Peptide **2** was a nonfluorogenic peptide that contained only the quenching chromophore at the amino terminus. Thus, the Tyr(NO_2) and Abz groups in peptide **1** were replaced by tyrosine and Tyr(NO_2), respectively. Peptide **3** was designed as a fluorogenic peptide, devoid of the quenching chromophore. The Abz moiety of peptide **1** was retained and the quencher Tyr(NO_2) was replaced by a non-quenching tyrosine (Tyr) residue. We contemplated that by mixing dilute solutions of peptides **2** and **3**, heterodimers would result and the fluorescence of the peptide **3** would be strongly quenched by the quenching chromophore of peptide **2**. A study of the rate of decay of the fluorescence by time-resolved fluorescence spectroscopy indeed confirmed that the dimerization occurred in a parallel manner.[15]

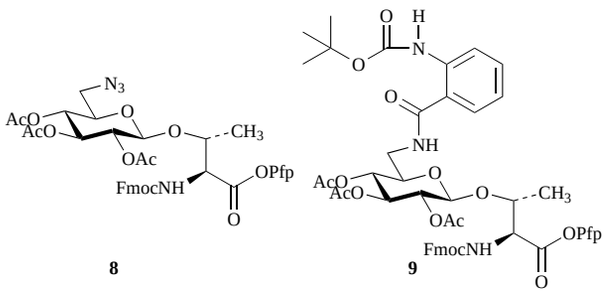
In order to investigate the influence of glycosylation on the conformation of peptide **1**, the glycopeptides **4** and **5** were designed. For the synthesis of the O-linked glycopeptide substrates **4**–**6** the method of choice was to synthesize glycosylated threonine building blocks and subsequently to incorporate them into the solid-phase peptide synthesis. In the glycopeptide **4**, a glycosylated threonine residue was introduced at position 15. The helical wheel representation of this glycopeptide [Fig. 1(*b*)] illustrates the position of the sugar residue with respect to the coiled-coil. In this position, the sugar is pointing outwards, away from the α-helical dimer, so that any effect on the tendency to form α-helical dimers can be assigned to the tendency to form the secondary structure and is not due to any influence on the dimerization. In the peptide **5** the site of glycosylation was shifted to position 13. The Leu13 in peptide **1** was replaced by a glycosylated threonine residue. This corresponds to the position 'd' of the second heptad. In this arrangement the sugar residue is positioned on the inner hydrophobic face of the α-helical dimer, directly between the coiled-coil [Fig. 1(*c*)].

FET varies inversely with the sixth power of the distance between the fluorescence donor and the fluorescence acceptor.[13] In order to vary the interprobe distance and to provide another handle for conformational studies, the glycopeptide **6** and the peptide **7** were synthesized. In the glycopeptide **6**, the fluorescent Abz group was positioned on the sugar moiety rather than the peptide backbone. For the peptide **7**, the quenching chromophore was moved from the fourth heptad to the third heptad. Leu19 was replaced by a Tyr(NO_2).

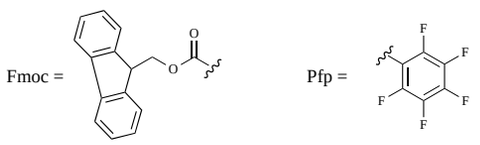
Design and synthesis of the building blocks

Two glycosylated building blocks, the non-fluorogenic com-

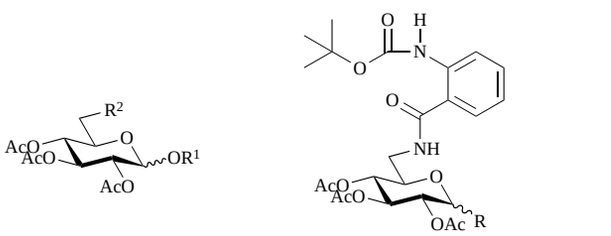
pound **8** and the fluorogenic compound **9**, were designed for the incorporation into solid-phase peptide synthesis. The synthesis of compounds **8** and **9** is presented below.



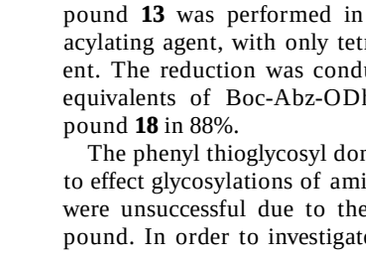
8 **9**



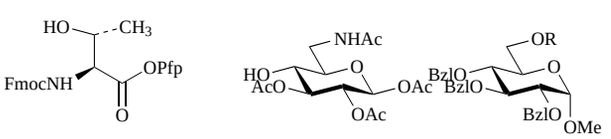
The selective tritylation of the 6-position of glucose with triphenylmethyl (trityl) chloride in pyridine followed by acetylation of the remaining hydroxy groups with acetic anhydride and pyridine afforded compound **10**.[16] Removal of the trityl group was effected with 80% aq. acetic acid at 50 °C in 2 h. The resulting compound **11**, upon treatment with trifluoromethanesulfonic anhydride and 2,6-di-*tert*-butyl-4-methylpyridine provided compound **12**[17] in 85% yield. The trifluoromethanesulfonate at the 6-position was displaced with sodium azide in *N*,*N*-dimethylformamide (DMF) to afford compound **13**[18] (91%).



	R^1	R^2
10	Ac	OTr
11	Ac	OH
12	Ac	OTf
13	Ac	N_3
14	H	N_3
15	α-C(=NH)CCl_3	N_3
17	Ac	NH_2



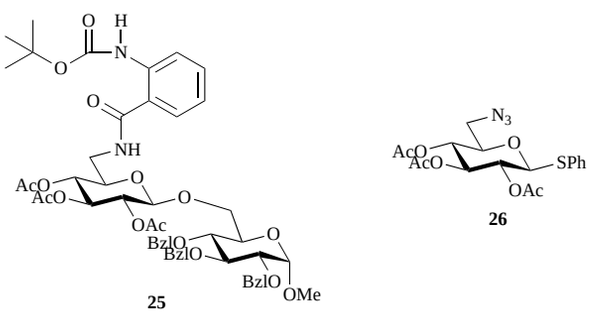
	R
18	OAc
20	β-SPh
23	OH
24	OC(=NH)CCl_3
27	β-SOPh



16

19

21 R = H
22 R = Ac



25

26

The glycosyl donor of choice for the synthesis of the building block was the glycosyl trichloroacetimidate **15**.[19] This was obtained from compound **13** by the selective removal of the anomeric acetate with hydrazine acetate[20] in DMF to provide the hemiacetals **14** in 82% yield, followed by their reaction with trichloroacetonitrile and potassium carbonate in dichloromethane.[19] The desired α-trichloroacetimidate **15** was obtained in a yield of 81%. The corresponding β-compound was also isolated, in 6% yield. N^{α}-Fmoc-L-threonine-OPfp **16** was obtained from its commercially available *tert*-butyl derivative, by treatment with trifluoroacetic acid for 1.5 h followed by crystallization from diethyl ether–hexane. Glycosylation of N^{α}-Fmoc-L-threonine-OPfp **16** with the glycosyl trichloroacetimidate **15** in the presence of 0.1 mol equiv. of trimethylsilyl trifluoromethanesulfonate as the promoter afforded the desired building block **8** in 85% yield.

The synthetic approach to compound **9** involved the introduction of the Abz-functionality at the 6-position, followed by activation of the anomeric centre and subsequent coupling with the protected amino acid. Initial efforts to reduce the 6-azido group of compound **13** and to introduce the Abz group with 3,4-dihydro-4-oxo-1,2,3-benzotriazol-3-yl 2-*tert*-butyloxycarbonylamino benzoate (Boc-Abz-ODhbt),[14] were not successful. The reduction of the azido group was performed in the presence of Pd/C in methanol and acetic acid. Attempts to isolate the amine **17**, and to treat it quickly with Boc-Abz-ODhbt did not afford the desired compound **18**. The major product **19**, was a result of the migration of the acetyl group from the 4- to the 6-position. To overcome this difficulty, we attempted to do the two-step reaction in a single step and to perform the reduction in the presence of Boc-Abz-ODhbt. This however was not effective. The use of zinc/acetic acid instead of hydrogen and Pd/C did not offer any advantage. However, improved reaction conditions were realized when the reduction of compound **13** was performed in the presence of Pd/C and the acylating agent, with only tetrahydrofuran (THF) as the solvent. The reduction was conducted in the presence of 3 mole equivalents of Boc-Abz-ODhbt to afford the desired compound **18** in 88%.

The phenyl thioglycosyl donor **20** was synthesized. Attempts to effect glycosylations of amino acids with this building block were unsuccessful due to the unreactive nature of the compound. In order to investigate other activating procedures, as well as other glycosyl donors, the reactive glycosyl acceptor **21**,[21] which contained a primary hydroxy group at the 6 position, was synthesized as a model acceptor. Glycosylation of the methyl glycoside **21** with the thioglycoside **20** was attempted under nitrosyl tetrafluoroborate promotion.[22] However, the major product was compound **22**, formed as a result of transesterification. A more reactive glycosyl donor was required and the glycosyl trichloroacetimidate approach was contemplated. A mixture of the α- and β-hemiacetal **23** was obtained by the treatment of compounds **18** with hydrazine acetate,[20] in 82% yield. Reaction of the hemiacetals **23** with trichloroacetonitrile and potassium carbonate afforded a mixture of the α- and β-glycosyl trichloroacetimidates **24** (87%) in the ratio of 2:1. These were used without separation. Glycosylation of the glycosyl acceptor **21** with glycosyl donor **24** was performed in the presence of a catalytic amount of trimethylsilyl trifluoromethanesulfonate. The desired disaccharide **25** was obtained stereoselectively in an unoptimized yield of 51%. The low yield was a result of transesterification side products and disaccharides with less than the expected number of acetyl protecting groups were also isolated. The next step was the glycosylation of the protected amino acid N^{α}-Fmoc-L-threonine-OPfp **16** with the glycosyl trichloroacetimidate **24**. This was also successful and afforded, stereoselectively, the building block **9** in 60% yield.

Synthesis of α-helical dimeric peptides and glycopeptides

The substrates **1**–**7** were synthesized by solid-phase peptide synthesis on the PEGA resin[23] in DMF. Two peptides–

glycopeptides were synthesized simultaneously in 2 parallel columns using an automated peptide synthesizer.[24] The *p*-(α-amino-2,4-dimethoxybenzyl)phenoxyacetic acid[25] (Rink) was employed as the linker. The linker was coupled to the resin by activation with O-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU)[26] and 4-ethylmorpholine (NEM). Amino acids were added as their N^{α}-Fmoc-protected Pfp ester derivatives. The use of the Fmoc group enables deprotection of the α-amino group under mild conditions of 20% piperidine in DMF. The Pfp esters[27] served to protect the carboxy group during glycosylations as well as to activate it during acylation.[28] 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhbtOH) was added as indicator and auxiliary nucleophile to enhance the reactivity of the Pfp-esters. Acylation can be followed by observing the disappearance of the yellow colour of $DhbtO^{-}$, either visually or with a solid-phase photometer.[24] The control peptide **2** was synthesized first. In this peptide the yellow Tyr(NO_2) residue was the final one to be incorporated into the chain and the synthesis of this peptide enabled the reaction times of each amino acid coupling to be noted and subsequently applied for the synthesis of analogue peptides **3**–**7**.

Cleavage of the N^{α}-Fmoc protecting group was accomplished by treatment with 20% piperidine in DMF for 15 min. The cleavage was monitored by UV spectroscopy at 320 nm. Tyr(NO_2) was coupled as its N^{α}-Fmoc derivative in the presence of TBTU and NEM. The coupling of subsequent amino acids was performed with their N^{α}-Fmoc-protected Pfp ester derivatives (3 mol equiv.). Dhbt-OH (1 mol equiv.) was added for each coupling as the auxiliary nucleophile to catalyse the acylation. Couplings were allowed to proceed for 1–3 h. Longer coupling times were required for the coupling of the threonine and the glutamine residues and these were allowed to proceed overnight. The glycosylated threonine building blocks **8** or **9** (2.5–3 mol equiv.) were allowed to react for 6 h. The final Abz group was introduced with Boc-Abz-ODhbt[14] and the coupling was allowed to proceed for 7 h. Following the final coupling, the resin was washed with piperidine (30 min). Substrates **2** and **6** were further treated with 10% acetic anhydride in DMF to acylate the free terminal amine. Cleavage of the substrates from the resin was carried out by treatment with 95% aq. TFA with concurrent removal of the side-chain-protecting groups on the amino acids. Removal of the *O*-acetyl groups on the carbohydrates provided the glycopeptides **4**–**6** by treatment with a catalytic amount of sodium methoxide in methanol at a controlled pH of 10 for 4–5 h. All compounds were purified by reversed-phase HPLC and analysed by amino acid analysis and electrospray mass spectroscopy (ES-MS). The pure peptides and glycopeptides **1**–**7** were obtained in yields of 60–80%.

Circular dichroism characterization of compounds 1–7

The CD spectra of the peptides **1**–**3** and **7** and the glycopeptides **4**–**6** were measured at 5 μM concentrations and the spectra exhibit minima at 220 and 207 nm as well as a maximum at 192 nm indicative of an α-helical conformation. The glycopeptide **5** showed less ellipticity at 207 and 220 nm, indicating less α-helical structure. In general the CD spectra cannot be used for quantification of the fraction of α-helical structure, as the CD signals from both the fluorescent donor acceptor pair and the sugar moiety are not known. This can indirectly be seen in the fact that using the normal signal intensity for a purely α-helical peptide for the 220 nm signal some of the peptides would have an estimated fraction of α-helical structure slightly more than 100%.

Stability of the α-helical homodimers and the heterodimers. In order to evaluate the influence of the carbohydrate moiety on the stability of α-helical peptides, it was decided to assess and compare the stability of the substrates **1**, **4** and **5** by monitoring their fluorescence intensity as a function of urea concentration at a fixed peptide concentration. Initial urea denaturation studies were performed on a mixture of the control peptides **2** and **3**. A mixture of the quenching peptide **2** and the fluorogenic peptide **3** is capable of forming the homodimers **2**–**2** and **3**–**3**, as well as the heterodimer **2**–**3** (Scheme 1). This

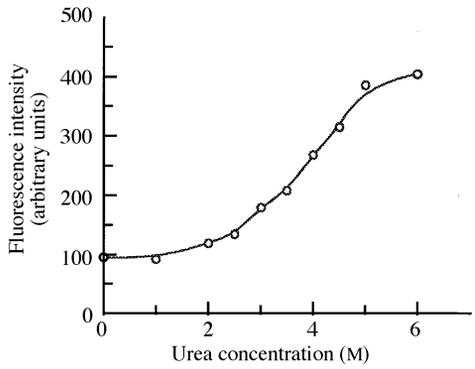
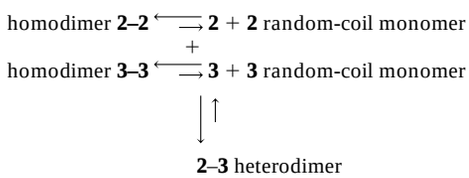


Fig. 2 Urea denaturation profile of a mixture of compounds **2** and **3** studied by fluorescence spectroscopy at substrate concentration of 5.0 μM in 50 mM TRIS chloride buffer, pH 8.5



Scheme 1

experiment furthermore probes the assumption that the peptides align in parallel fashion and not in an anti-parallel one, as only the parallel arrangement will position the fluorescence donor and quencher in close proximity to give a stronger quenching than that observed in the homodimers.

Peptides **2** and **3** were mixed in equimolar concentrations, the mixture was subjected to varying concentrations of urea (1.0–7.0 M) and the fluorescence intensity of the solution was monitored. As the urea concentration was increased, an increase in the fluorescence was observed. This corresponded to a shift in the equilibrium from the α-helical heterodimeric state to the random-coil monomeric state. When the fluorescence was plotted as a function of urea concentration, a typical sigmoidal urea denaturation curve was obtained (Fig. 2).

To obtain the urea denaturation curve of the internally quenched peptide **1**, the peptide was subjected to varying concentrations of urea and the changes in fluorescence were monitored. However, well defined denaturation curves were not obtained. At this point it was decided to study the urea denaturation of the heterodimer **1**–**2**, as opposed to the homodimer **1**–**1**, in a series of experiments similar to those performed for the control heterodimer **2**–**3**. In this case, the changes in fluorescence would be more pronounced and thus more easily observed. Indeed, the denaturation study of the heterodimer **1**–**2** afforded well defined denaturation curves. Similar experiments were repeated with the heterodimers **2**–**4** and **2**–**5**. The results are presented in Fig. 3.

A comparison of the urea denaturation profiles allowed us to comment on the relative stabilities of the heterodimers formed by the peptide **1** and the glycopeptides **4** and **5** with the control peptide **2**. The midpoints of the denaturation curve of peptide **1** (3.8 M) and the corresponding glycopeptide **4** (3.3 M) were separated by about 0.5 M urea, where the peptide **1** was more resistant to denaturation than the glycopeptide **4**. This result suggested that the sugar residue, on the external face of the coiled-coil (Fig. 1), had an adverse effect on the ability of the peptide to dimerize.

Interestingly, the fluorescence of the mixture of the glyco-

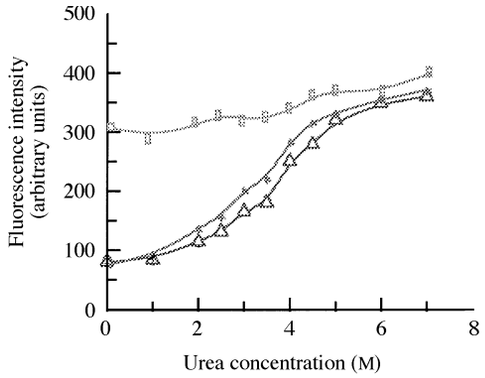


Fig. 3 Urea denaturation profiles of a mixture of compounds **2** + **1** (—△—), **2** + **4** (—*—), **2** + **5** (—□—), studied by fluorescence spectroscopy at substrate concentrations of 5.0 μM in 50 mM TRIS chloride buffer, pH 8.5

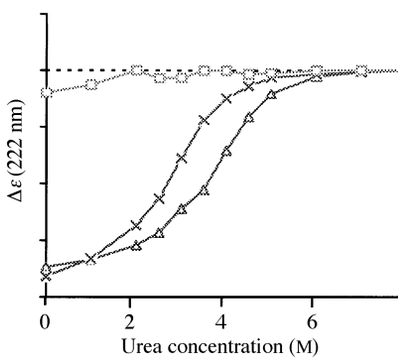


Fig. 4 Urea denaturation profiles of a solution of compounds **1** (—△—), **4** (—×—), **5** (—□—), studied by CD spectroscopy at substrate concentrations of 120 μM in 50 mM TRIS chloride buffer, pH 8.5

peptide **5** with **2** is substantially higher at 0 M urea than for the mixtures **2** + **3** and **2** + **4**. Moreover, the fluorescence remained constant in spite of the increased urea concentration. These results indicate the reduced ability of the glycopeptide **5** to form homodimers as well as heterodimers and that the dimerization of compounds **2** and **5** was indeed abolished when the sugar residue is positioned along the internal face of the coiled-coil (Fig. 1).

In addition to the fluorescence study, the stability of the heterodimers was also corroborated by CD spectroscopy (Fig. 4). The urea denaturation profile of compounds **1** and **3**–**5** revealed that the glycopeptide **4** was more easily denatured as compared to the parent peptide **1**; the midpoints of their denaturation curves were separated by ~1 M urea (2.8 and 3.8 M, respectively, at 5 μM peptide concentrations). These results are in agreement with the fluorescence study. The denaturation experiments investigated by monitoring changes in fluorescence involved hetero-aggregates. Hence the interaction of one sugar residue, between a pair of peptides in a coiled-coil, was examined. In the case of the CD experiments, the species under investigation were homo-aggregates and the effect of the sugar residue was doubled, demonstrating the destabilizing effect of the glycosylation to be additive.

For the glycopeptide **5**, well defined urea denaturation curves were not obtained. The ellipticity remained more or less constant as the concentration of urea was increased from 0 M to 8 M. This result, in conjunction with the fluorescence results, imply the inability of the glycopeptide **5** to aggregate, possibly due to the interaction of the sugar residues.

In order to detect the interaction between the peptide **2** and the compounds **1** and **3**–**5** upon mixing, and to investigate the kinetics of dimerization, the following experiments were performed. Peptide **2** was mixed, in turn, with an equimolar concentration of compounds **1** and **3**–**5**, in either 2-amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) buffer or 2 M urea solutions. The intensity of fluorescence at the beginning of the experiment was lower for the mixtures **2** + **1**, **2** + **4**, **2** + **5** as compared to **2** + **3**. Since compounds **1**, **4**, **5** possess a fluorogenic group as well as a quenching chromophore, their initial fluorescence was lower due to the internal quenching factor. For peptide **3**, the only mode of quenching is external, hence the fluorescence intensity was comparatively elevated.

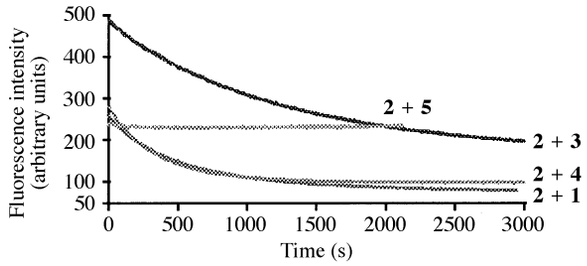


Fig. 5 Formation of 'dimers': **2** + **1**, **2** + **3**, **2** + **4**, **2** + **5**, studied by fluorescence spectroscopy at substrate concentrations of 5.0 μM in 50 mM TRIS chloride buffer, pH 8.5

The fluorescence of the solutions was monitored over time (Fig. 5). For the mixtures **2** + **1**, **2** + **3** and **2** + **4**, in pure buffer as well as in 2 M urea (data not shown), the fluorescence decreased with time. This can be attributed to the formation of hetero-aggregates, where the fluorescence of compounds **1**, **3** and **4** was quenched by the quenching peptide **2**. Interestingly, the fluorescence of the glycopeptide **5** remained constant in spite of the glycopeptide being mixed with the quenching peptide **2**. This result supports the conclusion that the presence of the sugar residue at site 13 on glycopeptide **5** hinders the formation of dimers or higher aggregates.

A comparison of the curves obtained in buffer alone (Fig. 5) with those obtained in 2 M urea indicated that the rate of formation of hetero-aggregates from homo-aggregates is higher in the latter situation. In 2 M urea, the equilibrium is shifted slightly towards the random-coil monomeric state (Scheme 1) and thereby enables faster heterodimerization.

In conclusion a set of analogues of 4 heptad repeat coiled-coil double-stranded peptides and glycopeptides were successfully synthesized in order to study peptide alignment and dynamics of the formation of the coiled-coil dimer. The peptide analogue **6** containing a fluorescent label in the glycan linked to a central amino acid residue was prepared through several routes, and the route using azido protection during the glycosylation and reduction of azide in the presence of acylating agents seems more general. An alternative route involving reduction of the azido group after incorporation on the solid phase has recently been described.[29]

The fluorescence and CD data obtained clearly demonstrate that the introduction of a single O-linked carbohydrate unit at the threonine of the solvent-exposed face of a coiled-coil peptide reduces the tendency to form α-helical dimers. In analogy to the study by O'Neill and DeGrado[6] this demonstrates that the inherent tendency to form α-helical structure is lowered by the glycosylation. The introduction of a carbohydrate moiety at the internal face of the coiled-coil peptide effectively destroys the dimerization. A further investigation using time-resolved fluorescence measurements in order to obtain more detailed information on the structure and dynamics of the dimeric peptides is currently being performed.

Experimental

General

Analytical TLC was performed on Merck Silica Gel 60 F_{254} aluminium sheets with detection by UV light and by charring with sulfuric acid. Vacuum liquid chromatography (VLC) was

performed on an open glass column with a sintered filter packed dry with suction using Merck silica gel H60 and equilibrated with a mixture of EtOAc and hexanes (1:1). 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 fraction boiling at 60–80 °C. Dichloromethane was distilled over P_2O_5. DMF was freshly distilled by fractional distillation at reduced pressure and kept over 4 Å molecular sieves. Concentrations were performed under reduced pressure at temperatures <40 °C (bath). 4-(Hydroxymethyl)benzoic acid (HMBA) and suitably protected N^{α}-Fmoc amino acids were purchased from NovaBiochem (Switzerland), DhbtOH, NEM and TBTU from Fluka (Switzerland). N^{α}-Fmoc-Tyr(NO_2)-OH and Boc-Abz-ODhbt were prepared as previously described.[14] 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. ES-MS spectra were recorded in the positive mode on a VG Quattro Mass Spectrometer from Fisons, with 50% aq. acetonitrile as the liquid phase. Matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Finnigan Mat 2000 mass spectrometer using a matrix of α-cyano-4-hydroxycinnamic acid. NMR spectra were recorded on a Bruker AM-500 or a Bruker AMX-600 MHz spectrometer. For all compounds, the assignment of ^{1}H NMR 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 $CDCl_3$ (reference: $CDCl_3$ at δ_H 7.3 and δ_C 77.0). *J*-Values are given in Hz. 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 cm^3 min^{-1} pump heads. The system was fitted with a switchable Delta Pak (25 × 200 mm; 10 cm^3 min^{-1}) and a preparative radial pack column (50 × 300 mm; 20 cm^3 min^{-1}), both packed with reversed-phase C_{18} or a Shodex DS-2013 column (20 × 300 mm; 4 cm^3 min^{-1}). Analytical HPLC was performed using a Waters RCM 8 × 10 module with a Waters 8 NV C_{18} (4 μm) column (1 cm^3 min^{-1}). The solvent system was buffer A: 0.1% aq. 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 Buchi melting point apparatus and are uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter; $[a]_D$-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).

Fluorescence measurements were performed on a Perkin-Elmer luminescence spectrometer LS50. The substrates were excited at 320 nm and their fluorescence was monitored at 420 nm, both with 5 nm slits. All measurements were performed at 25 °C.

The CD measurements were carried out as previously described on a Jobin Yvon Model IV dischrograph at NOVO Nordisk, Bagsværd, Denmark.[30]

Solid-phase peptide synthesis. General procedure

Synthesis of the peptides/glycopeptides **1**–**7** was performed on the PEGA resin[23] in DMF. Two peptides/glycopeptides were synthesized simultaneously in two parallel columns using an automated peptide synthesizer.[24] PEGA resin (200 mg, 0.04 mmol) was transferred into each column. The resin was allowed to swell in DMF for 2 h and was then treated with 20% piperidine in DMF for 10 min to remove any chloride ions and to expose the free amino groups on the resin. The resin was derivatized with the Rink linker[25] (54 mg, 0.1 mmol) in the presence of TBTU (32 mg, 0.09 mmol) and NEM (25 mm^3, 0.2 mmol).[26] Cleavage of the N^{α}-Fmoc protecting group was accomplished by treatment with 20% piperidine in DMF for 15 min. The cleavage was monitored by UV spectroscopy at 320 nm. This was followed by washing with DMF (30 cm^3 for 18 min). Tyr(NO_2) was coupled as its N^{α}-Fmoc derivative (52 mg, 0.12 mmol) in the presence of TBTU (35 mg, 0.11 mmol), and NEM (30 mm^3, 0.24 mmol). A coupling time of 3 h was allowed. Coupling of subsequent amino acids was performed with their N^{α}-Fmoc-protected Pfp ester derivatives (3 mol equiv.). The side chains on the glutamic acid were protected with *tert*-butyl groups. Glutamine was used as its trityl derivative, lysine as its *tert*-butoxycarbonyl (Boc) derivative, and threonine as its *tert*-butyl derivative. DhbtOH (1 mol equiv.) was added for each Pfp ester coupling as the auxiliary nucleophile to catalyse the acylation. Couplings were allowed to proceed for 1–3 h. Longer coupling times were required for the coupling of the threonine and the glutamine residues and these were allowed to proceed overnight. Building blocks **8** and **9** (2.5–3 mol equiv.) were employed and the coupling was allowed to proceed for 6 h. Finally the Abz group was introduced with Boc-Abz-ODhbt[14] and the coupling was allowed to proceed for 7 h. Following the final coupling, the resin was washed with piperidine (30 min). Resins with protected peptides **2** and **6** were further treated with 10% acetic anhydride in DMF (30 min) to acylate the free terminal amine. The resin was transferred into a syringe and washed with DMF (×10) and CH_2Cl_2 (×10), dried for 2 h and lyophilized overnight. Cleavage of the peptides from the resin was carried out by treatment with 95% aq. TFA (5–6 cm^3) for 3 h. This effected concomitant removal of the side-chain-protecting groups on the amino acids. The resin was transferred into a sintered funnel, washed with 95% TFA followed by 95% aq. acetic acid (×4). The filtrate was concentrated and lyophilized. All compounds were purified by reversed-phase HPLC and were analysed by amino acid analysis and ES-MS. For the deprotection of glycopeptides **4**–**6** they were dissolved in anhydrous methanol (1 cm^3 per 2 mg) and a freshly prepared solution of sodium methoxide in methanol (0.2 M) was added dropwise (to pH 10–11). The reaction mixtures were stirred for 4–5 h and neutralized with acetic acid. The solutions were concentrated, lyophilized, purified by reversed-phase HPLC and analysed by amino acid analysis and ES-MS.

Abz-Glu-Leu-Glu-Ala-Leu-Glu-Lys-Lys-Leu-Ala-Ala-Leu-Glu-Thr-Lys-Leu-Gln-Ala-Leu-Glu-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Tyr(NO_2)-Gly-NH_2 1

The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 10% B, then a linear gradient of 10–90% B during 70 min; retention time 52.0 min) to give pure compound **1** (120.0 mg, 87%). Amino acid analysis is presented in Table 1 {Found (ES-MS): *m/z*, 1146.6 $[M + 3\ H]^{3+}$. 860.4 $[M + 4\ H]^{4+}$; (MALDI) 3436.6 $(M + H)^+$. $C_{155}H_{256}N_{38}O_{49}$ requires *M*, 3435.9}.

Ac-Tyr(NO_2)-Glu-Leu-Glu-Ala-Leu-Glu-Lys-Lys-Leu-Ala-Ala-Leu-Glu-Thr-Lys-Leu-Gln-Ala-Leu-Glu-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Tyr-Gly-NH_2 2

The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 10% B, then a linear gradient of 10–100% B during 70 min; retention time 53.0 min) to give pure compound **2** (99.0 mg, 72 %). Amino acid analysis is presented in Table 1 {Found: (MALDI) 3562.5 $(M + K)^+$. $C_{159}H_{262}N_{38}O_{51}$ requires *M*, 3522.1}.

Abz-Glu-Leu-Glu-Ala-Leu-Glu-Lys-Lys-Leu-Ala-Ala-Leu-Glu-Thr-Lys-Leu-Gln-Ala-Leu-Glu-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Tyr-Gly-NH_2 3

The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 20% B, then a linear gradient of 20–90% B during 80 min; retention time 62.0 min) to give pure compound **3** (132.0 mg, 78%). Amino acid analysis is presented

Table 1 Amino acid analysis of glycopeptides and peptides **1–7**. Theoretical values are given in parentheses

Compound	Thr	Glu	Gly	Ala	Leu	Tyr	Tyr(NO_2)	Lys
1	0.93 (1)	7.89 (8)	1.22 (1)	4.70 (5)	8.50 (8)		0.97 (1)	4.85 (5)
2	0.87 (1)	8.04 (8)	1.11 (1)	5.05 (5)	8.16 (8)	0.87 (1)	0.96 (1)	4.91 (5)
3	0.91 (1)	7.91 (8)	1.18 (1)	4.70 (5)	8.60 (8)		0.91 (1)	4.92 (5)
4	0.88 (1)	8.01 (8)	1.11 (1)	5.10 (5)	8.50 (8)		0.88 (1)	4.72 (5)
5	2.00 (2)	8.50 (8)	1.20 (1)	4.80 (5)	6.61 (7)		0.90 (1)	4.80 (5)
6	0.79 (1)	7.94 (8)	1.13 (1)	5.26 (5)	8.34 (8)		0.83 (1)	4.72 (5)
7	0.86 (1)	8.14 (8)	1.10 (1)	4.40 (4)	8.60 (8)	0.87 (1)	0.93 (1)	4.85 (5)

in Table 1 {Found (ES-MS): *m/z*, 1131.6 $[M + 3\ H]^{3+}$; 858.7 $[M + Na + 3\ Li]^{4+}$; 849.06 $[M + 4\ H]^{4+}$; (MALDI) 3392.1 $(M + H)^+$. $C_{155}H_{257}N_{37}O_{47}$ requires *M*, 3391.0}.

Abz-Glu-Leu-Glu-Ala-Leu-Glu-Lys-Lys-Leu-Ala-Ala-Leu-Glu-Thr(6-azido-6-deoxy-β-D-glucopyrannosyl)-Lys-Leu-Gln-Ala-Leu-Glu-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Tyr(NO_2)-Gly-NH_2 4

The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 15% B, then a linear gradient of 15–85% B during 60 min; retention time 52.0 min) to give pure protected glycopeptide (105.0 mg, 70%). The protected substrate (25 mg) was dissolved in anhydrous methanol (15 cm^3) and a freshly prepared solution of sodium methoxide in methanol (0.2 M) was added dropwise (to pH 10–11), the reaction mixtures were stirred for 3.5 h, at which point analytical HPLC indicated complete deacetylation. The reaction mixture was neutralized with acetic acid and concentrated. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 15% B, then a linear gradient of 15–85% B during 70 min; retention time 56.0 min) to give pure compound **4** (15 mg, 63%). Amino acid analysis is presented in Table 1 {Found (ES-MS): *m/z*, 1812.62 $[M + 2\ H]^{2+}$; 1222.2 $[M + 2\ Li + Na]^{3+}$; 1215.9 $[M + Na + 2\ H]^{3+}$; 1208.8 $[M + 3\ H]^{3+}$; (MALDI) 3623.6 $(M + H)^+$. $C_{161}H_{265}N_{41}O_{53}$ requires *M*, 3623.1}.

Abz-Glu-Leu-Glu-Ala-Leu-Glu-Lys-Lys-Leu-Ala-Ala-Thr(6-azido-6-deoxy-β-D-glucopyrannosyl)-Glu-Thr-Lys-Leu-Gln-Ala-Leu-Glu-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Tyr(NO_2)-Gly-NH_2 5

The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 15% B, then a linear gradient of 15–85% B during 70 min; retention time 68.0 min) to give pure protected glycopeptide (130.0 mg, 87%). The protected substrate (80 mg) was dissolved in anhydrous methanol (35 cm^3) and a freshly prepared solution of sodium methoxide in methanol (0.2 M) was added dropwise (to pH 9–10); the reaction mixture was stirred for 4 h, at which point analytical HPLC indicated complete deacetylation. The reaction mixture was neutralized with acetic acid and concentrated. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 15% B, then a linear gradient of 15–85% B during 60 min; retention time 49.0 min) to give pure compound **5** (70 mg, 90%). Amino acid analysis is presented in Table 1 {Found (ES-MS): *m/z*, 1204.5 $[M + 3\ H]^{3+}$; 903.9 $[M + 4\ H]^{4+}$; 723.4 $[M + 5\ H]^{5+}$. $C_{159}H_{261}N_{41}O_{54}$ requires *M*, 3611.1}.

Abz-Glu-Leu-Glu-Ala-Leu-Glu-Lys-Lys-Leu-Ala-Ala-Leu-Glu-Thr[6-(2′-aminobenzoylamino)-6-deoxy-β-D-glucopyrannosyl]-Lys-Leu-Gln-Ala-Leu-Glu-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Tyr(NO_2)-Gly-NH_2 6

The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 15% B, then a linear gradient of 15–85% B during 70 min; retention time 52.0 min) to give pure protected glycopeptide (90.0 mg, 60%). The protected substrate (20 mg) was dissolved in anhydrous methanol (8 cm^3) and a freshly prepared solution of sodium methoxide in methanol (0.2 M) was added dropwise (to pH 10–11). The reaction mixture was stirred for 5 h, at which point analytical HPLC indicated complete deacetylation. The reaction mixture was neutralized with acetic acid and concentrated. The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 20% B, then a linear gradient of 20–80% B during 70 min; retention time 52.5 min) to give pure compound **6** (21 mg, 83%). Amino acid analysis is presented in Table 1 {Found (ES-MS): *m/z*, 1228.1 $[M + Na + 3\ Li]^{3+}$; (MALDI) 3639.5 $(M + H)^+$. $C_{163}H_{269}N_{39}O_{54}$ requires *M*, 3639.2}.

Abz-Glu-Leu-Glu-Ala-Leu-Glu-Lys-Lys-Leu-Ala-Ala-Leu-Glu-Thr-Lys-Leu-Gln-Tyr(NO_2)-Leu-Glu-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Tyr-Gly-NH_2 7

The crude product was purified by semipreparative HPLC (Delta Pak column; 10 min 15% B, then a linear gradient of 15–85% B during 70 min; retention time 52.0 min) to give pure compound **7** (120.0 mg, 85%). Amino acid analysis is presented in Table 1 {Found (ES-MS): *m/z*, 1765.5 $[M + 2\ H]^{2+}$; 1177.1 $[M + 3\ H]^{3+}$. $C_{161}H_{260}N_{38}O_{50}$ requires *M*, 3528.1}.

1,2,3,4-Tetra-*O*-acetyl-6-*O*-trifluoromethylsulfonyl-β-D-glucopyranose 12

1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose **11**[17,31,32] (1.20 g, 3.45 mmol) was added portionwise to a solution of trifluoromethanesulfonic anhydride (1.36 g, 0.81 cm^3, 4.82 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (1.00 g, 4.88 mmol) in dichloromethane (60 cm^3). The mixture was stirred at 25 °C for 2 h and then was poured into cold, saturated aq. $NaHCO_3$. The organic phase was separated and the aqueous phase was extracted with dichloromethane. The combined organic phases were washed successively with water and brine, dried ($MgSO_4$) and concentrated. Recrystallization of the residue from dichloromethane–hexanes then gave the trifluoromethanesulfonate **12** (1.36 g) as needles, mp 95–96 °C (decomp.) [lit.,[17] 85 °C (decomp.)] $[\alpha]_D$ +5.3 (*c* 0.8, $CHCl_3$). The mother liquors were subjected to vacuum liquid chromatography (VLC) [EtOAc–hexanes (1:1)] to give a further 0.20 g of product. Total yield: 1.56 g (94%); ($CDCl_3$) 20.4, 20.5, 20.7 and 20.8 (4 × $COCH_3$), 72.7 (C-6), 67.8 (C-4), 69.9 (C-2), 72.4 (C-3), 71.9 (C-5), 91.4 (C-1), 118.5 (CF_3, J_{CF} 319.8) and 168.7, 169.1, 169.3 and 170.0 (4 × $COCH_3$); δ_H($CDCl_3$) 2.06, 2.08, 2.11 and 2.16 (12 H, 4 s, 4 × Ac), 4.55 (1 H, dd, $J_{5,6a}$ 4.4, $J_{6a,6b}$ 11.3, H^a-6), 4.58 (1 H, dd, $J_{5,6b}$ 3.1, H^b-6), 4.00 (1 H, ddd, $J_{4,5}$ 9.4, H-5), 5.12 (1 H, t, $J_{3,4}$ 9.2, H-4), 5.17 (1 H, dd, $J_{1,2}$ 8.2, $J_{2,3}$ 9.2, H-2), 5.31 (1 H, t, H-3) and 5.78 (1 H, d, H-1).

1,2,3,4-Tetra-*O*-acetyl-6-azido-6-deoxy-β-D-glucopyranose 13

To a mixture of the trifluoromethanesulfonate **12** (3.04 g, 6.33 mmol) and sodium azide (2.0 g, 30.8 mmol) was added DMF (40 cm^3) and the mixture then was stirred at 25 °C for 1 h before being poured into water and extracted with ethyl acetate. The extracts were washed successively with water and brine, dried ($MgSO_4$) and concentrated. Recrystallization of the residue from diethyl ether–hexanes then gave the *azide* **13** (1.81 g) as needles, mp 86 °C; $[\alpha]_D$ +9.5 (*c* 2.3, $CHCl_3$); δ_C($CDCl_3$) 20.4, 20.6, 20.6 and 20.8 (4 × $COCH_3$), 50.6 (C-6), 68.9 (C-4), 70.1 (C-2), 72.6 (C-3), 73.8 (C-5), 91.5 (C-1) and 168.7, 169.4, 169.6 and 170.2 (4 × $COCH_3$); δ_H($CDCl_3$) 2.04, 2.07, 2.09 and 2.23 (12 H, 4 s, 4 × Ac), 3.37 (1 H, dd, $J_{5,6a}$ 5.3, $J_{6a,6b}$ 13.5, H^a-6), 3.40 (1 H, dd, $J_{5,6b}$ 3.3, H^b-6), 3.84 (1 H, ddd, $J_{4,5}$ 9.4, H-5), 5.10 (1 H, t, $J_{3,4}$ 9.4, H-4), 5.16 (1 H, dd, $J_{1,2}$ 10.1, $J_{2,3}$ 9.4, H-2), 5.27 (1 H,

t, H-3) and 5.75 (1 H, d, H-1) (Found: C, 45.1; H, 5.0. $C_{14}H_{19}N_3O_9$ requires C, 45.0; H, 5.1%). The mother liquors were subjected to VLC [EtOAc–hexanes (2:3)] to give a further crop (0.44 g) of product. Total yield: 2.25 g (95%).

Phenyl 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-1-thio-β-D-gluco-pyranoside 26

A mixture of compound **13** (373 mg, 1.0 mmol), thiophenol (220 mg, 0.204 cm^3, 2.0 mmol), boron trifluoride–diethyl ether (710 mg, 0.628 cm^3, 5.0 mmol) and 3 Å molecular sieves in dichloromethane (2 cm^3) was stirred at 25 °C for 21 h. The mixture was then diluted with dichloromethane and poured into saturated aq. $NaHCO_3$. The organic phase was separated, washed successively with water and brine, dried ($MgSO_4$) and concentrated. VLC [EtOAc–hexanes (2:3)] of the residue then gave the *thioglycoside* **26** (359 mg, 85%) as needles, mp 120–121 °C (from CH_2Cl_2–hexanes); $[\alpha]_D$ −11 (*c* 0.6, $CHCl_3$); $\delta_C(CDCl_3)$ 20.6 and 20.7 (3 × $COCH_3$), 51.3 (C-6), 69.2 (C-4), 69.9 (C-2), 73.8 (C-3), 77.0 (C-5), 85.7 (C-1), 128.7–133.8 (arom. C) and 169.2, 169.4 and 170.2 (3 × $COCH_3$); $\delta_H(CDCl_3)$ 2.03, 2.06 and 2.14 (9 H, 3 s, 3 × Ac), 3.34 (1 H, dd, $J_{5,6a}$ 6.5, $J_{6a,6b}$ 13.4, H^a-6), 3.40 (1 H, dd, $J_{5,6b}$ 2.9, H^b-6), 3.70 (1 H, ddd, $J_{4,5}$ 9.7, H-5), 4.76 (1 H, d, $J_{1,2}$ 10.1, H-1), 4.99 (1 H, dd, $J_{2,3}$ 9.7, H-2), 5.00 (1 H, t, $J_{3,4}$ 9.3, H-4) and 5.25 (1 H, t, H-3) (Found: C, 51.0; H, 5.0. $C_{18}H_{21}N_3O_7S$ requires C, 51.0; H, 5.0%).

Phenyl 2,3,4-tri-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)-benzoylamino]-6-deoxy-1-thio-β-D-glucopyranoside 20

The thioglycoside **26** (1.27 g, 3.0 mmol) was suspended in dry methanol (25 cm^3). Methanolic sodium methoxide (1 cm^3; 1 mol dm^{-3}) was then added and the mixture was stirred at room temperature until dissolution was complete (10 min). The mixture was then stirred for a further 1 h, neutralized (Amberlite IRC-50, H^+), filtered and concentrated. The residue was redissolved in methanol (25 cm^3), triethylamine (1.21 g, 1.67 cm^3, 12.0 mmol) and dithiothreitol (1.85 g, 12.0 mmol) were added, and the mixture then was stirred at room temperature for 16 h before being concentrated, the residue was dissolved in THF (25 cm^3) and Boc-Abz-ODhbt (1.26 g, 3.3 mmol) was added. The mixture was stirred for 1.5 h and then acetic anhydride (5 cm^3) and pyridine (5 cm^3) were added. The mixture was stirred overnight and then was poured into water and extracted with ethyl acetate. The extracts were washed successively with dil. HCl, water, saturated aq. $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated. VLC [EtOAc–hexanes (1:4–3:7)] of the residue then gave the *title compound* **20** (1.56 g, 84% from **26**) as plates, mp 145–146 °C (from EtOAc–hexanes); $[\alpha]_D$ +4 (*c* 0.5, $CHCl_3$); $\delta_C(CDCl_3)$ 20.5, 20.6 and 20.7 (3 × $COCH_3$), 28.3 [$C(CH_3)_3$], 39.7 (C-6), 69.2 (C-4), 70.1 (C-2), 73.6 (C-3), 76.4 (C-5), 80.2 [$C(CH_3)_3$], 85.3 (C-1), 119.0–140.5 (Ar), 153.0 [$C(O)C(CH_3)_3$] and 168.7, 169.3, 169.8 and 170.1 (C=O); $\delta_H(CDCl_3)$ 1.54 (9 H, s, Bu^t), 2.03, 2.12 and 2.13 (9 H, 3 s, 3 × Ac), 3.41 (1 H, ddd, $J_{5,6a}$ 6.6, H^a-6), 3.96 (1 H, m, $J_{5,6b}$ 2.7, $J_{6a,6b}$ 14.3, H^b-6), 3.74 (1 H, ddd, $J_{4,5}$ 9.8, H-5), 4.80 (1 H, d, $J_{1,2}$ 10.1, H-1), 4.96 (1 H, t, $J_{3,4}$ 9.4, H-4), 5.00 (1 H, dd, $J_{2,3}$ 9.7, H-2), 5.29 (1 H, t, H-3), 6.53 (1 H, br t, NH^{Abz}), 6.98–8.44 (9 H, ArH) and 10.14 (1 H, s, NH^{Boc}) (Found: C, 58.5; H, 5.9. $C_{30}H_{36}N_2O_{10}S$ requires C, 58.4; H, 5.9%).

2,3,4-Tri-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)benzoyl-amino]-6-deoxy-1-phenylsulfinyl-β-D-glucopyranose 27

To a solution of the sulfide **20** (123 mg, 0.20 mmol) at −78 °C was added 3-chloroperbenzoic acid (MCPBA) (49 mg of 85% from Fluka, 0.24 mmol). The mixture was allowed to warm to −20 °C over a period of 1.5 h and then was poured into saturated aq. $NaHCO_3$. The organic phase was separated and the aqueous phase was extracted with dichloromethane. The combined organic phases were washed successively with water and brine, dried ($MgSO_4$) and concentrated. VLC [EtOAc–hexanes (1:1)] of the residue then gave the *sulfoxide* **27** (118 mg, 94%) as a solid 5.5:1 mixture of diastereomers, used without further purification; *m/z* 633.3 $(M + H)^+$ and 655.3 $(M + Na)^+$ (Found: C, 56.9; H, 5.9. $C_{30}H_{36}N_2O_{11}S$ requires C, 57.0; H, 5.7%).

A small portion was recrystallized from EtOAc–Et_2O to give the major diastereomer as needles, mp 142–143 °C; $\delta_C(CDCl_3)$ 20.6 (3 × $COCH_3$), 28.3 [$C(CH_3)_3$], 39.8 (C-6), 67.6 (C-2), 68.9 (C-4), 73.5 (C-3), 77.2 (C-5), 80.2 [$C(CH_3)_3$], 90.5 (C-1), 119.8–140.3 (Ar), 152.9 [$C(O)C(CH_3)_3$] and 168.7, 168.9, 169.6 and 170.3 (C=O); $\delta_H(CDCl_3)$ 1.55 (9 H, s, Bu^t), 2.07, 2.11 and 2.13 (9 H, 3 s, 3 × Ac), 3.26 (1 H, ddd, $J_{5,6a}$ 7.6, H^a-6), 3.64 (1 H, ddd, $J_{4,5}$ 9.6, H-5), 3.89 (1 H, m, $J_{5,6b}$ 2.5, $J_{6a,6b}$ 14.4, H^b-6), 4.30 (1 H, d, $J_{1,2}$ 9.9, H-1), 5.01 (1 H, t, $J_{3,4}$ 9.2, H-4), 5.37 (1 H, t, H-3), 5.44 (1 H, dd, $J_{2,3}$ 9.5, H-2), 6.22 (1 H, br t, NH^{Abz}), 7.02–8.42 (9 H, ArH) and 9.95 (1 H, s, NH^{Boc}).

2,3,4-Tri-*O*-acetyl-6-azido-6-deoxy-α-D-glucopyranosyl trichloroacetimidate 15

A mixture of α- and β-peracetylated 6-azido-6-deoxyglucopyranoside **13** (0.6 g, 1.6 mmol) was dissolved in DMF (16 cm^3). Hydrazinium acetate (0.2 g, 2.4 mmol) was added and the reaction mixture was stirred under argon for 3 h. The reaction was quenched with ethyl acetate (20 cm^3), diluted with dichloromethane (20 cm^3), stirred for 5 min, and washed with aq. sodium chloride (5%; 20 cm^3). The organic extracts were dried over magnesium sulfate, filtered and concentrated. The syrup was purified by column chromatography with hexane–ethyl acetate (1:1) as eluent (R_f 0.4). A mixture of 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α/β-D-glucopyranose **14** was obtained as a foam (0.43 g, 81%).

Compounds **14** (0.3 g, 0.9 mmol) were dissolved in CH_2Cl_2 (4.5 cm^3). Trichloroacetonitrile (0.9 cm^3, 9.0 mmol) and potassium carbonate (1.0 g, 7.2 mmol) were added and the reaction mixture was stirred under argon for 12 h before being filtered through Celite and concentrated. The residue was chromatographed with hexane–ethyl acetate (1.75:1) as eluent [R_f α-isomer 0.5; β-isomer 0.35]. The *title compound* was obtained as a foam (α-isomer: 0.33 g, 75%, β-isomer: 0.26 g, 6%). α-Isomer: $[\alpha]_D^{20}$ 91.7 (*c* 9.3, CH_2Cl_2); $\delta_C(CDCl_3)$ 20.4, 20.6 and 20.7 (3 × $COCH_3$), 50.6 (C-6), 68.9 (C-4), 69.7 (C-3, -2), 71.1 (C-5), 92.7 (C-1), 160.7 (C=N) and 169.5, 169.8 and 170.0 (3 × $COCH_3$); $\delta_H(CDCl_3)$ 2.06, 2.08 and 2.10 (9 H, 3 s, 3 × Ac), 3.36 (1 H, dd, $J_{5,6a}$ 5.4, $J_{6a,6b}$ 13.5, H^a-6), 3.45 (1 H, dd, $J_{5,6b}$ 2.9, H^b-6), 4.22 (1 H, ddd, $J_{4,5}$ 10.2, H-5), 5.16 (1 H, dd, $J_{1,2}$ 3.7, $J_{2,3}$ 10.2, H-2), 5.18 (1 H, t, $J_{3,4+4,5}$ 19.4, H-4), 5.5 (1 H, t, $J_{2,3+3,4}$ 19.6, H-3), 6.63 (1 H, d, $J_{1,2}$ 3.7, H-1) and 8.76 (1 H, s, $NHCCl_3$) (Found: C, 35.4; H, 3.7; N, 11.6. Calc. for $C_{14}H_{17}N_3O_8Cl_3$: C, 35.4; H, 3.6; N, 11.8%).

***N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*O*-(2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-glucopyranosyl)-L-threonine pentafluorophenyl ester 8**

A mixture of 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-glucopyranosyl trichloroacetimidate **15** (0.10 g, 0.20 mmol), N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester **16** (0.13 g, 0.25 mmol), freshly activated 4 Å molecular sieves in anhydrous CH_2Cl_2 (1 cm^3) was stirred under argon for 1 h. The reaction mixture was cooled to −78 °C and trimethylsilyl trifluoromethanesulfonate (3 mm^3, 0.015 mmol) was added. After 0.5 h the reaction mixture was warmed to room temperature during 10 min to ensure completion of the reaction. The mixture was recooled to −78 °C and quenched with 1 drop of triethylamine. The reaction mixture was filtered, concentrated and subjected to column chromatography on a short column of pre-dried silica gel with hexane–ethyl acetate (1.5:1) as eluent (R_f 0.4). The title compound was obtained as a powder (0.15 g, 85%), $[\alpha]_D^{20}$ 4.6 (*c* 9.9, CH_2Cl_2); $\delta_C(CDCl_3)$ 14.2 (Thr^g), 20.6 ($COCH_3$), 47.1 ($Fmoc^{\beta}$), 50.9 (C-6), 58.5 (Thr^{α}), 67.4 ($Fmoc^{\alpha}$), 69.4 (C-4), 71.4 (C-2), 72.2 (C-3), 73.3 (Thr^{β}), 73.5

(C-5), 97.6 (C-1), 119.9–143.3 (Ar), 156.5 (CONH), 166.3 [*C*(O)OPfp] and 169.3, 169.4 and 170.2 ($COCH_3$); $\delta_H(CDCl_3)$ 1.35 (3 H, d, $J_{Thr^{\beta},Thr^{g}}$ 6.3, Thr^{g}), 2.07, 2.08 and 2.09 (9 H, 3 s, 4 × $COCH_3$), 3.28 (1 H, dd, $J_{5,6a}$ 2.2 $J_{6a,6b}$ 13.0, H^{a}-6), 3.35 (1 H, dd, $J_{5,6b}$ 6.7, $J_{6a,6b}$ 13.0, H^{b}-6), 3.70 (1 H, m, $J_{4,5}$ 9.0, H-5), 4.30 (1 H, t, $J_{Fmoc^{\alpha},Fmoc^{\beta}}$ 7.0, Fmoc$^{\beta}$), 4.48 (1 H, t, $J_{Fmoc^{\alpha},Fmoc^{\alpha}}$ 10.0, $J_{Fmoc^{\alpha},Fmoc^{\beta}}$ 6.8, Fmoc$^{\alpha}$), 4.54 (1 H, t, Fmoc$^{\alpha}$), 4.64–4.70 (2 H, m, Thr$^{\beta}$ and H-1), 4,79 (1 H, dd, $J_{NH,Thr^{\alpha}}$ 9.1, $J_{Thr^{\alpha},Thr^{\beta}}$ 2.0, Thr$^{\alpha}$), 5.0 (1 H, t, $J_{1,2+2,3}$ 18.0, H-2), 5.04 (1 H, t, $J_{3,4+4,5}$ 18.8, H-4), 5.28 (1 H, t, $J_{2,3+3,4}$ 19.0, H-3), 5.63 [1 H, d, $J_{NH,Thr^{\alpha}}$ 9.2, C(O)NH] and 7.20–7.85 (8 H, ArH); ES-MS 827.0 $[M + Li]^+$. $C_{37}H_{33}F_5N_4O_{12}$ requires *M*, 820.2 (Found: C, 53.91; H, 4.10; N, 6.86. Calc. for $C_{37}H_{33}N_4O_{12}F_5$: C, 54.15; H, 4.05; N, 6.83%).

1,2,3,4-Tetra-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)-benzoylamino]-6-deoxy-α/β-D-glucopyranose 18

A mixture of 1,2,3,4-tetra-*O*-acetyl-6-azido-6-deoxy-β-D-glucopyranose **13** (0.6 g, 1.6 mmol) and Boc-Abz-ODhbt (2.0 g, 5.2 mmol) was dissolved in anhydrous THF (36 cm^3) and Pd/C (0.12 g) was added. The reaction mixture was stirred under a positive pressure of hydrogen for 18 h, filtered through Celite and concentrated. The residue was chromatographed with hexane–ethyl acetate (1:1.5) as eluent (R_f 0.3). The inseparable impurity was crystallized out with hexane–ethyl acetate (1:2) and the concentrated filtrate was chromatographed with hexane–ethyl acetate (1:1.5) to afford the title compounds **18** (0.8 g, 88%).

α-Isomer: $\delta_C(CDCl_3)$ 20.4–20.9 (3 × $COCH_3$), 29.0 [C(CH_3)$_3$], 39.09 (C-6), 68.9 (C-4), 69.4 (C-2), 69.7 (C-3), 70.6 (C-5), 80.2 [*C*(CH_3)$_3$], 89.1 (C-1), 119.3–140.5 (Ar), 153.1 [*C*(O)C(CH_3)$_3$] and 168.9–170.2 (C=O); $\delta_H(CDCl_3)$ 1.55 (9 H, s, Bu^t), 2.05, 2.06, 2.14 and 2.2 (12 H, 4 s, 4 × Ac), 3.54 (1 H, m, H^{a}-6), 3.85 (1 H, m, H^{b}-6), 4.14 (1 H, m, H-5), 5.05 (1 H, t, $J_{3,4+4,5}$ 19.0, H-4), 5.10 (1 H, dd, $J_{1,2}$ 3.8 $J_{2,3}$ 10.3, H-2), 5.53 (1 H, t, $J_{2,3+3,4}$ 19.9, H-3), 6.35 (1 H, d, H-1), 6.54 (1 H, br t, NH^{Abz}), 7.0–8.4 (4 H, ArH) and 10.01 (1 H, s, NH^{Boc}).

β-Isomer: $\delta_C(CDCl_3)$ 20.4–20.9 (3 × $COCH_3$), 29.0 [C(CH_3)$_3$], 39.3 (C-6), 68.8 (C-4), 70.4 (C-2), 72.7 (C-3), 73.5 (C-5), 80.2 [*C*(CH_3)$_3$], 89.1 (C-1), 119.3–140.5 (Ar), 153.1 [*C*(O)C(CH_3)$_3$] and 168.9–170.2 (C=O); $\delta_H(CDCl_3)$ 1.55 (9 H, s, Bu^t), 2.057, 2.08, 2.159 and 2.16 (12 H, 4 s, 4 × Ac), 3.57 (1 H, m, H^{a}-6), 3.85 (2 H, m, H-5 and H^{b}-6), 5.03 (1 H, t, $J_{3,4+4,5}$ 20.0, H-4), 5.16 (1 H, dd, $J_{1,2}$ 8.2, $J_{2,3}$ 9.3, H-2), 5.31 (1 H, t, $J_{2,3+3,4}$ 18.8, H-3), 5.73 (1 H, d, H-1), 6.57 (1 H, br t, NH^{Abz}), 7.0–8.4 (4 H, ArH) and 10.06 (1 H, s, NH^{Boc}); ES-MS (Found: 589.4 $[M + Na]^+$. $C_{26}H_{34}N_2O_{12}$ requires *M*, 566.3).

2,3,4-Tri-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)benzoyl-amino]-6-deoxy-α/β-D-glucopyranosyl trichloroacetimidate 24

A mixture of 1,2,3,4-tetra-*O*-acetyl-6-[2′-(*tert*-butoxycarbonyl-amino)benzoylamino]-6-deoxy-α/β-D-glucopyranose **18** (0.75 g, 1.3 mmol) was dissolved in DMF (13 cm^3). Hydrazinium acetate (0.18, 2.0 mmol) was added and the reaction mixture was stirred under argon for 2 h. The reaction was quenched with ethyl acetate (20 cm^3), diluted with dichloromethane (20 cm^3), stirred for 5 min, and washed with aq. sodium chloride (5%; 20 cm^3). The organic extracts were dried over magnesium sulfate, filtered and concentrated. The syrup was purified by column chromatography with hexane–ethyl acetate (1:1) as eluent (R_f 0.45). A 2.5:1 mixture of α- and β-2,3,4-tri-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)benzoylamino]-6-deoxy-D-gluco-pyranose **23** (0.57 g, 82%) was obtained. α-Isomer: $\delta_C(CDCl_3)$ 20.5, 20.7 and 21.0 (3 × $COCH_3$), 26.2 [C(CH_3)$_3$], 39.2 (C-6), 67.6 (C-5), 69.5 (C-3), 69.7 (C-4), 71.3 (C-2), 80.4 [*C*(CH_3)$_3$], 119.5–140.0 (Ar), 153.1 [*C*(O)C(CH_3)$_3$], 169.9, 170.1, 170.2 and 170.4 (C=O); $\delta_H(CDCl_3)$ 1.55 (9 H, s, Bu^t), 2.04, 2.09 and 2.14 (9 H, 3 s, 4 × Ac), 3.36 (1 H, dt, $J_{5,6a}$ 5.5, $J_{6a,6b}$ 14.3, $J_{6a,NH}$ 6.5, H^{a}-6), 3.84 (1 H, ddd, $J_{5,6b}$ 2.8, $J_{6b,NH}$ 6.5, H^{b}-6), 4.28 (1 H, ddd, $J_{4,5}$ 10.0, H-5), 4.87 (1 H, dd, $J_{1,2}$ 3.7, $J_{2,3}$ 10.1, H-2), 4.95 (1 H, t, $J_{3,4+4,5}$ 19.6, H-4), 5.48 (1 H, d, H-1), 5.60 (1 H, t, $J_{2,3+3,4}$ 19.5, H-3), 6.79 (1 H, t, NH^{Abz}), 7.0–8.4 (4 H, ArH) and 10.00 (1 H, s, NH^{Boc}).

β-Isomer: $\delta_H(CDCl_3)$ 1.55 (9 H, s, Bu^t), 2.038, 2.10 and 2.13 (9 H, 3 s, 3 × Ac), 3.50 (1 H, dt, $J_{6a,6b}$ 14.5, $J_{6a,6b+6a,NH}$ 11.9, H^{a}-6), 3.74 (1 H, m, H-5), 3.88 (1 H, m, H^{b}-6), 4.80 (1 H, d, $J_{1,2}$ 8.0, H-1), 4.90 (1 H, dd, $J_{1,2}$ 8.0, $J_{2,3}$ 9.5 H-2), 4.99 (1 H, t, $J_{3,4+4,5}$ 18.8, H-4), 5.29 (1 H, t, $J_{2,3+3,4}$ 19.0, H-3), 6.79 (1 H, m, NH^{Abz}), 7.0–8.4 (4 H, ArH) and 9.96 (1 H, s, NH^{Boc}).

A mixture of α- and β-2,3,4-tri-*O*-acetyl-6-[2′-(*tert*-butoxy-carbonylamino)benzoylamino]-6-deoxy-D-glucopyranose **23** (0.6 g, 1.0 mmol) was dissolved in CH_2Cl_2 (5.5 cm^3). Trichloro-acetonitrile (0.72 cm^3, 7.3 mmol) and potassium carbonate (0.5 g, 3.5 mmol) were added and the reaction was stirred under argon for 5 h. The reaction mixture was filtered through Celite and concentrated. The residue was chromatographed with hexane–ethyl acetate (1.2:1) as eluent (R_f 0.44). The *title compounds* **24** were obtained as a foam (0.6 g, 87%; α/β = 2).

α-Isomer: $\delta_C(CDCl_3)$ 20.4, 20.47 and 20.5 (3 × $COCH_3$), 28.3 [C(CH_3)$_3$], 39.1 (C-6), 68.9 (C-4), 69.7 (C-3), 69.9 (C-2), 70.6 (C-5), 80.2 [*C*(CH_3)$_3$], 92.7 (C-1), 119.1–140.4 (Ar), 152.9 [*C*(O)C(CH_3)$_3$], 160.9 (C=N) and 168.8–169.9 (C=O); δ_H-($CDCl_3$) 1.56 (9 H, s, Bu^t), 2.06, 2.07 and 2.17 (9 H, 3 s, 3 × Ac), 3.53 (1 H, dt, $J_{6a,6b}$ 14.5, $J_{5,6a+6a,NH}$ 12.0, H^{a}-6), 3.87 (1 H, ddd, $J_{5,6b}$ 2.6, $J_{6b,NH}$ 6.7, H^{b}-6), 4.24 (1 H, ddd, $J_{4,5}$ 10.3, $J_{5,6a}$ 6.1, H-5), 5.06 (1 H, t, $J_{3,4+4,5}$ 19.9, H-4), 5.13 (1 H, t, $J_{1,2}$ 3.8 $J_{2,3}$ 10.0, H-2), 5.62 (1 H, t, $J_{2,3+3,4}$ 19.8, H-3), 6.56 (1 H, d, H-1), 6.57 (1 H, t, $J_{NH,6}$ 6.3, NH^{Abz}), 7.0–8.4 (4 H, ArH), 8.69 (1 H, s, $NHCCl_3$) and 10.05 (1 H, s, NH^{Boc}).

β-Isomer: $\delta_C(CDCl_3)$ 95.8 (C-1); $\delta_H(CDCl_3)$ 1.55 (9 H, s, Bu^t), 2.069, 2.07 and 2.15 (9 H, 3 s, 3 × Ac), 3.90–3.97 (3 H, m, H-5 and H_2-6), 5.30–5.37 (3 H, m, H-2,-3 and -4), 5.86 (1 H, d, $J_{1,2}$ 7.5, H-1), 6.61 (1 H, NH^{Abz}), 7.0–8.4 (4 H, ArH), 8.75 (1 H, s, $NHCCl_3$) and 10.03 (1 H, s, NH^{Boc}) (Found: C, 46.55; H, 4.99; N, 6.07. $C_{26}H_{32}N_3O_{11}Cl_3$ requires C, 46.69; H, 4.82; N, 6.28%).

Methyl 2,3,4-tri-*O*-benzyl-6-*O*-{2′,3′,4-tri-*O*-acetyl-6′-[2′-(*tert*-butoxycarbonylamino)benzoylamino]-6-deoxy-β-D-gluco-pyranosyl}-α-D-glucopyranoside 25

A mixture of 2,3,4-tri-*O*-acetyl-6-[2′-*tert*-butoxycarbonyl-amino)benzoylamino]-6-deoxy-α/β-D-glucopyranosyl trichloro-acetimidate **24** (0.1 g, 0.15 mmol), methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside **21** (0.1 g, 0.22 mmol) and freshly activated 4 Å molecular sieves in anhydrous CH_2Cl_2 (2 cm^3) was stirred under argon for 1 h. The reaction mixture was cooled to −78 °C and trimethylsilyl trifluoromethanesulfonate (3 mm^3, 0.015 mmol) was added. After 10 min the reaction mixture was warmed to room temperature and was stirred for 5 h. The mixture was recooled to −30 °C and quenched with 1 drop of triethylamine before being filtered, concentrated and subjected to column chromatography with hexane–ethyl acetate (1:1) as eluent (R_f 0.35). The *title compound* **25** was obtained as a syrup (0.07 g, 51%); $\delta_C(CDCl_3)$ 20.7 ($COCH_3$), 28.3 [C(CH_3)$_3$], 39.3 (C-6′), 55.2 (OCH_3), 68.5 (C-6), 69.0 (C-4′), 69.6 (C-5′), 71.3 (C-2′), 72.4 (C-5), 72.8 (C-3′), 73.4, 74.9 and 75.7 ($CH_2C_6H_5$), 77.5 (C-4), 79.7 (C-2), 80.2 [*C*(CH_3)$_3$], 81.9 (C-3), 98.2 (C-1′), 100.9 (C-1), 121.4–141.0 (Ar), 153.0 [*C*OC(CH_3)$_3$] and 169.0, 169.6 and 170.2 (C=O); $\delta_H(CDCl_3)$ 1.55 (9 H, s, Bu^t), 1.98, 2.03 and 2.12 (9 H, 3 s, 3 × Ac), 3.37 (3 H, s, OCH_3), 3.47 (1 H, t, $J_{3,4+4,5}$ 18.7, H-4), 3.53 (1 H, dd, $J_{1,2}$ 3.5, $J_{2,3}$ 9.8, H-2), 3.63–3.80 (5 H, m, H-5,-5′, H_2-6′ and H^{a}-6), 4.0 (1 H, t, $J_{2,3+3,4}$ 18.5, H-3), 4.07 (1 H, m, H^{b}-6), 4.55 (1 H, d, *J* 11.0, C*H*HC_6H_5), 4.56 (1 H, d, $J_{1',2'}$ 8.0, H-1′), 4.59 (1 H, d, H-1), 4.67 (1 H, d, *J* 12.0, C*H*HC_6H_5), 4.81 (1 H, d, *J* 12.0, CH*H*C_6H_5), 4.82 (1 H, d, *J* 10.9, C*H*HC_6H_5), 4.89 (1 H, d, *J* 11.0, CH*H*C_6H_5), 4.99 (1 H, d, *J* 10.9, C*H*HC_6H_5), 5.00 (1 H, t, $J_{3',4'+4',5'}$ 18.9, H-4′), 5.08 (1 H, t, $J_{2',3'}$ 9.8, H-2′), 5.23 (1 H, t, $J_{2',3'+3',4'}$ 19.0, H-3′), 6.55 (1 H, t, $J_{NH,6}$ 5.9, NH^{Abz}), 7.0–8.4 (19 H, ArH) and 10.0 (1 H, s, NH^{Boc}); ES-MS 977.6 $[M + Li]^+$ (Found: C, 64.01; H, 6.50; N,

2.92%. $C_{52}H_{62}N_2O_{16}$ requires *M*, 970.4; C, 64.36; H, 6.44; N, 2.89%).

N$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*O*-{2,3,4-tri-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)benzoylamino]-6-deoxy-β-D-glucopyranoxy}-L-threonine pentafluorophenyl ester 9

A mixture of 2,3,4-tri-*O*-acetyl-6-[2′-(*tert*-butoxycarbonylamino)benzoylamino]-6-deoxy-α/β-D-glucopyranosyl trichloroacetimidate **24** (0.10 g, 0.20 mmol), *N*$^{\alpha}$-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester **16** (0.15 g, 0.30 mmol) and freshly activated 4 Å molecular sieves in anhydrous CH_2Cl_2 (2 cm^3) was stirred under argon for 1 h. The reaction mixture was cooled to −78 °C and trimethylsilyl trifluoromethanesulfonate (3 mm^3, 0.015 mmol) was added. After 0.5 h the reaction mixture was warmed to room temperature and was stirred for 1 h before being recooled to −78 °C, quenched with 1 drop of triethylamine, filtered, concentrated and subjected to column chromatography on a short column of pre-dried silica gel with hexane–ethyl acetate (1:1) as eluent (R_f 0.4). The title compound **9** was obtained as a foam (0.09 g, 60%), $[a]_D^{20}$ −27.3 (*c* 3.3, CH_2Cl_2); $\delta_C(CDCl_3)$ 17.1 (Thr^g), 20.6 and 20.7 ($COCH_3$), 28.3 [C(CH_3)$_3$], 39.9 (C-6), 47.1 (Fmoc$^{\beta}$), 58.7 (Thr$^{\alpha}$), 67.2 (Fmoc$^{\alpha}$), 69.4 (C-2), 71.35 (C-4), 72.1 (C-3), 72.9 (C-5), 75.1 (Thr$^{\beta}$), 80.2 [*C*(CH_3)$_3$], 99.3 (C-1), 119.5–144.0 (Ar), 152.9 [*C*OC(CH_3)$_3$], 156.2 (CONH), 167.0 [*C*(O)OPfp] and 168.8, 169.2, 169.8 and 170.1 (C=O); $\delta_H(CDCl_3)$ 1.33 (3 H, d, $J_{Thr^{\beta},Thr^{g}}$ 6.4, Thr^g), 1.56 (9 H, s, Bu^t), 2.07, 2.09 and 2.16 (9 H, 3 s, 3 × Ac), 3.07 (1 H, m, H^a-6), 3.67 (1 H, m, H-5), 3.92 (1 H, m, H^b-6), 4.25 (1 H, t, $J_{Fmoc^{\alpha},Fmoc^{\beta}}$ 6.0, Fmoc$^{\beta}$), 4.47 (1 H, m, Thr$^{\beta}$), 4.52 (2 H, m, Fmoc$^{\alpha}$), 4.60 (1 H, d, $J_{1,2}$ 8.0, H-1), 4.75 (1 H, dd, $J_{Thr^{\alpha},Thr^{\beta}}$ 3.0, $J_{Thr^{\alpha},NH}$ 8.4, Thr$^{\alpha}$), 4.95 (1 H, t, $J_{1,2+2,3}$ 19.5, H-2), 4.99 (1 H, t, $J_{3,4+4,5}$ 17.3, H-4), 5.27 (1 H, t, $J_{2,3+3,4}$ 19.1, H-3), 5.82 [1 H, d, $J_{NHThr^{\alpha}}$ 8.4, NH^{Thr}], 6.67 (1 H, br t, $J_{NH,6}$ 5.8, NH^{Abz}), 6.8–8.4 (12 H, ArH) and 10.15 (1 H, s, NH^{Boc}); ES-MS 1020.3 [M + Li]$^+$ ($C_{39}H_{48}F_5N_3O_{15}$ requires *M*, 1013.9).

Fluorescence experiments

Stock solutions of compounds **1**–**5** were prepared in 50 mmol TRIS chloride buffer at pH 8.5, to give substrate concentrations of ~500 μM. For urea-denaturation studies, a series of aliquots of ~10 mm^3 of the stock solution were transferred into vials containing solutions of urea in TRIS buffer (0, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 and 7.0 M) to make the final volume equal to 1 cm^3, and the final concentration of the peptide equal to 5.0 μM. The solutions were allowed to equilibrate for 2 h at 25°C before fluorescence measurements were commenced.

For the comparison of the rate of heterodimer formation of peptide **2** with the peptides and glycopeptides **1** and **3**–**5** in TRIS and 2 M urea, ~10 mm^3 of the stock solution of compound **2** (500 μM) and ~10 mm^3 of the stock solutions of one of the compounds **1**, **3**–**5** (500 μM) was added to an appropriate volume of either TRIS or 2 M urea in a cuvette to make the final volume 1 cm^3 and a final concentration of the compounds equal to 5.0 μM. Fluorescence measurements were commenced 30 s after mixing of any two substrates.

CD experiments

For the CD spectra, solutions of the peptide **1**–**7** were prepared in degassed water (~0.5 mg cm^{-3}) and concentrations were determined accurately by UV spectroscopy. For the denaturation studies stock solutions of the substrates **1**, **4** and **5** were prepared in 50 mM TRIS perchlorate buffer at pH 8.5 at concentrations of 120 μM.

Acknowledgements

Amino acid analysis was performed by Dr Ib Svendsen, and Dr Anita M. Jansson performed the ES-MS. Support in obtaining the CD data was provided by Niels Kaarsholm at NOVO Nordisk A/S.

References

1 H. Lis and N. Sharon, *Eur. J. Biochem*, 1993, **218**, 1; H. C. Joao and R. A. Dwek, *Eur. J. Biochem*. 1993, **218**, 239; J. R. Rasmussen, *Curr. Opin. Struct. Biol.*, 1992, **2**, 682; R. B. Parekh, *Curr. Opin. Struct. Biol.*, 1991, **1**, 750; R. A. Dwek, *Chem. Rev.*, 1996, **96**, 683.
2 F. C. Grochee, M. J. Gramer, D. C. Andersen, J. B. Bahr and J. R. Rasmussen, *Frontiers in Bioprocessing II*, ed. C. P. Todd, S. K. Sikdar and M. Bier, ACS-series, Washington, DC, 1992, p. 199.
3 T. Arakawa and S. N. Timasheff, *Methods Enzymol.*, 1985, **114**, 49.
4 K. Olden, J. B. Parent and S. L. White, *Biochim. Biophys. Acta*, 1982, **650**, 209; A. D. Elbein, *Methods Enzymol.*, 1987, **138**, 661.
5 M. A. Titus, *Curr. Opin. Cell Biol.*, 1993, **5**, 77; A. Columbus, *Curr. Opin. Cell Biol.*, 1993, **5**, 17.
6 K. T. O'Neil and W. F. DeGrado, *Science*, 1990, **250**, 646.
7 L. Otvos, J. Thurin, E. Kollat, L. Urge, H. M. Mantsch and M. Hollosi, *Int. J. Pept. Protein Res.*, 1991, **38**, 476; J. P. Aubert, N. Helbecque and M. H. Loueheux-Lefebvre, *Arch. Biochem. Biophys.*, 1981, **208**, 20.
8 H. C. Joao, I. G. Scragg and R. A. Dwek, *FEBS Lett.*, 1992, **307**, 343; J. T. Davis, S. Hirani, C, Bartlett and B. R Reid, *J. Biol. Chem.*, 1994, **269**, 3331.
9 K. G. Rice, P. Wu, L. Brand and Y. C. Lee, *Biochemistry*, 1993, **32**, 7264; 1991, **30**, 6646; B. Imperiali and K. W. Rickert, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 97.
10 P. Y. Chou and G. D. Fasman, *Annu. Rev. Biochem*., 1978, **47**, 251; N. E. Zhou, C. M. Kay and R. S. Hodges, *J. Mol. Biol.*, 1994, **237**, 500.
11 S. Y. M. Lau, A. K. Taneja and R. S. Hodges, *J. Biol. Chem.*, 1984, **259**, 13 253.
12 V. T. Förster, *Ann. Phys.*, 1948, **6**, 55.
13 P. Well and L. Brand, *Anal. Biochem*., 1994, **218**, 1.
14 M. Meldal and K. Breddam, *Anal. Biochem*., 1991, **195**, 141.
15 J. Ø. Duus, J. Winkler and M. Meldal, manuscript in preparation.
16 R. L. Whistler, L. W. Doner and M. Kosik, *Methods Carbohydr. Chem*., 1972, **6**, 411.
17 M. G. Ambrose and R. W. Binkley, *J. Org. Chem*., 1983, **48**, 674.
18 Z. Gyorgydeak and L. Szilagyi, *Leibigs Ann. Chem*., 1987, 235.
19 R. R. Schmidt, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, 212.
20 G. Excoffier, D. Gagnaire and J.-P. Utille, *Carbohydr. Res.*, 1975, **39**, 368.
21 P. J. Garegg, T. Iverson and S. Oscarson, *Carbohydr. Res.*, 1976, **50**, c12.
22 V. Pozgay and H. Jennings, *J. Org. Chem*., 1987, **52**, 4635; 1988, **53**, 4042.
23 M. Meldal, *Tetrahedron Lett*., 1992, **33**, 3077.
24 A. Dryland and R. C. Sheppard, *Tetrahedron*, 1988, **44**, 859; M. Meldal, A. Holm and O. Buchardt, *PCT Int.Appl. WO* 90 07,975 (*Chem. Abstr.*, 1991, **114**, 82556v); L. R. Cameron, J. L. Holder, M. Meldal and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1988, 2895.
25 H. Rink, *Tetrahedron Lett.*, 1987, **28**, 3787.
26 R. Knorr, A. Trzeciak, W. Bannwarth and D. Gillessen, *Tetrahedron Lett.*, 1989, **30**, 1927.
27 E. Atherton, L. R. Cammeron and R. C. Sheppard, *Tetrahedron*, 1988, **44**, 843.
28 M. Meldal and K. J. Jensen, *J. Chem. Soc., Chem. Commun.*, 1990, 438.
29 E. Meinjohanns, M. Meldal, T. Jensen, O. Werdelin, L. Galli-Stampino, S. Mouritsen and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1997, 871.
30 I. Christiansen-Brams, M. Meldal and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1993, 1461.
31 K. Dax, W. Wolflehner and H. Weidmann, *Carbohydr. Res.*, 1978, **65**, 132.
32 T. Utamura, K. Kuromatsu, K. Suwa, K. Koizumi and T. Shingu, *Chem. Pharm. Bull.*, 1986, **34**, 2341.

Paper 6/07624F
Received 8*th November* 1996
Accepted 20*th January* 1997