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Influence of glycosylation on the conformational preferences of folded oligopeptides

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Abstract—Synthesis, characterization, and conformational analysis by FT-IR absorption, ^{1}H NMR and X-ray diffraction techniques are described for a series of side-chain *O*-glycosylated Thr peptides of different main-chain length rich in the helicogenic Aib residue. The results obtained, compared with those of related peptides containing side-chain protected Thr and Ser residues and host Aib homo-oligomers, also reported in this work, provided new information on the preferred conformation of the naturally occurring antifreeze glycopeptides. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Antifreeze glycoproteins (AFGPs), present in the serum of polar and deep-sea fish, allow them to survive in seas where the temperature is sub-zero by depressing the freezing temperature of the blood without increasing the osmotic pressure.[1] The primary structure of these proteins consist of repeating glycotripeptide units, $(Thr–Ala–Ala)_n$, with β-D-galactopyranosyl-(1→3)-α-D-*N*-acetylgalactosamine [Gal(β1→3)GalNAcα1] disaccharide groups extending from the Thr side chains. Different fractions of AFGPs have been isolated, which range from the large AFGP 1 (32 kD) to the smallest AFGP 8, consisting of only four glyco-tripeptide units (2.7 kD). In order to elucidate their mechanism of action a number of efforts have been made to characterize their structure in solution. Bush and Feeney[2] proposed an attractive 3D-model for the physiological action of antifreeze glycoproteins, namely that in water they form an extended, left-handed threefold helix of $poly(Pro)_n$ II type. In this structure all of the sugar moieties are on one side of the helix. This disposition allows for hydrophilic interactions with water molecules to take place, particularly through the action of the hydroxyl groups, thus blocking the growth of ice crystals.

CD and NMR studies in water, carried out in our laboratory on the sequential glycopeptides $[Thr(Galβ1)–Ala–Ala]_n$ (n=2–7) as models of natural AFGPs, showed no evidence for the presence of an ordered structure, at least in the temperature range from −2 to 25°C.[3] Nevertheless, for the 21-residue glycopeptide $[Thr(Galβ1)–Ala–Ala]_7$, the CD pattern in water at temperatures higher than 50°C or in 2,2,2-trifluoroethanol (TFE) was compatible with the presence of a small amount of helical structure, suggesting that an ordered conformation (more specifically, a 3_{10}-helix[4]) might form in sufficiently long segments of glycosylated peptides in a structure supporting solvent. In such a case the model proposed by Bush and Feeney[2] for the antifreezing action of AFGPs would still hold, at least partially, as in the right-handed, threefold 3_{10}-helix all sugar side chains would be located on one side of the rod-like structure.

In order to prepare well-characterized models of such a helical structure, we have decided to exploit the known peculiarity of $C^α$-tetrasubstituted α-amino acids to stabilize helical peptide conformations,[5,6] by designing repeating glycotripeptide sequences of the AFGPs in which the Ala residues are replaced by the prototypical $C^α$ -tetrasubstituted α-amino acid α-aminoisobutyric acid (Aib). Indeed, previous work from our as well as from other laboratories[5,6] has firmly established that, in solvent of low polarity, homo-oligomers based on Aib (and on other $C^α$-tetrasubstituted α-amino acids as well)[7] form fully developed 3_{10}-helices at approximately the octamer level, and even from pentamer to heptamer the amount of 3_{10}-helix is already significant. In the crystal state, however, all terminally blocked Aib homo-oligomers, beginning from the tripeptide, form either type III(III′) β-turns,[8–10] the basic structural unit of the 3_{10}-helix, or regular 3_{10}-helices. Even in hetero-peptides containing Aib and $C^α$-trisubstituted protein amino acids, Aib residues are strong promoters of the 3_{10}-helical conformation.[5,6,11] Indeed, 3_{10}-helices are predominantly adopted by short (<10 residues) sequences with at least 50% Aib residues and containing isolated protein amino acids.

With the aim of determining the influence, if any, of glycosylation on the preferred conformation of folded (helical)

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Table 1. Primary structures of peptides investigated in this work

Symbol	Peptide
3TG	Z–Thr(GalAc$_4$β1) –Aib–Aib–O*t*Bu[a]
4TG	Z–Aib–Thr(GalAc$_4$β1) –Aib–Aib–O*t*Bu
5TG	Z–Aib–Aib–Thr(GalAc$_4$β1) –Aib–Aib–O*t*Bu
6TG	Z–Thr(GalAc$_4$β1) –Aib–Aib–Thr(GalAc$_4$β1) –Aib–Aib–O*t*Bu
8TG	Z–Aib–Aib–Thr(GalAc$_4$β1) –Aib–Aib–Thr(GalAc$_4$β1) –Aib–Aib–O*t*Bu
4′TG	Z–Thr(GalAc$_4$β1) –Aib–Aib–Aib–OMe[b]
3T	Z–Thr(*t*Bu) –Aib–Aib–OMe[c]
4T	Z–Aib–Thr(*t*Bu) –Aib–Aib–OMe
5T	Z–Aib–Aib–Thr(*t*Bu) –Aib–Aib–OMe
3S	Z–Ser(*t*Bu) –Aib–Aib–OMe
5S	Z–Aib–Aib–Ser(*t*Bu) –Aib–Aib–OMe
4U	Z–Aib–Aib–Aib–Aib–O*t*Bu
5U	Z–Aib–Aib–Aib–Aib–Aib–O*t*Bu
6U	Z–Aib–Aib–Aib–Aib–Aib–Aib–O*t*Bu
8U	Z–Aib–Aib–Aib–Aib–Aib–Aib–Aib–Aib–O*t*Bu

[a] Z, benzyloxycarbonyl; Ac, acetyl; O*t*Bu, *tert*-butoxy.
[b] OMe, methoxy.
[c] *t*Bu, *tert*-butyl.

oligopeptides, we have synthesized a series of terminally protected, *O*-glycosylated, Aib-rich, Thr peptides (Table 1) and, for comparison, two sets of non-glycosylated oligopeptides containing side-chain protected Thr or Ser residues. Their conformational preferences have been investigated by FT-IR absorption, ^{1}H NMR and, whenever possible, by X-ray diffraction, and compared with those of the previously described Aib homo-oligomers.[5,6] Preliminary accounts of a limited part of this work have been reported.[12,13]

2. Results and discussion

2.1. Synthesis and characterization

Peptides and glycopeptides were synthesized in solution, preferentially by the step-by-step approach, starting from the C-terminal H–Aib–OMe (OMe, methoxy)[19] or H–Aib–O*t*Bu (O*t*Bu, *tert*-butoxy)[14] derivative. Z–Aib–OH (Z, benzyloxycarbonyl) was activated by the symmetrical anhydride method,[15,16] whereas HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate)[17] was used as condensing reagent in couplings involving a chiral C$^\alpha$-trisubstituted amino acid, including Z–Thr(GalAc$_4$-β1)OH.[18] In order to avoid premature removal of the Ac (acetyl) protecting groups from the sugar, in the synthesis of the Thr glycopeptides (**TG**) coupling reactions were stopped after 24 h, even if the amino component was still detectable. The bis-Thr glycosylated octapeptide **8TG** and the Ser(*t*Bu) (*t*Bu, *tert*-butyl) pentapeptide **5S** were obtained by fragment condensation of Z–Aib–Aib–OH[19] on the N$^\alpha$-deprotected compounds **6TG** and **3S**, respectively, in the presence of HATU. The Z-protecting group was removed by catalytic hydrogenation.

Tripeptides were obtained in a pure form by crystallization and all other compounds were purified by medium-pressure liquid chromatography. Peptides and glycopeptides were characterized by melting point determination, optical rotation, thin-layer chromatography (TLC), mass spectra (MALDI–TOF MS), elemental analysis and ^{1}H NMR. The synthesis and characterization of the Aib(U) homopeptides have already been reported.[14]

2.2. Crystal structure analysis

The 3D-structure of Z–Aib–Aib–Thr(*t*Bu)– Aib–Aib–OMe (**5T**) was determined by X-ray diffraction. Fig. 1 compares the molecular structure of **5T** with that recently reported[13] for the related glycosylated tripeptide Z–Thr(GalAc$_4$β1)– Aib–Aib–O*t*Bu (**3TG**). Relevant N$^\alpha$-protecting group, backbone and side-chain torsion angles, as well as the intra- and intermolecular H-bond parameters, for pentapeptide **5T** are given in Tables 2 and 3, respectively.

Bond lengths and bond angles are in general agreement with

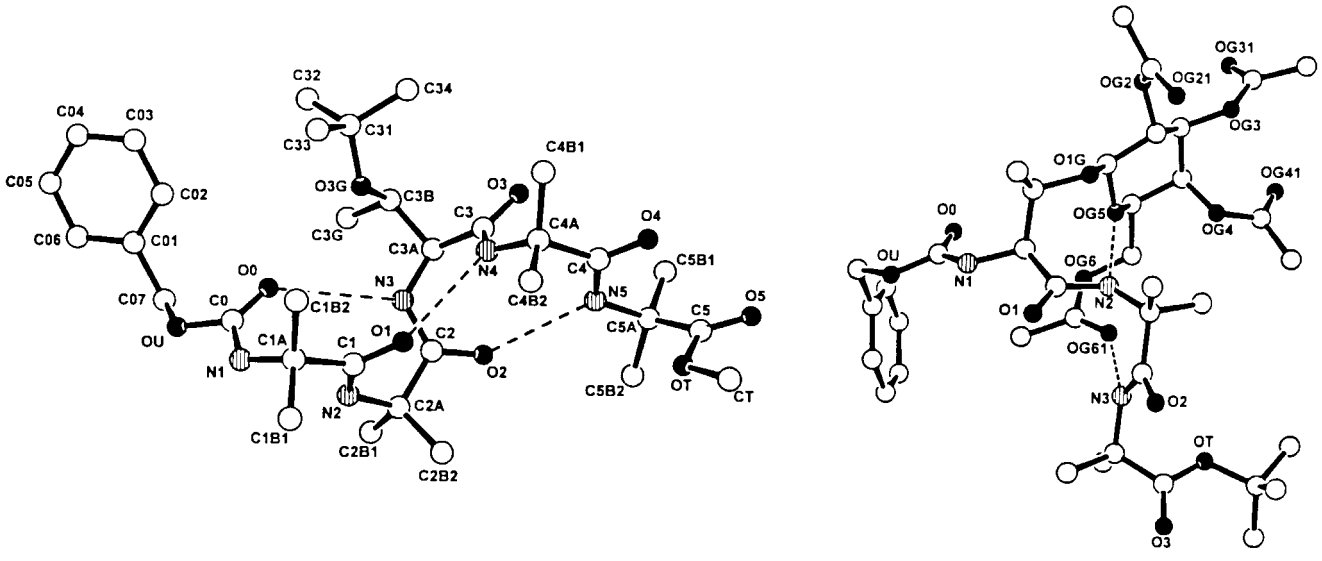

Figure 1. X-ray diffraction structures of Z–Aib–Aib–Thr(*t*Bu)–Aib–Aib–OMe (**5T**) (left) and Z–Thr(GalAc$_4$β1)–Aib–Aib–O*t*Bu (**3TG**) (Ref. 13) (right). The intramolecular H-bonds are represented by dashed lines.

Table 2. Selected N^{α}-protecting group, backbone and side-chain torsion angles (deg) [the torsion angles for rotation about bonds of the peptide backbone (ϕ, ψ, ω) and side-chains (χ) are described in Ref. 44. For the torsion angles for rotation about bonds of the Z-protecting group (θ) see Ref. 20] for Z–Aib–Aib–Thr(*t*Bu) –Aib–Aib–OMe (**5T**)

Torsion angle	Peptide **5T**	Torsion angle	Peptide **5T**
θ^{2}	−98.7(3)	ϕ_4	−56.4(3)
θ^{1}	−167.5(3)	ψ_4	−25.7(4)
ω_0	−170.2(3)	ω_4	178.2(3)
ϕ_1	−56.7(4)	ϕ_5	47.0(4)
ψ_1	−29.4(4)	ψ_5[a]	47.0(3)
ω_1	−175.5(3)	ω_5[b]	−176.3(3)
ϕ_2	−54.7(4)	$\chi^{1,1}{}_{3}$[c]	−60.8(3)
ψ_2	−27.7(4)	$\chi^{1,2}{}_{3}$[d]	61.1(3)
ω_2	−177.9 (2)	$\chi^{2}{}_{3}$	139.0(2)
ϕ_3	−66.7(3)		
ψ_3	−14.0(3)		
ω_3	−172.9(2)		

[a] N5-C5A-C5-OT torsion angle.
[b] C5A-C5-OT-CT torsion angle.
[c] N3-C3A-C3B-C3G torsion angle.
[d] N3-C3A-C3B-O3G torsion angle.

previously reported values for the geometry of the benzyloxycarbonylamino moiety,[20] the methyl ester group,[21] the Aib residue,[22,23] and the peptide unit.[24,25] The N3–C3A–C3 bond angle of the Thr(*t*Bu) residue is significantly expanded relative to the regular tetrahedral value, 114.2(2)°, presumably to help accomodate the bulky ether side chain.

The backbone of the pentapeptide is folded in a right-handed 3_{10}-helical structure.[4] Peptide groups N3–H3 to N5–H5 and O0═C0 to O2═C2 participate in three consecutive 1←4 (type III β-turn[8–10]) C═O···H–N intramolecular H-bonds, appropriate for a 3_{10}-helix. The range of observed N···O distances, 3.008(3)– 3.187(3) Å, and that of N–H···O angles, 164.1–174.4°, are those usually seen for such intramolecular H-bonds.[26–28] Also the C-terminal Aib residue adopts a conformation in the helical region, but it has a handedness opposite to that shown by the preceding residues, a common observation for Aib-rich 3_{10}-helical peptides.[6]

In the pentapeptide no significant deviation of the ω torsion angles (>10°) from the ideal value of the *trans* planar urethane, peptide, and ester units (180°) is observed. The *trans* arrangement of the θ^1 torsion angle of the benzyloxycarbonylamino moiety is that usually reported for Z-amino acids and peptides.[20] Also the value of θ^2, −98.7(3)°, is found in one of the expected regions (±90°, 180°).[20] The methyl ester conformation with respect to preceding C^{α}–N bond is intermediate between the *anticlinal* and *antiperiplanar* conformations.[29] The set of χ^1 torsion angles found for the Thr side chain ($g^- g^+$, with the O^{γ} atom in the g^+ position) is that preferred by Thr peptides.[30]

The molecules of the helical pentapeptide are held together in a head-to-tail fashion along the *a*-direction, in rows stabilized by (urethane) N1–H1···O4═C4 (peptide) intermolecular H-bonds. The additional potential donor N2–H2 group does not seem to be involved in the intermolecular H-bonding scheme.

Initial information on the influence of glycosylation on the preferred conformation of peptides rich in the strong turn/helical forming Aib residue[5,6,11] is provided by a comparison of the X-ray diffraction structures of the non-glycosylated pentapeptide **5T** and its glycosylated, C-terminal tripeptide short sequence (**3TG**)[13] (Fig. 1). It is clear that the presence of an internal Thr(*t*Bu) residue does not significantly perturb the crystal-state helical structure of **5T**, despite the bulkiness and H-bonding acceptor properties of its ether side-chain moiety. By contrast, owing to the occurrence of side-chain to main-chain, carbohydrate-to-peptide intramolecular H-bonds, the peptide backbone of the tripeptide with an N-terminal Thr($GalAc_4\beta1$) residue is forced to adopt a more extended conformation than the expected regularly folded structure. It remains to be seen whether this dramatic conformational influence will also be related to the length of the peptide (i.e. to the intrinsic stability of the turn/helix) in general and to the position of the glycosylated residue in the peptide main chain in particular.

2.3. Solution conformational analysis

The conformational preferences of the four series of terminally and side-chain protected Aib-rich peptides containing *O*-glycosylated Thr (**3-6TG**, **8TG** and **4′TG**), Thr (**3-5T**) and Ser (**3S** and **5S**), respectively, were investigated in a solvent of low polarity ($CDCl_3$) at different concentrations (in the range 10–0.1 mM) by using FT-IR absorption and ^1H NMR techniques. Figs. 2 and 3 show the most relevant FT-IR absorption spectra in the conformationally informative 3500–3200 cm^{-1} region (amide N–H stretching or amide A) region. Using Mizushima's dilution method[31] we have been able to show that at 1 mM concentration self-association via N–H···O═C intermolecular H-bonding is negligible for all oligopeptides (spectra not shown). As a consequence, for all peptides investigated, the H-bonding observed at 1 mM concentration should be interpreted as arising from intramolecular interactions only.

In the TG series, Fig. 2(I), the curves are complex and characterized by several absorptions.[32] We assign: (i) the band (shoulder) at about 3430 cm^{-1}, present only in the spectra of the peptides with an N-terminal Aib residue, to free, solvated NH groups; (ii) the band(s) in the 3410–3390 cm^{-1} region, relatively stronger in the peptides with an N-terminal Thr($GalAc_4\beta1$) residue, to weakly H-bonded NH groups typical of the fully-extended

Table 3. Intra- and intermolecular H-bond parameters for Z–Aib–Aib–Thr(*t*Bu)–Aib–Aib-OMe (**5T**)

Donor D–H	Acceptor A	Symmetry operation	Distance (Å) D…A	Distance (Å) H…A	Angle (deg) D–H…A
N3–H3	O0	*x*, *y*, *z*	3.187(3)	2.33	174.4
N4–H4	O1	*x*, *y*, *z*	3.008(3)	2.17	164.1
N5–H5	O2	*x*, *y*, *z*	3.102(3)	2.26	165.6
N1–H1	O4	*x*+1, *y*, *z*	2.826(3)	1.99	164.0

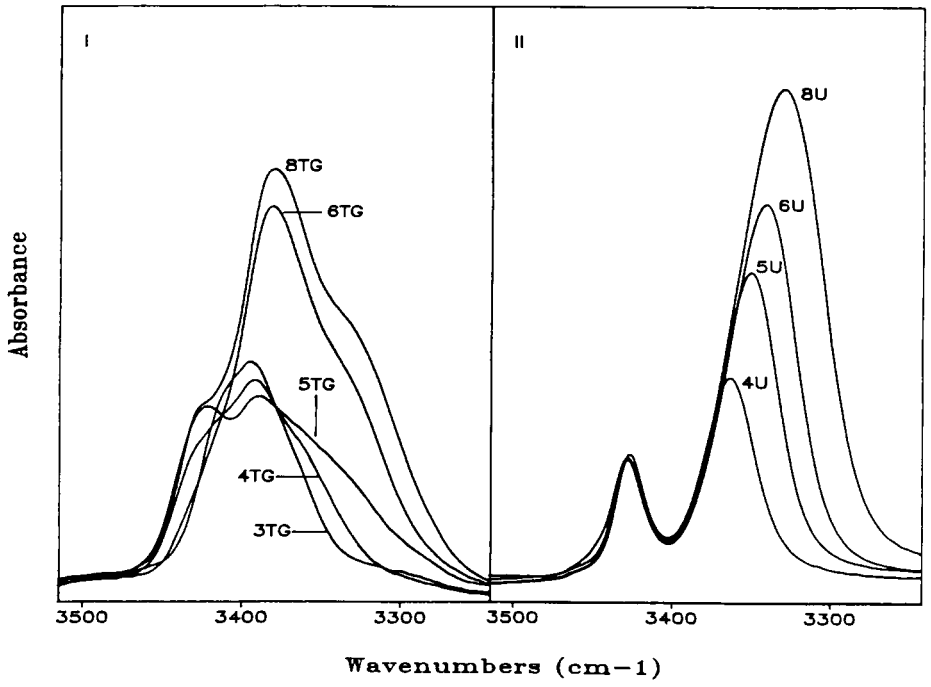

Figure 2. FT-IR absorption spectra (N–H stretching region) of the glycosylated peptides **3TG**, **4TG**, **5TG**, **6TG** and **8TG** (I) and the Aib homo-peptides **4U**, **5U**, **6U** and **8U** (II) in $CDCl_3$ solution. Peptide concentration: 1 mM.

(C_5) conformation[9,33] and, probably, of side-chain to main-chain interactions as well;[34,35] (iii) the pronounced shoulder at 3360–3330 cm^{-1}, shown by the **4TG–8TG** peptides, to strongly H-bonded NH groups typical of folded conformations. Conversely, the spectra of the Aib homo-oligomers, Fig. 2(II), are much simpler, exhibiting only the two bands at about 3430 and 3350 cm^{-1}, indicative of an increasing amount of folded forms with main-chain elongation.[36,37]

An inspection of the spectra of peptides with the same main-chain length (either tetra- or pentapeptides), illustrated in Fig. 3, allow a more stringent comparison of the conformational propensities of the various series to be made. Clearly, the observed decreasing rank order of the intensity of the $\approx$3350 cm^{-1} band relative to the bands of free/weakly H-bonded NH groups at 3430–3390 cm^{-1} (Aib>Ser(*t*-Bu)>Thr(*t*Bu)$\gg$Thr($GalAc_4\beta1$)) strongly supports the

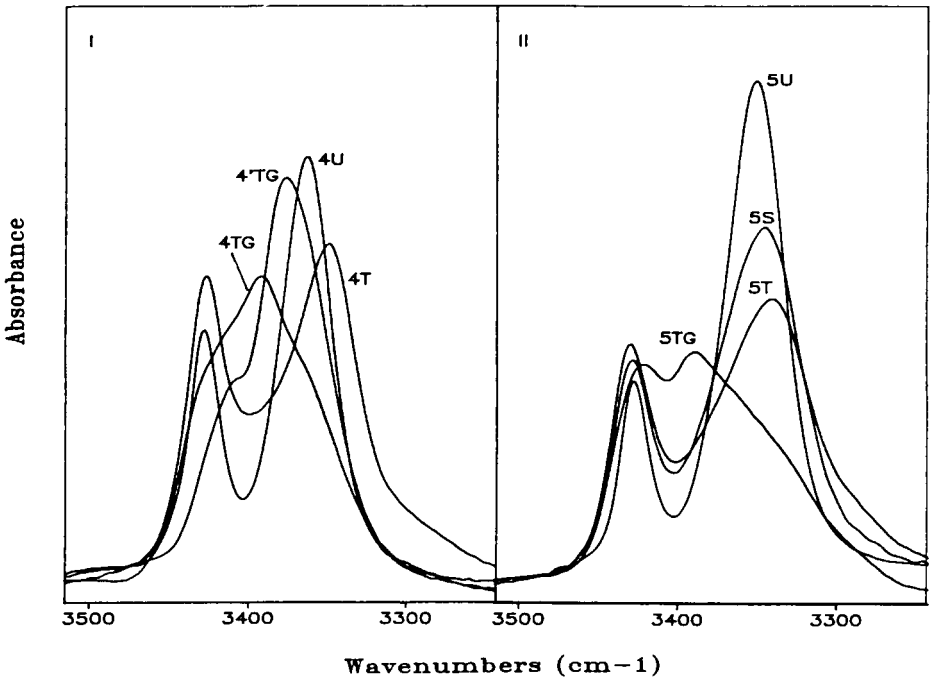

Figure 3. FT-IR absorption spectra (N–H stretching region) of the tetrapeptides **4TG**, **4′TG**, **4T** and **4U** (I) and the pentapeptides **5TG**, **5T**, **5S** and **5U** (II) in $CDCl_3$ solution. Peptide concentration: 1 mM.

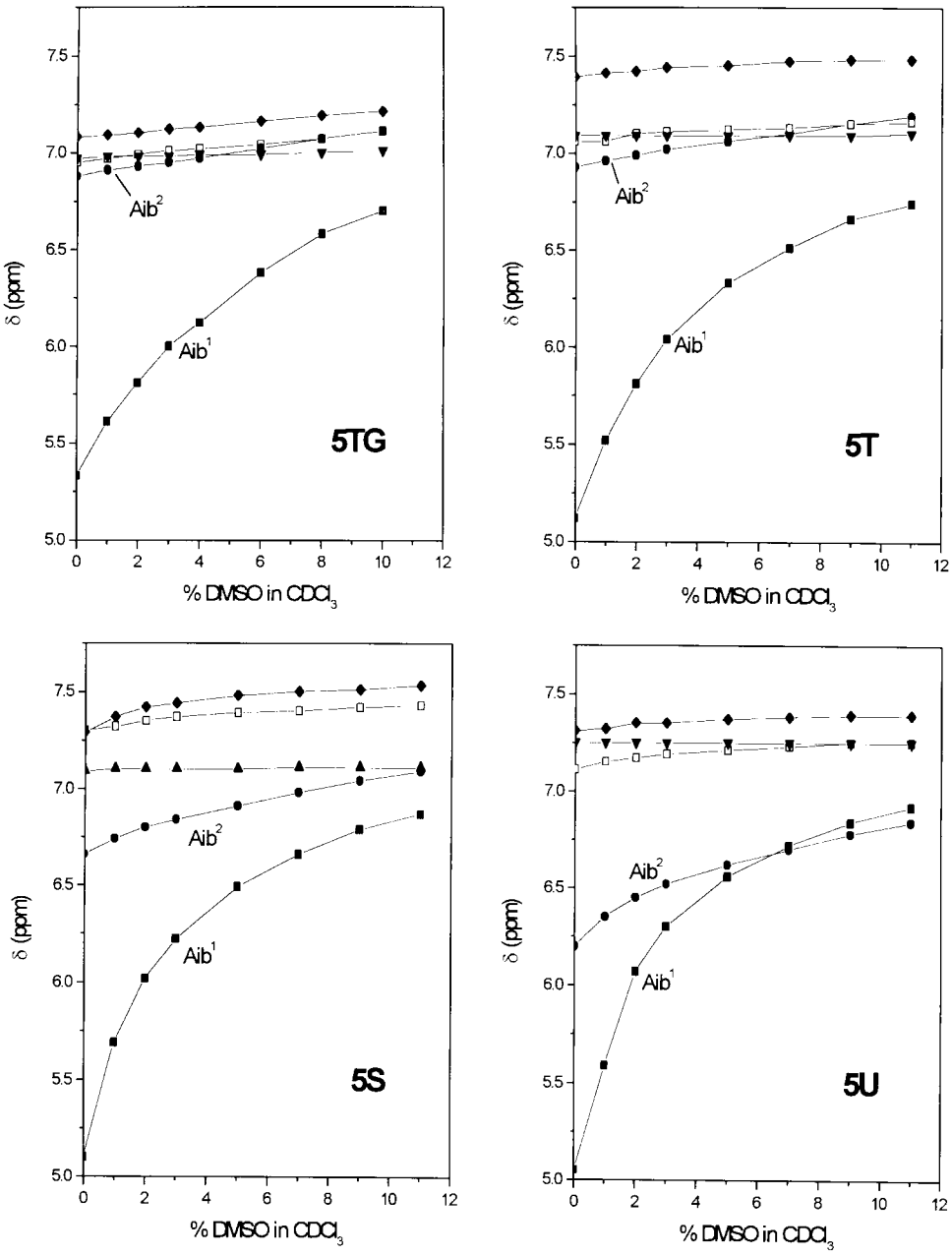

Figure 4. Plot of NH chemical shifts (δ) in the ^{1}H NMR spectra of the pentapeptides **5TG**, **5T**, **5S** and **5U** as a function of increasing percentages of DMSO-d_6 added to the $CDCl_3$ solution (v/v). Peptide concentration: 1 mM.

view that the Thr(GalAc$_4\beta$1) residues effectively interfere with the propensity of the Aib-rich segments to fold into turn/helical structures. This phenomenon is slightly more significant when the glycosylated Thr residue is positioned at the N-terminus of the peptide chain (compare the curves of the two isomeric tetrapeptides **4TG** and **4′TG**). In conclusion, a combination of side-chain bulkiness and appropriate location of H-bonding acceptor groups seems to drastically reduce the extent of turn/helix formation even in a structure supporting solvent and in Aib-based peptides.

To obtain more detailed information on the conformational preferences of the four peptide series in $CDCl_3$ solution, we carried out a 400 MHz ^{1}H NMR investigation. The delineation of inaccessible (or intramolecular H-bonded) NH groups by ^{1}H NMR was performed using solvent dependence of NH chemical shifts, by adding increasing amounts of the strong H-bonding acceptor solvent dimethylsulphoxide (DMSO) to the $CDCl_3$ solution (peptide concentration 1 mM).[38,39] As typical examples, Figs. 4 and 5 illustrate the behaviour of the NH resonances of the penta-, hexa-, and octapeptides. Assignment of the conformationally critical N(1)H and N(2)H protons was achieved by chemical shift analysis (the position of the N-terminal urethane proton is always largely upfield with respect to the peptide protons)[32] and ROESY experiments.

In all of the peptides shown in Figs. 4 and 5, except **6TG**, two classes of NH protons were observed. Class (a) [N(1)H

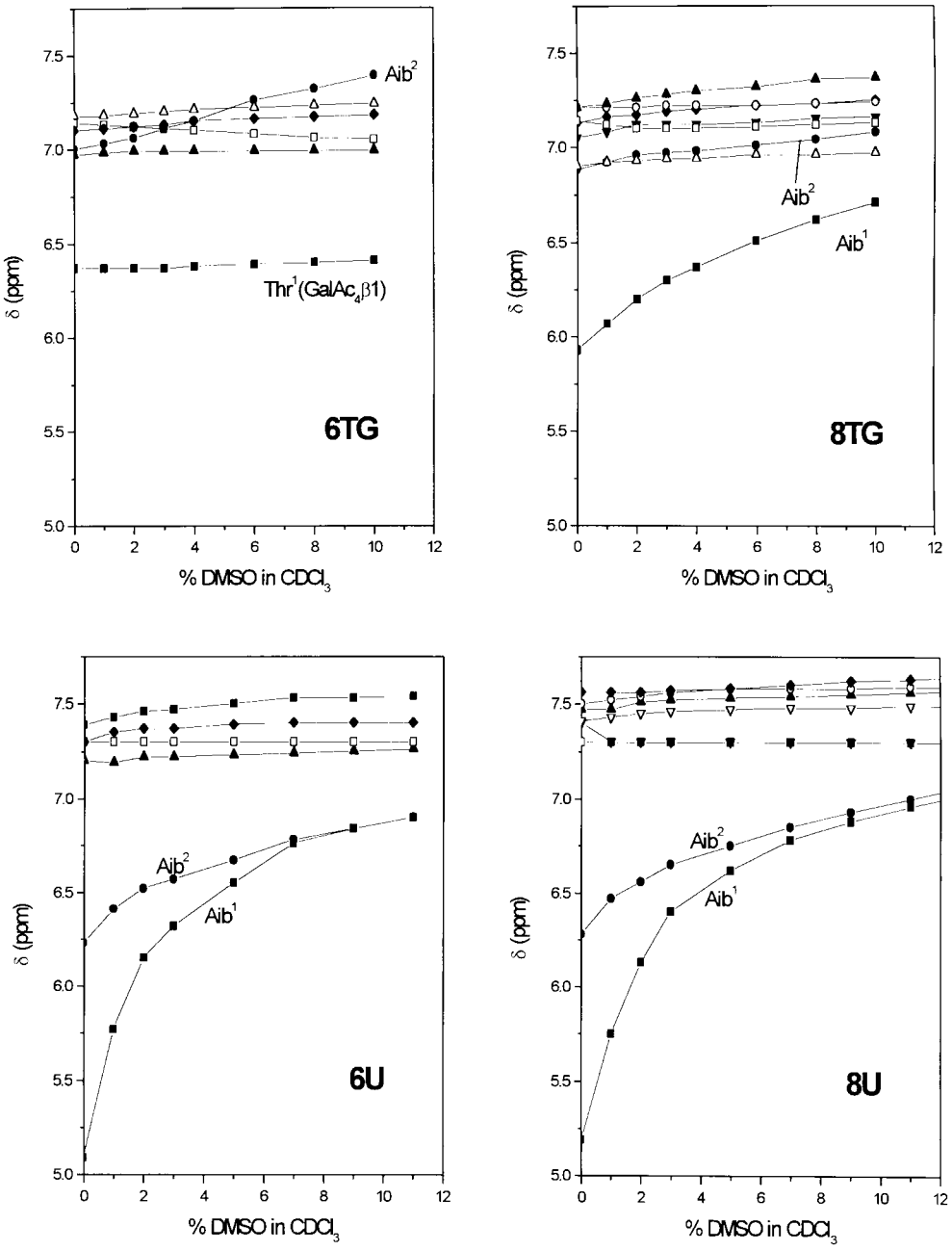

Figure 5. Plot of NH chemical shifts (δ) in the ^{1}H NMR spectra of the hexapeptides **6TG** and **6U**, and the octapeptides **8TG** and **8U** as a function of increasing percentages of DMSO-d_6 added to the $CDCl_3$ solution (v/v). Peptide concentration: 1 mM.

and N(2)H protons] includes protons whose chemical shifts are sensitive to the addition of DMSO. Interestingly, the sensitivity of the N(1)H proton is much higher than that of the N(2)H proton. Class (b) [all other NH protons] includes those displaying a behaviour characteristic of shielded protons (relative insensitivity of chemical shifts to solvent composition). However, in hexapeptide **6TG**, with a glycosylated Thr residue at the *N*-terminus, even the chemical shift of the N(1)H proton does not change significantly upon addition of DMSO. Between 0 and 10% DMSO in $CDCl_3$ (v/v) the decreasing rank orders of Δ ppm values for N(1)H protons of peptides of the same main-chain length are as follows: **5U**>**5S**>**5T**>**5TG**; **6U**≫>**6TG**; **8U**≫**8TG**. The same trend is seen for the Δ ppm values of the N(2)H protons.

In summary, these ^{1}H NMR results allow us to conclude that in the structure-supporting solvent $CDCl_3$ the N(3)H protons and the following NH protons in the main-chain of the four peptide series investigated are almost inaccessible to the perturbing agent and are therefore most probably intramolecularly H-bonded. From the analysis of the behaviour of the N(1)H and N(2)H protons, however, it seem safe to conclude that the stability of the regular 3_{10}-helical structure typical of the $(Aib)_n$ homo-oligomers (where only the two N-terminal NH protons do not participate in the intramolecular H-bonding scheme) is only slightly reduced by the presence of one Ser(*t*Bu) or Thr(*t*Bu) residue, whereas one (or two) Thr($GalAc_4\beta1$) residue(s) is (are) highly detrimental, particular when located at the N-terminus of the peptide main-chain. It is worth noting that these conclusions

of the ^{1}H NMR study agree well with those extracted from the FT-IR absorption investigation discussed above.

3. Conclusions

Clearly, the repetition of a triplet, –Thr(O–glycosyl)– Ala–Ala–, in the sequential peptides with antifreeze activity from Antartic fish[1,2] implies a ternary helix as a reasonable conformation. Of the two most extensively described ternary helices in peptides and proteins, the extended poly(Pro)$_n$ II helix and the folded 3_{10}-helix,[4] recent conformational work[40] tends to favour the former as the characteristic structural motif of the antifreeze peptides. However, the contribution of the latter type of helix could not be completely discarded.[3]

With the aim of shedding light on the contribution, if any, of the 3_{10}-helix to the 3D-structural ensemble of antifreeze peptides, in this work we examined the solution preferred conformations of peptides containing one (or two) Thr(GalAc$_4$β1) residue(s) and characterized by a high amount of Aib residues. The incorporation of this strongly helicogenic amino acid[5,6] was intended to drastically bias the peptide conformation towards the formation of a 3_{10}-helical structure. In our model compounds the acetoxy groups of the peracetylated, *O*-glycosylated Thr side chain are expected to mimic the acetamido group of the GalNAc moiety of the disaccharide unit present in the antifreeze peptides.[1,2]

Taken together, the results described here, combined with our crystallographic data illustrated in Ref. 13, are strongly in favour of the thesis that the presence of a glycosylated Thr residue tends to discourage the formation of a 3_{10}-helical structure even in an Aib-rich peptide, particularly when the glycosylated residue is located at the N-terminus of the main chain, and that the major factor operative in the helix destabilization is the competitive role of side-chain to main-chain H-bonding. In our view this conclusion implies that the occurrence of the 3_{10}-helix to a significant extent in the conformational equilibrium mixture of the antifreeze peptides, where the much less helicogenic Ala residues are found in lieu of the Aib residues, should be regarded as a remote possibility.

4. Experimental

4.1. General procedures and materials

HATU and HOAt (1-hydroxy-7-azabenzotriazole) were purchased from PerSeptive Biosystems Gmbh. All other chemicals were of the best grade commercially available. TLC was performed on Merck silica gel 60 F_{254} precoated plates using the following solvent systems: E6: $CHCl_3$/MeOH (methanol)/acetic acid (90:8:2); E8: EtOAc (ethyl acetate).

Amino acid derivatives and peptides were detected by one or more of the following procedures: ninhydrin, 10% sulfuric acid in ethanol (100°C) or UV light. Medium-pressure liquid chromatography (MPLC) was performed on Merck silica gel 60 (0.040–0.063 mm) using a Büchi 688 chromatographic pump connected with a Büchi UV/Vis filter photometer detector and a Knauer recorder (see text for elution conditions). Melting points were taken on a Büchi model 150 apparatus in open capillary, at 20°C, and are not corrected. Optical rotations were recorded on a Perkin–Elmer 241 polarimeter. NMR spectra were obtained either on a Bruker AM-400 or on a Bruker AC-250F spectrometer at 298 K in $CDCl_3$. Chemical shifts are given in ppm and referred to tetramethylsilane as the internal standard. For the hexa- and octaglycopeptides the assignments of the ^{1}H NMR resonances were based on 2D proton–proton shift correlation spectra. MALDI–TOF MS was carried out on a Bruker Reflex TOF instrument, operating in the linear mode at nominal accelerating potential of +20 kV (matrix: α-cyano-4-hydroxycinnamic acid).

4.1.1. *N*-(Benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid *tert*-butyl ester [Z–Thr(GalAc$_4$β1)– Aib–Aib–O*t*Bu] (3TG). To an ice-cooled solution of Z–Thr(GalAc$_4$β1)–OH[18] (2.95 g, 5 mmol) and HATU (1.96 g, 5 mmol) in CH_2Cl_2/DMF (4:1, 5 ml), 2.4 ml of diisopropylethylamine (DIEA), H–Aib–Aib–O*t*Bu[14] (0.81 g, 3.3 mmol) and HOAt (0.46 g, 3.3 mmol) were consecutively added and the reaction mixture was stirred at room temperature. After 24 h the solvent was evaporated in vacuo, the residue taken up with EtOAc and washed with 0.5 M citric acid (2×25 ml), saturated NaCl (1×25 ml), 5% $NaHCO_3$ (3×25 ml), and H_2O (2×25 ml). The organic layer was dried over Na_2SO_4 and evaporated to dryness. The title compound was obtained as a white solid after purification by MPLC (1: 1 EtOAc/petroleum ether). Yield 1.5 g (57%); R_f (E6) 0.71; R_f (E8) 0.80; mp 160–161°C; (Found: C, 56.29; H, 6.78; N, 5.15%. $C_{38}H_{55}N_3O_{16}$ requires C, 56.36; H, 6.85; N, 5.19%); $[\alpha]_D$=+9.4 (c=1.0, MeOH); δ_H (400 MHz, $CDCl_3$): 7.34 (5H, m, Ph C*H*), 7.04 (1H, s, Aib N*H*), 6.86 (1H, s, Aib N*H*), 5.88 (1H, d, J=6.0 Hz, Thr N*H*), 5.41 (1H, d, J=3.0 Hz, Gal H_4), 5.18–5.05 (2H, m, Gal H_2 and H_3), 5.08 (2H, s, CH_2Ph), 4.72 (1H, d, J=7.9 Hz, Gal H_1), 4.43 (1H, m, Thr C$^\alpha$*H*), 4.29 (1H, m, Gal H_6), 4.10 (3H, m, Thr C$^\beta$*H*, Gal $H_{6'}$, Gal H_5), 2.13, 2.05, 2.00, 1.99 (12H, 4s, 4 GalAc CH_3–CO), 1.59–1.46 (12H, 4s, 4 Aib C$^\beta H_3$), 1.44 (9H, s, O*t*Bu CH_3), 1.13 (3H, d, J=6.4 Hz, Thr C$^\gamma H_3$); MALDI–TOFMS: [M+Na]$^+$, found 832. $C_{38}H_{55}N_3O_{16}$ requires 809.8.

4.1.2. *N*-(Benzyloxycarbonyl)-α-aminoisobutyryl-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid *tert*-butyl ester [Z–Aib–Thr(GalAc$_4$β1)– Aib–Aib–O*t*Bu] (4TG). To a chilled solution of N$^\alpha$-deprotected tripeptide **3TG** (1.1 g, 1.6 mmol) in 10 ml CH_2Cl_2, (Z–Aib)$_2$O[15,16] (0.90 g, 1.9 mmol) and DIEA (0.34 ml) were added and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed in vacuo and the oily residue taken up with EtOAc (30 ml) and worked up as described for compound **3TG**. Purification by MPLC (75: 25 EtOAc/petroleum ether) yielded 110 mg (53%) of the title compound as a white solid. R_f (E6) 0.67; R_f (E8) 0.60; mp 132–133°C; (Found: C, 56.35; H, 6.95; N, 6.25%. $C_{42}H_{62}N_4O_{17}$ requires C, 56.37; H, 6.98; N, 6.26%); $[\alpha]_D$=−2.9 (c=1.0, MeOH); δ_H (400 MHz, $CDCl_3$): 7.34

(5H, m, Ph C*H*), 7.05 (2H, m, Thr N*H* and Aib N*H*), 7.00 (1H, s, Aib N*H*), 5.41 (1H, d, *J*=3.0 Hz, Gal H_4), 5.25 (1H, s, Aib N*H*), 5.16 (1H, dd, *J*=3.0, 10.5 Hz, Gal H_3), 5.09 (2H, s, CH_2Ph), 5.04 (1H, dd, *J*=7.8, 10.5 Hz, Gal H_2), 4.66 (1H, d, *J*=7.8 Hz, Gal H_1), 4.45 (1H, m, Thr C$^{\alpha}H$), 4.28 (1H, m, Gal H_6), 4.20 (1H, m, Thr C$^{\beta}H$), 4.09 (1H, m, Gal $H_{6'}$), 4.03 (1H, m, Gal H_5), 2.09, 2.05, 2.03, 2.00 (12H, 4s, 4 GalAc CH_3–CO), 1.54, 1.51, 1.50, 1.49, 1.47, 1.46 (18H, 6s, 6 Aib C$^{\beta}H_3$), 1.44 (9H, s, O*t*Bu CH_3), 1.10 (3H, d, *J*=6.0 Hz, Thr C$^{\gamma}H_3$); MALDI–TOFMS: $[M+Na]^+$, 918. $C_{42}H_{62}N_4O_{17}$ requires 894.9.

4.1.3. *N*-(Benzyloxycarbonyl)-α-aminoisobutyryl-α-aminoisobutyryl-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid *tert*-butyl ester [Z–Aib–Aib–Thr(GalAc$_4$β1)– Aib–Aib–O*t*Bu] (5TG). *(a) Stepwise procedure.* The N$^{\alpha}$-deprotected tetrapeptide **4TG** (0.30 g, 0.39 mmol) was acylated with (Z–Aib)$_2$O and the crude product was worked up as described above for **4TG**. The title compound was obtained as a white solid after MPLC purification (7:3 EtOAc/petroleum ether). Yield 0.146 g (38%); R_f (E6) 0.66; R_f (E8) 0.60; mp 95–97°C; (Found: C, 56.24; H, 7.15; N, 6.99%. $C_{46}H_{69}N_5O_{18}$ requires C, 56.37; H, 7.10; N, 7.15%); $[\alpha]_D$=+8.5 (*c* =0.9, MeOH); δ_H (400 MHz, $CDCl_3$): 7.35 (5H, m, Ph C*H*), 7.09 (1H, s, Aib N*H*), 6.98 (1H, s, Aib N*H*), 6.95 (1H, d, Thr N*H*), 6.89 (1H, s, Aib N*H*), 5.38 (1H, d, *J*=3.2 Hz, Gal H_4), 5.31 (1H, s, Aib N*H*), 5.13–5.08 (3H, m, Gal H_2 and CH_2Ph), 5.02 (1H, dd, *J*=3.3, 10.5 Hz, Gal H_3), 4.65 (1H, d, *J*=7.8 Hz, Gal H_1), 4.43 (1H, m, Thr C$^{\alpha}H$), 4.28 (1H, m, Thr C$^{\beta}H$), 4.20 (1H, dd, Gal H_6), 4.08 (1H, dd, Gal $H_{6'}$), 4.02 (1H, m, Gal H_5), 2.09, 2.04, 2.03, 1.98 (12H, 4s, 4 GalAc CH_3–CO), 1.58–1.45 (24H, m, 8 Aib C$^{\beta}H_3$), 1.43 (9H, s, O*t*Bu CH_3), 1.14 (3H, d, *J*=6.2 Hz, Thr C$^{\gamma}H_3$); MALDI–TOFMS: $[M+Na]^+$ found 1003. $C_{46}H_{69}N_5O_{18}$ requires 980.0.

(b) Fragment condensation. Z–Aib–Aib–O*t*Bu[14] (0.2 g, 0.54 mmol) was dissolved in 5 ml of ice-cooled 90% aq TFA (trifluoroacetic acid) and stirred at room temperature for 30 min. The solvent was evaporated in vacuo and the residue was triturated several times with diethyl ether (Et_2O) and dried. The resulting Z–Aib–Aib–OH[19] (0.16 g, 0.40 mmol) was added to a chilled solution of N$^{\alpha}$-deprotected tripeptide **3TG** (0.18 g, 0.27 mmol), HATU (0.16 g, 0.42 mmol) and DIEA (0.14 ml) in 5 ml of CH_2Cl_2/DMF (4:1). The reaction mixture was stirred at room temperature, by keeping the pH at 7–8 by addition of DIEA. After 24 h the solvent was removed under reduced pressure and the residue worked up as described for compound **3TG**. The title compound was obtained, after MPLC purification, as a white solid, in 26% yield.

4.1.4. *N*-(Benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyryl-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid *tert*-butyl ester [Z–Thr(GalAc$_4$β1)– Aib–Aib–Thr(GalAc$_4$β1)– Aib–Aib–O*t*Bu] (6TG). *(a) Stepwise procedure.* The N$^{\alpha}$-deprotected pentapeptide **5TG** (80 mg, 0.10 mmol) was reacted with Z–Thr(GalAc$_4$β1)–OH (90 mg, 0.16 mmol) as described above for **3TG**, and the resulting hexapeptide was obtained, after purification by MPLC (75:25 EtOAc/petroleum ether),as a white solid. Yield 110 mg (84%); R_f (E6) 0.57; R_f (E8) 0.57; mp 119–121°C; (Found: C, 54.26; H, 6.73; N, 6.17%. $C_{64}H_{94}N_6O_{29}$ requires C, 54.46; H, 6.71; N, 5.95%); $[\alpha]_D$=+10.9 (*c*=1.0, MeOH); δ_H (400 MHz, $CDCl_3$): 7.34 (5H, m, Ph C*H*), 7.27 (1H, s, Aib N*H*), 7.15 (1H, d, *J*=7.0 Hz, Thr^A N*H*), 7.12 (1H, s, Aib N*H*), 6.99 (2H, broad s, 2 Aib N*H*), 6.38 (1H, d, Thr^B N*H*), 5.43 (1H, d, *J*=2.9 Hz, Gal^A H_4), 5.39 (1H, d, *J*=3.2 Hz, Gal^B H_4), 5.20–5.00 (6H, m, Gal^A H_2, Gal^B H_2, Gal^A H_3, Gal^B H_3, CH_2Ph), 4.73 (1H, d, *J*=7.8 Hz, Gal^A H_1), 4.70 (1H, d, *J*=7.6 Hz, Gal^B H_1), 4.57 (2H, m, Thr^A C$^{\alpha}H$, Gal^A H_6), 4.35 (1H, m, Thr^B C$^{\alpha}H$), 4.24 (2H, m, Thr^A C$^{\beta}H$, Gal^A H_5), 4.10–4.03 (4H, m, Thr^B C$^{\beta}H$, Gal^B H_6, Gal^B $H_{6'}$, Gal^A $H_{6'}$), 3.91 (1H, m, Gal^B H_5), 2.14, 2.13, 2.11, 2.04, 2.03, 2.00, 1.98, 1.90 (24H, 8s, 8 GalAc CH_3–CO), 1.55–1.41 (24H, m, 8 Aib C$^{\beta}H_3$), 1.43 (9H, s, O*t*Bu CH_3), 1.20 (3H, d, *J*=6.8 Hz, Thr^A C$^{\gamma}H_3$); 1.11 (3H, d, *J*=5.7 Hz, Thr^B C$^{\gamma}H_3$); MALDI–TOFMS: $[M+Na]^+$ found 1435. $C_{64}H_{94}N_6O_{29}$ requires 1411.5.

(b) Fragment condensation. Compound **3TG** (0.15 g, 0.19 mmol) was dissolved in 5 ml of ice-cooled 90% aq TFA and stirred at room temperature for 60 min. The solvent was evaporated in vacuo and the oily residue was taken up with EtOAc (20 ml), washed several times with water, dried (Na_2SO_4) and evaporated to dryness. The resulting Z–Thr(GalAc$_4$β1)– Aib–Aib–OH (0.13 g, 0.40 mmol) was coupled with N^{α}-deprotected **3TG** as described above for the synthesis of **5TG** by fragment condensation (b). The title compound was obtained, after purification by MPLC, as a white solid, in 62% yield.

4.1.5. *N*-(Benzyloxycarbonyl)-α-aminoisobutyryl-α-aminoisobutyryl-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyryl-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid *tert*-butyl ester [Z–Aib–Aib–Thr(GalAc$_4$β 1)– Aib–Aib–Thr(GalAc$_4$β1)– Aib–Aib–O*t*Bu] (8TG). The title compound was prepared from Z–Aib–Aib–OH (87 mg, 0.27 mmol) and the N^{α}-deprotected hexapeptide **6TG** (100 mg, 0.08 mmol) as described above for the synthesis of **5TG** by fragment condensation (b), and obtained, after purification by MPLC (9:1 EtOAc/petroleum ether), as a white solid. Yield 32 mg (25%); R_f (E6) 0.61; R_f (E8) 0.22; mp 125–128°C; (Found: C, 54.42; H, 7.00; N, 7.05%. $C_{72}H_{108}N_8O_{31}$ requires C, 54.68; H, 6.88; N, 7.08%); $[\alpha]_D$=+9.2 (*c* =1.0, MeOH); δ_H (400 MHz, $CDCl_3$): 7.35 (5H, m, Ph C*H*), 7.23 (1H, s, Aib N*H*), 7.16 (2H, m, Aib N*H*, Thr^A N*H*), 7.08 (2H, m, Aib N*H*, Thr^B N*H*), 6.90 (2H, broad s, 2 Aib N*H*), 5.97 (1H, s, Aib N*H*), 5.40 (2H, m, Gal^A H_4, Gal^B H_4), 5.15–5.08 (4H, m, Gal^A H_2, Gal^B H_2, CH_2Ph), 5.03 (2H, m, Gal^A H_3, Gal^B H_3), 4.79 (1H, d, *J*=7.9 Hz, Gal^A H_1), 4.65 (1H, broad d, Thr^A C$^{\alpha}H$, Gal^B H_1), 4.52 (1H, m, Gal^A H_5), 4.36 (1H, m, Thr^B C$^{\alpha}H$,), 4.17 (2H, m, Thr^A C$^{\beta}H$, Thr^B C$^{\beta}H$), 4.09 (2H, m, Gal^B H_5, Gal^A H_6), 3.96 (1H, m, Gal^A $H_{6'}$), 3.90 (1H, m, Gal^B H_6), 2.13–1.99 (24H, 8s, 8 GalAc CH_3–CO), 1.54–1.36 (36H, m, 12 Aib C$^{\beta}H_3$), 1.43 (9H, s, O*t*Bu CH_3), 1.18 (3H, d, *J*=6.8 Hz, Thr^A C$^{\gamma}H_3$); 1.10 (3H, d, *J*=6.8 Hz, Thr^B C$^{\gamma}H_3$); MALDI–TOFMS: $[M+Na]^+$ found 1605. $C_{72}H_{108}N_8O_{31}$ requires 1581.7.

4.1.6. *N*-(Benzyloxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyryl-α-aminoisobutyric acid methyl ester [Z–Thr(GalAc$_4$β1)– Aib–Aib–Aib–OMe] (4′TG). Z–Aib–Aib–Aib–OMe[19] (150 mg, 0.35 mmol) was N^{α}-deprotected, dissolved in 2 ml of CH_2Cl_2 and added to a chilled solution of Z–Thr(GalAc$_4$β1)– OH (306 mg, 0.52 mmol), HATU (199 mg, 0.52 mmol), HOAt (42 mg, 0.31 mmol) and DIEA (0.22 ml) in 3 ml of CH_2Cl_2/DMF (2:1). After 48 h the solvent was evaporated in vacuo and the oily residue worked up as described above for **3TG**. The title compound was obtained as a white solid after purification by MPLC (8:2 EtOAc/petroleum ether). Yield 214 mg (75%); R_f (E6) 0.93; R_f (E8) 0.62; mp 96–98°C; (Found: C, 54.79; H, 6.90; N, 6.42%. $C_{39}H_{56}N_4O_{17}$ requires C, 54.92; H, 6.62; N, 6.57%]; $[\alpha]_D$=+3.8 (c =1.0, MeOH); δ_H (400 MHz, $CDCl_3$): 7.35 (5H, m, Ph C*H*), 7.20, 6.96, 6.80 (3H, 3s, 3 Aib N*H*), 5.73 (1H, d, J=5.7 Hz, Thr N*H*), 5.44 (1H, d, J=3.0 Hz, Gal H_4), 5.17 (1H, m, Gal H_2), 5.09 (2H, s, CH_2Ph), 5.07 (1H, dd, J=15.0 Hz, J=16.0 Hz, Gal H_3), 4.72 (1H, d, J=8.0 Hz, Gal H_1), 4.57 (1H, m, Gal H_6), 4.41 (1H, m, Thr C$^{\alpha}H$), 4.07 (2H, m, Thr C$^{\beta}H$, Gal H_5), 3.94 (1H, dd, Gal $H_{6'}$), 3.69 (3H, s, OMe CH_3), 2.13, 2.05, 2.00, 1.98 (12H, 4s, 4 GalAc CH_3–CO), 1.51–1.44 (18H, m, 6 Aib C$^{\beta}H_3$), 1.13 (3H, d, J=6.3 Hz, Thr C$^{\gamma}H_3$); MALDI–TOFMS: $[M+Na]^+$ found 875. $C_{39}H_{56}N_4O_{17}$ requires 852.9.

4.1.7. *N*-(Benzyloxycarbonyl)-*O*-(*tert*-butyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid methyl ester [Z–Thr(*t*Bu)– Aib–Aib–OMe] (3T). The title compound was prepared from Z–Thr(*t*Bu)– OH[41] (0.82 g, 2.67 mmol) and H–Aib–Aib–OMe[19] (0.54 g, 2.67 mmol) as described above for **3TG** and purified by MPLC (99.5: 0.5 CH_2Cl_2/MeOH). Yield 0.93 g (64%); colourless oil; R_f (E6) 0.97; R_f (E8) 0.93; (Found: C, 60.95; H, 8.10; N, 8.27%. $C_{25}H_{39}N_3O_7$ requires C, 60.83; H, 7.96; N, 8.51%); $[\alpha]_D$=+25.8 (c =1.1, MeOH); δ_H (250 MHz, $CDCl_3$): 7.35 (5H, m, Ph C*H*), 7.30 (2H, s, 2 Aib N*H*), 5.90 (1H, d, J=4.8 Hz, Thr N*H*), 5.12 (2H, q, CH_2Ph), 4.15 (2H, m, Thr C$^{\alpha}H$, Thr C$^{\beta}H$), 3.70 (3H, s, OMe CH_3), 1.54, 1.52.1.50, 1.49 (12H, 4s, 4 Aib C$^{\beta}H_3$), 1.30 (9H, s, *t*Bu CH_3), 1.07 (3H, d, J=6.1 Hz, Thr C$^{\gamma}H_3$).

4.1.8. *N*-(Benzyloxycarbonyl)-α-aminoisobutyryl-*O*-(*tert*-butyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid methyl ester [Z–Aib–Thr(*t*Bu)– Aib–Aib–OMe] (4T). The N^{α}-deprotected tripeptide **3T** (0.39 g, 1.10 mmol) was reacted with (Z–Aib)$_2$O according to the procedure described above for **4TG**, and the crude product was purified by MPLC (98: 2 CH_2Cl_2/MeOH) and obtained as a white solid. Yield 0.57 g (89%); R_f (E6) 0.92; R_f (E8) 0.81; mp 135–136°C; (Found: C, 60.41; H, 8.04; N, 9.49%. $C_{29}H_{46}N_4O_8$ requires C, 60.19; H, 8.01; N, 9.68%); $[\alpha]_D$=+6.5 (c =0.9, MeOH); δ_H (250 MHz, $CDCl_3$): 7.39 (1H, s, Aib N*H*), 7.35 (5H, m, Ph C*H*), 7.19 (1H, d, J=4.8 Hz, Thr N*H*), 7.11 (1H, s, Aib N*H*), 5.25 (1H, s, Aib N*H*), 5.09 (2H, q, CH_2Ph), 4.28 (1H, m, Thr C$^{\beta}H$), 4.04 (1H, m, Thr C$^{\alpha}H$), 3.70 (3H, s, OMe CH_3), 1.59–1.49 (18H, m, 6 Aib C$^{\beta}H_3$), 1.18 (9H, s, *t*Bu CH_3), 1.12 (3H, d, J=6.0 Hz, Thr C$^{\gamma}H_3$); MALDI–TOFMS: $[M+Na]^+$ found 601. $C_{29}H_{46}N_4O_8$ requires 578.7.

4.1.9. *N*-(Benzyloxycarbonyl)-α-aminoisobutyryl-α-aminoisobutyryl-*O*-(*tert*-butyl)-L-threonyl-α-aminoisobutyryl-α-aminoisobutyric acid methyl ester [Z–Aib–Aib–Thr(*t*Bu)– Aib–Aib–OMe] (5T). The N^{α}-deprotected tetrapeptide **4T** (0.30 g, 0.70 mmol) was reacted with (Z–Aib)$_2$O according to the procedure described above for **4TG**, and the resulting pentapeptide was obtained as a white solid, after purification by MPLC (98: 2 CH_2Cl_2/MeOH). Yield 0.27 g (60%); R_f (E6) 0.82; R_f (E8) 0.77; mp 199–200°C; (Found: C, 59.76; H, 8.27; N, 10.35%. $C_{33}H_{53}N_5O_9$ requires C, 59.71; H, 8.05; N, 10.55%); $[\alpha]_D$=+21.3 (c=1.0, MeOH); δ_H (400 MHz, $CDCl_3$): 7.40 (1H, s, Aib N*H*), 7.36 (5H, m, Ph C*H*), 7.08 (2H, m, Aib N*H*, Thr N*H*), 6.93 (1H, broad s, Aib N*H*), 5.18 (1H, s, Aib N*H*), 5.12 (2H, q, CH_2Ph), 4.33 (1H, m, Thr C$^{\beta}H$), 4.01 (1H, m, Thr C$^{\alpha}H$), 3.69 (3H, s, OMe CH_3), 1.56–1.46 (24H, m, 8 Aib C$^{\beta}H_3$), 1.15 (9H, s, *t*Bu CH_3), 1.12 (3H, d, J=6.5 Hz, Thr C$^{\gamma}H_3$); MALDI–TOFMS: $[M+Na]^+$ found 686. $C_{33}H_{53}N_5O_9$ requires 663.8.

4.1.10. *N*-(Benzyloxycarbonyl)-*O*-(*tert*-butyl)-L-seryl-α-aminoisobutyryl-α-aminoisobutyric acid methyl ester [Z–Ser(*t*Bu)– Aib–Aib–OMe] (3S). This compound was prepared from Z–Ser(*t*Bu)– OH[44] (0.80 g, 2.72 mmol) and H–Aib–Aib–OMe (0.55 g, 2.72 mmol) as described above for **3TG**, and purified by MPLC (99.5:0.5 CH_2Cl_2/MeOH). Yield 1.25 g (95%); colourless oil; R_f (E6) 0.96; R_f (E8) 0.57; (Found: C, 60.05; H, 7.81; N, 8.70%. $C_{24}H_{37}N_3O_7$ requires C, 60.11; H, 7.78; N, 8.76%); $[\alpha]_D$=+8.4 (c=0.6, MeOH); δ_H (400 MHz, $CDCl_3$): 7.36 (5H, m, Ph C*H*), 7.12, 6.86 (2H, 2s, 2 Aib N*H*), 5.66 (1H, broad s, Ser N*H*), 5.12 (2H, q, CH_2Ph), 4.13 (1H, m, Ser C$^{\alpha}H$), 3.79 (1H, m, Ser C$^{\beta}H$), 3.70 (3H, s, OMe CH_3), 3.41 (1H, m, Ser C$^{\beta}H'$), 1.58, 1.52, 1.50, 1.47 (12H, 4s, 4 Aib C$^{\beta}H_3$), 1.20 (9H, s, *t*Bu CH_3).

4.1.11. *N*-(Benzyloxycarbonyl) α-aminoisobutyryl-α-aminoisobutyryl-*O*-(*tert*-butyl)-L-seryl-α-aminoisobutyryl-α-aminoisobutyric acid methyl ester [Z–Aib–Aib–Ser(*t*Bu)– Aib–Aib–OMe] (5S). The title compound was prepared from Z–Aib–Aib–OH (300 mg, 0.93 mmol) and the N^{α}-deprotected tripeptide **3S** (320 mg, 0.93 mmol) as described for the synthesis of **5TG** by fragment condensation (b), and obtained as a white solid after purification by MPLC (98: 2 CH_2Cl_2/MeOH). Yield 120 mg (20%); R_f (E6) 0.76; R_f (E8) 0.86; mp 183–184°C; (Found: C, 58.97; H, 8.14; N, 10.56%. $C_{32}H_{51}N_5O_9$ requires C, 59.15; H, 7.91; N, 10.78%); $[\alpha]_D$=+19.5 (c =0.7, MeOH); δ_H (400 MHz, $CDCl_3$): 7.36 (6H, m, Ph C*H*, Ser N*H*), 7.30, 7.10, 6.67, 5.18 (4H, 4s, 4 Aib N*H*), 5.12 (2H, q, CH_2Ph), 4.15 (1H, m, Ser C$^{\alpha}H$), 3.81 (1H, m, Ser C$^{\beta}H$), 3.68 (3H, s, OMe CH_3), 3.64 (1H, m, Ser C$^{\beta}H'$), 1.54–1.45 (24H, m, 8 Aib C$^{\beta}H_3$), 1.15 (9H, s, *t*Bu CH_3); MALDI–TOFMS: $[M+Na]^+$ found 673. $C_{32}H_{51}N_5O_9$ requires 649.8.

FT-IR absorption. FT-IR absorption spectra were recorded with a Perkin–Elmer model 1720X spectrophotometer flushed with nitrogen, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 16 scans for 10 and 1.0 mM sample concentrations, or 64 scans for 0.1 mM sample concentration. Solvent (baseline) spectra were recorded under the same conditions. Cells with path lengths of 0.1, 1.0, and 10 mm (with

CaF_2 windows) were used. Spectrograde deuterochloroform (99.8% D) was purchased from Merck. Elaboration of the spectra with subtraction of the baseline and the second derivative was carried out with the SpectraCalc (Galactic) program.

Nuclear magnetic resonance. The ^{1}H NMR spectra were recorded at 298 K, with a Bruker model AM-400 spectrometer. Measurements were carried out in $CDCl_3$ (99.96% D, CIL) and deuterated DMSO (99.9% d_6, Acros Organics) with tetramethylsilane as the internal standard.

X-Ray diffraction. Single crystals of Z–Aib–Aib–Thr(*t*Bu)– Aib–Aib–OMe (**5T**) were obtained by slow evaporation at room temperature from an ethanol solution. Intensity data collection was performed using a Philips PW 1100 four-circle diffractometer. Graphite-monochromated Cu Kα radiation (λ=1.54178 Å) and $\theta/2\Theta$ scan mode were used. Cell parameters were obtained by least-squares refinements of the angular setting of 48 carefully centred high angle reflections. The structure was solved by direct methods (SHELXS 97 [42]program). Refinement was carried out by full-matrix block least-squares on F^2, using all data, with all non-H atom anisotropic, by application of the SHELXL 97 [43] program. The positional parameters and the anisotropic displacement parameters of the non H-atoms were allowed to refine at alternate cycles. H-atoms were calculated at idealized positions and during the refinement they were allowed to ride on their carrying atom with U_{iso} set equal to 1.2 (or 1.5 for methyl groups) times the U_{eq} of the parent atom. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk) and copies can be obtained on request, free of charge, by quoting the publication citation and the deposition number 152568.

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