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Influence of Sequential Modifications and Carbohydrate Variations in Synthetic AFGP Analogues on Conformation and Antifreeze Activity

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Abstract: Certain Arctic and Antarctic ectotherm species have developed strategies for survival under low temperature conditions that, among others, consist of antifreeze glycopeptides (AFGP). AFGP form a class of biological antifreeze agents that exhibit the ability to inhibit ice growth in vitro and in vivo and, hence, enable life at temperatures below the freezing point. AFGP usually consist of a varying number of $(Ala\text{-}Ala\text{-}Thr)_n$ units ($n=4$–55) with the disaccharide β-D-galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine glycosidically attached to every threonine side chain hydroxyl group. AFGP have been shown to adopt polyproline II helical conformation. Although this pattern is highly conserved among different species, microheterogeneity concerning the amino acid composition usually occurs; for example, alanine is occasionally replaced by proline in smaller AFGP. The influence of minor and major sequence mutations on conformation and antifreeze activity of AFGP analogues was investigated by replacement of alanine by proline and glycosylated threonine by glycosylated hydroxyproline. The target compounds were prepared by using microwave-enhanced solid phase peptide synthesis. Furthermore, artificial analogues were obtained by copper-catalyzed azide–alkyne cycloaddition (CuAAC): propargyl glycosides were treated with polyproline helix II-forming peptides comprising $(Pro\text{-}Azp\text{-}Pro)_n$ units ($n=2$–4) that contained 4-azidoproline (Azp). The conformations of all analogues were examined by circular dichroism (CD). In addition, microphysical analysis was performed to provide information on their inhibitory effect on ice recrystallization.

Keywords: bioorganic chemistry · circular dichroism · glycopeptides · ice recrystallization · microwave chemistry

Introduction

A variety of ectotherm marine species in polar and subpolar regions have developed strategies to survive at temperatures below the colligative freezing point of their body fluids. This phenomenon relies on regulation of ice nucleation and ice crystal growth. The sera of such fish contain a high concentration of salts, carbohydrates and amino acids. However, an additional protective effect leading to a higher freezing point depression is accomplished by macromolecules such as antifreeze proteins (AFP) and antifreeze glycoproteins (AFGP).[1] The latter have been found in blood, body tissues and the intestines of several Arctic and Antarctic fish at concentrations that largely vary with the season and can differ in the range 5–35 $mg\,mL^{-1}$.[1,2] These glycoproteins are supposed to directly interact with the ice surface with implications on ice crystal growth. Besides the inhibition of ice recrystallization, heterogeneous nucleation is suppressed and a change in crystal habitus occurs.[3] The non-equilibrium hysteresis freezing point (HFP) is depressed, whereas the equilibrium freezing point (EFP) is changed only marginally. The difference between the HFP and EFP is referred to as the thermal hysteresis (TH).[1b,4]

In contrast to AFP that are accessible by recombinant techniques and, hence, have been investigated thoroughly,[5] AFGP can be obtained only in small amounts as pure samples from natural sources. Chemical and biochemical approaches for obtaining homogeneous AFGP are much more demanding. Consequently, the molecular mechanism of action and nature of the interface between the proteins and the ice surface has not yet been fully elucidated.[1b] Recently AFGP were implicated to disturb long-range hydration dynamics of the ice surface.[6]

AFGP are iterative peptides and proteins comprising of a tripeptide unit $(Ala\text{-}Ala\text{-}Thr)_n$ ($n=4$–55) in a molecular mass range between 2.6 and 33.7 kDa. They are glycosylated with β-D-galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine at each threonine hydroxyl group.[1b,4c,7] Interestingly, the same glycosyl residue is also found in mammals where it is known as a tumor-associated carbohydrate antigen (T-antigen). Although the primary structure is largely conserved in AFGP,

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some isoforms have been characterized. For instance, alanine occasionally is substituted by proline, particularly in small AFGP.[8] Threonine sometimes is replaced by arginine in the *Northern notothenoides*.[9] Other modifications were found to be tolerated, too; for example, *N*-acetylglucosamine was discovered as the exclusive carbohydrate component in *Pleagranna antarcticum*.[10] There is evidence that the antifreeze activity depends on several essential features, such as free hydroxyl groups and the *galacto* configuration of the carbohydrate moieties, the acetamido group in C2 position, the α-glycosidic bond between the D-galactosamine and the threonine residue, and the γ-methyl group in the side chain of every threonine residue.[11] The presence of the second galactosyl residue is not crucial for freezing point depression, but significantly improves the antifreeze activity.[11]

The first synthetic approach to AFGP analogues was accomplished by Tsuda and Nishimura who used Ala-Ala-Thr tripeptide fragments, which were glycosylated with a benzylidene and acetate protected disaccharide fluoride. Subsequently, the fully deprotected tripeptide precursors were polymerized with diphenylphosphoryl azide (DPPA) to give peptides with molecular masses in the range of 6.0–7.3 kDa ($n=10$–12).[12] The same group further elaborated this synthetic strategy and slightly modified the protecting group approach. Here the benzyl protected disaccharide halide was coupled to Ala-Thr-Ala tripeptide units, which were polymerized providing glycopeptides with a molecular mass of about 6.6 kDa.[13] However, this fragment oligomerization approach does not allow for variations in sequence and peptide length and homogenous product is obtained only after tedious separation.

An alternative synthetic route relies on the preparation of a glycosylated amino acid building block, that is incorporated by solid phase peptide synthesis.[14] The T-antigen precursor (Gal-GalNAc) could either be generated by coupling of a carbohydrate to T_N-antigen (GalNAc) or the amino acid is glycosylated with a disaccharide donor. The latter approach was communicated by us recently.[15] A *tert*-butyldiphenylsilyl (TBDPS) protected galactal was converted into a glycosyl donor by azidochlorination, and coupled subsequently to orthogonally protected threonine forming the desired α-anomeric product. After conversion of the azide moiety in C2 position into the acetamido group and changes in the protecting group scheme the Fmoc-protected and disaccharide-substituted threonine building block was introduced by microwave-enhanced SPPS.[15] Several further approaches regarding mutations in primary structure, usage of different carbohydrate moieties and bond motifs apart from *O*-glycosides were evaluated to explore the molecular mechanism of action between AFGP and the ice surface. Recently, we published the synthesis of peptoids with triazol-linked carbohydrate moieties, which showed no significant antifreeze activity.[16] Peltier et al. reported on the analysis of monosaccharide-substituted AFGP analogues with alanine residues being replaced by proline. Both the lack of the second galactosyl component and the sequential variations resulted in decreased antifreeze activity.[17] We published the synthesis of AFGP analogues containing three to four (Ala-Ala-Thr) repeating units substituted by the monosaccharide T_N-antigen. Besides naturally occurring AFGP, analogues with amino acid replacements with glycine, proline and serine residues instead of alanine were synthesized. The glycopeptides with mutated sequence showed less activity than the (Ala-Ala-Thr)-glycopeptides due to aperiodic turn formations caused by Pro, Gly and Ser.[18] Miller et al. reported on the synthesis of azido-functionalized amino acids or peptides, which were glycoconjugated by CuAAC under microwave conditions with a propargylated *N*-acetylgalactosamine giving triazole-linked AFGP analogues.[19] However, no recrystallization assays were conducted to gain the activity in ice growth inhibition.

Conformational analysis by using NMR spectroscopy, CD experiments, Raman and IR spectroscopy suggest that low-molecular AFGP adopt highly flexible left-handed helices similar to a polyproline II (PPII) helix.[20] Bush and Feeney stated that the carbohydrate residues are aligned at the same side forming the hydrophilic face and the methyl groups of the alanine and threonine residues represent the hydrophobic face. Such peptides represent amphipathic molecules enabling binding to the ice surface with the hydrophilic side.[11,21] The larger glycopeptides AFGP1–4 are supposed to behave like flexible rods showing a high segmental mobility.[22]

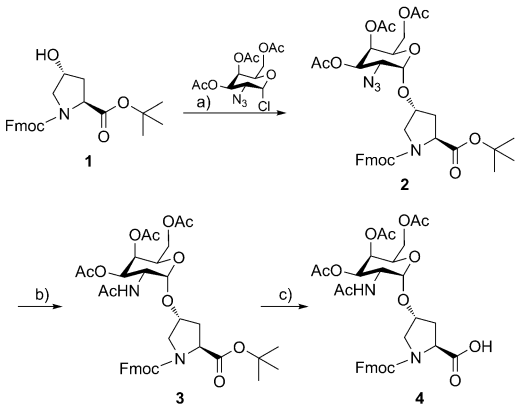
Results and Discussion

We recently communicated a novel synthetic strategy for preparation of suitably glycosylated threonine building blocks either the T- or T_N-antigen. It can be incorporated into peptides by microwave-enhanced SPPS providing AFGP analogues predefined in length and sequence. We adapted this approach to different amino acids, such as hydroxyproline (Hyp), and other compounds, like propargyl alcohol. Glycopeptides and glycopeptide analogues containing these various glycosylated derivatives were prepared. Alanine residues were replaced by different amino acids, such as proline. The main target is to associate the modified structure of these glycopeptides with a change in conformation and antifreeze activity. Conformational analysis of these peptides was done by using CD spectroscopy. The antifreeze activity of the synthetic analogues was tested in recrystallization assays.

Monosaccharide-substituted AFGP analogues with partial replacement of alanine by proline as observed in peptides from natural sources were synthesized. As AFGP are supposed to adopt a polyproline II helix, a series of chimeric peptides displaying features of PPII helix-forming collagen (Gly-Pro-Hyp tripeptide repeating unit) was synthesized. A monosaccharide-substituted hydroxyproline residue was incorporated as a precursor into a sequence composed of Gly-Pro-Hyp(GalNAc) tripeptide units, mimicking the collagen motif.

As an additional modification, propargyl glycosides were conjugated by copper catalyzed azide–alkyne cycloaddition (CuAAC) to oligoprolines containing 4-azidoproline (Azp) in every third position [(Pro-Azp-Pro)$_n$]. Such azidoproline containing oligoprolines are known to form PPII helix and are easily functionalizable.[23]

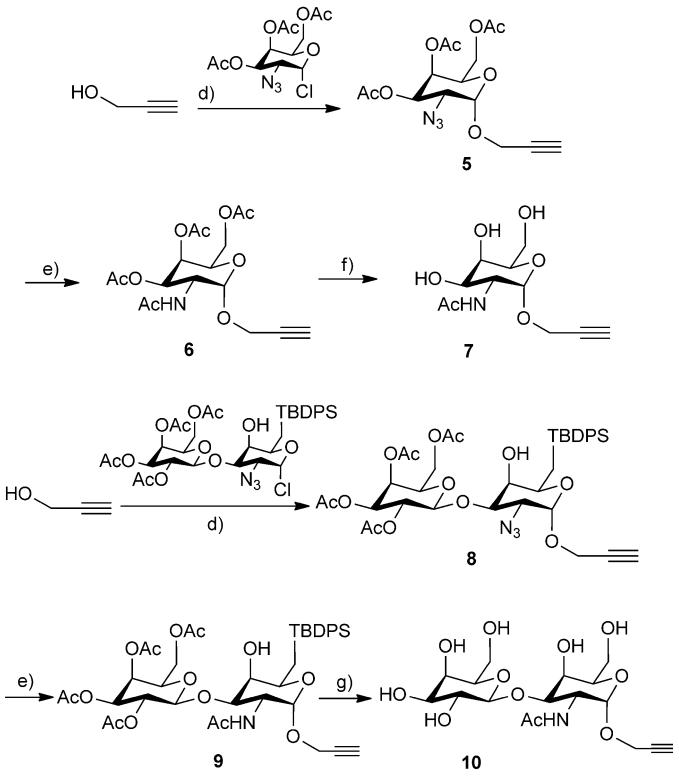
The synthesis of glycosylated threonine was accomplished as published previously by us.[15,18,24] This protocol was adapted for glycosylation of hydroxyproline (Scheme 1).



Scheme 1. Synthesis of the monosaccharide-substituted Hyp building block for the synthesis of AFGP analogues. a) Fmoc-L-*trans*-Hyp-O*t*Bu, Ag_2CO_3, $AgClO_4$, CH_2Cl_2/toluene (1:1), room temperature, 40 h, 56%; b) AcSH/pyridine (2:1), toluene, 50°C, 30 min, 54%; c) TFA/TIS/H_2O (95:2.5:2.5), 30 min, 69%.

The azidochloride[24] served as Koenigs–Knorr donor and was coupled to the orthogonally protected amino acids Fmoc-L-Thr-O*t*Bu and Fmoc-L-*trans*-Hyp-O*t*Bu. The glycosylated Thr building block was obtained in an anomeric ratio α/β of 3:1, whereas glycosylated Hyp had an anomeric ratio α/β of 9:1. Simultaneous azide reduction and *N*-acetylation succeeded smoothly for both amino acid derivatives and the anomers could be separated on column chromatography yielding exclusively α-configured product. After cleavage of the *t*Bu ester the monosaccharide-substituted Thr building block was used without further purification in solid phase peptide synthesis (SPPS). In the case of the monosaccharide-substituted Fmoc-Hyp-O*t*Bu ester cleavage with TFA and water both in the absence and presence of TIS resulted in anomerization to give an α/β ratio of about 3:1. The separation of the diastereomers was achieved by column chromatography.

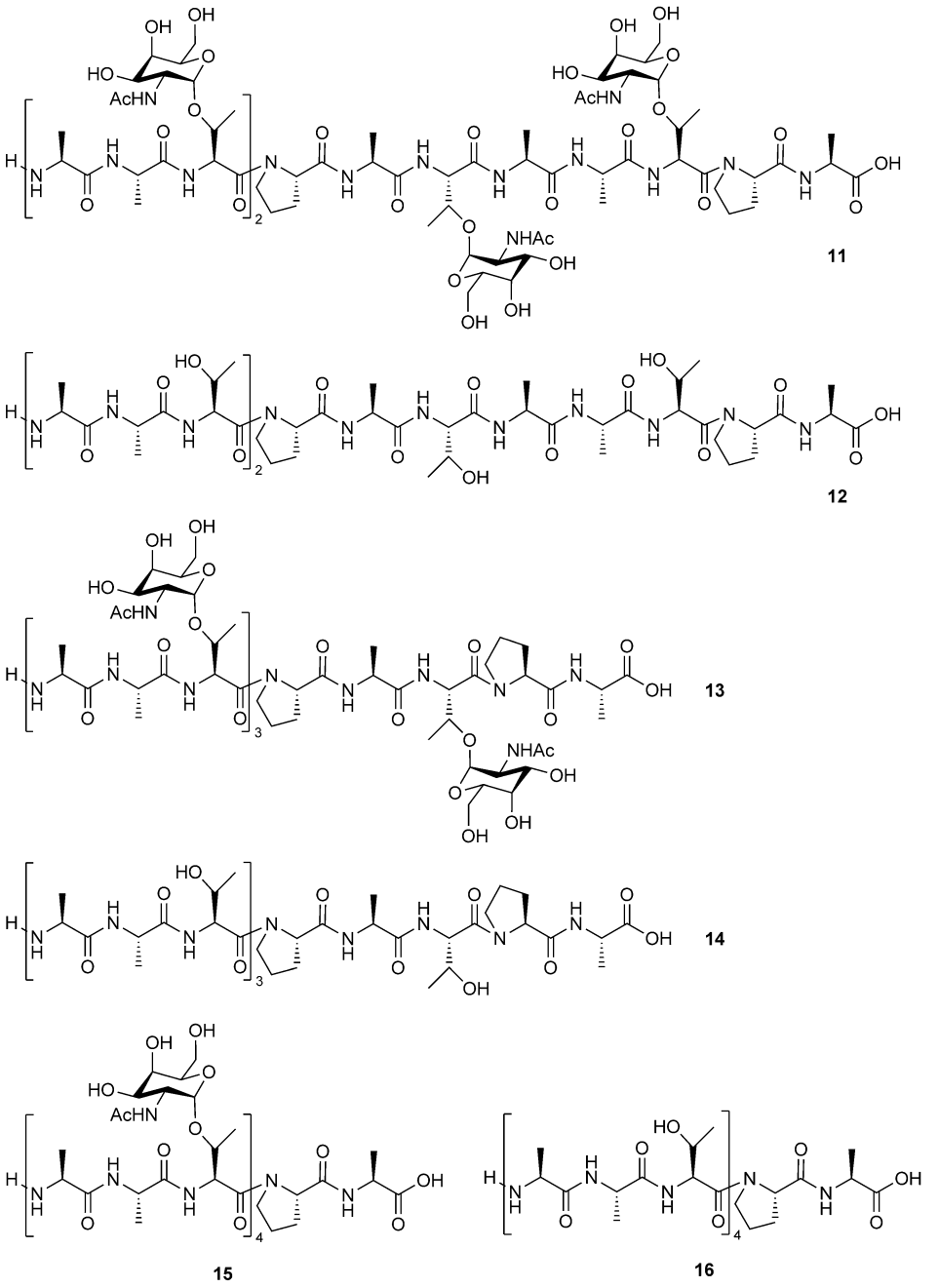
The glycosylation step and the subsequent introduction of the *N*-acetyl group in C2 position of the propargyl glycosides comprising the mono- and disaccharide succeeded as mentioned for the Thr and Hyp building blocks (Scheme 2).[15,24] The anomeric ratio α/β was determined to be 5:1 for the propargyl monosaccharide derivative and 3:1 for the propargyl disaccharide, whereas the glycosylation of threonine with a TBDPS protected disaccharide donor solely gave α-anomeric product due to the steric demand of



Scheme 2. Synthesis of the glycosylated propargyl alcohol building block for the synthesis of triazole-linked AFGP analogues. d) Ag_2CO_3, $AgClO_4$, CH_2Cl_2/toluene (1:1), room temperature, 40 h, 49% (**5**), 60% (**8**); e) AcSH/pyridine (2:1), toluene, 50°C, 30 min, 43% (**6**), 51% (**9**); f) cat. NaOMe, MeOH, room temperature, 20 h, 95% (**7**); g) 1. 1 M TBAF in THF, room temperature, 1 h, 2. cat. NaOMe, MeOH, room temperature, 20 h, 49% (**10**).

the TBDPS protecting group in C6 position of the disaccharide moiety and of Fmoc-L-Thr-O*t*Bu as the glycosyl acceptor.[15] In the case of the smaller propargyl alcohol the bulky TBDPS group did not have the ability to direct the glycosylation exclusively to α-configured product. After conversion of the azide in C2 position into the acetamide the anomers could be successfully separated by column chromatography. Cleavage of the acetate groups in the propargyl monosaccharide was carried out by using catalytic amounts of sodium methanolate in methanol. In the case of the propargyl disaccharide the TBDPS group was cleaved with TBAF in THF prior to cleavage of the acetate groups.[25]

The syntheses of the AFGP analogues containing Pro residues or Gly-Pro-Hyp tripeptide units (Scheme 3 and Scheme 4) were conducted by stepwise microwave-assisted SPPS.[15,18,24] The best results in the semi-automated peptide synthesis were obtained by using 2-chlorotrityl resin loaded with Fmoc-L-Ala-OH and an optimized microwave irradiation protocol limiting the temperature to 40°C. HATU and HOAt were employed for coupling of the heavily glycosylated amino acid building blocks to safeguard an efficient coupling. The corresponding aglycons were synthesized according to a standard protocol with a maximum temperature of 78°C.



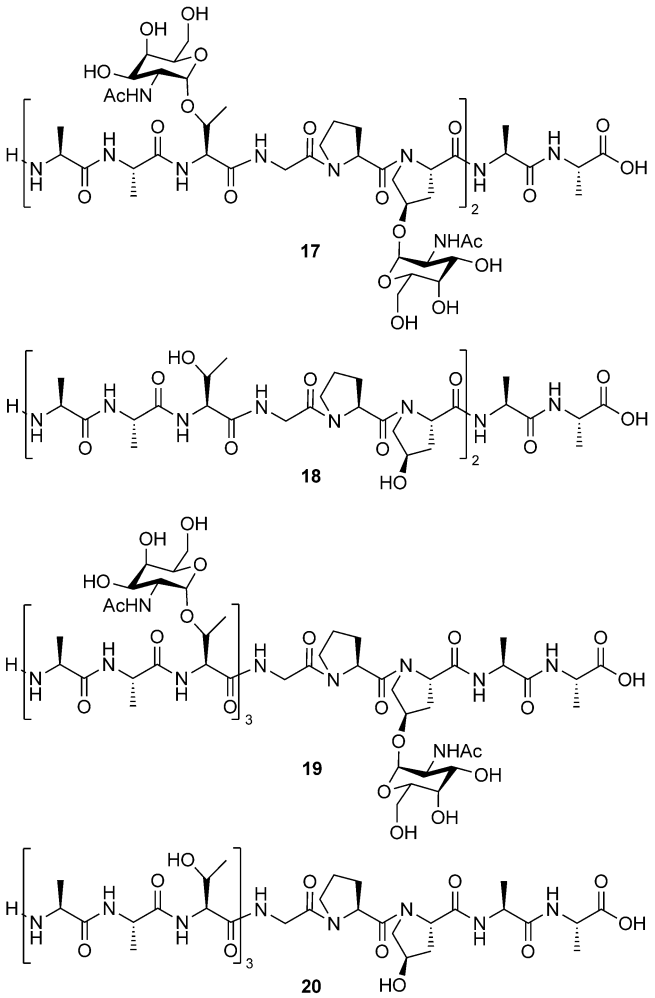
Scheme 3. Synthetic AFGP analogues obtained containing monosaccharide-substituted Thr together with Pro residues in different positions (**11**, **13**, **15**) and the corresponding aglycons (**12**, **14**, **16**).

The synthesis of the AFGP analogues with triazole-linked mono- and disaccharide moieties (Scheme 5) was accomplished by adapting a previously published synthetic procedure.[23b–d,26] The Fmoc-protected amino acids Pro and Azp were successively coupled to the resin with HCTU and *i*Pr_2NEt. The Fmoc-protecting group was cleaved after each coupling step by treatment with piperidine in DMF.

The solid phase bound aglycon peptide was treated with the propargyl saccharides (2.5 equiv propargyl monosaccharide derivative per azide function; 1.5 equiv propargyl disaccharide derivative per azide) in the presence of substoichiometric amounts of $[Cu(MeCN)_4]PF_6$ and TBTA (tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine)[27] under microwave irradiation at 60 °C for 2 h.[26] After a few washing steps of the resin with a mixture of sodium diethyldithiocarbamate in DMF with *i*$PrNEt_2$, DMF and methylene chloride, the peptides were cleaved from the resin by adding a mixture of TFA/H_2O/TIS (95:2.5:2.5). The peptides were precipitated and dried under reduced pressure, dissolved in water and lyophilized to give white foams. The residue was purified by preparative HPLC to give pure AFGP analogues with triazole-linked mono- and disaccharide moieties (**21**–**28**).

CD experiments were conducted to gain insight into the conformational properties of the synthetic AFGP analogues and their corresponding aglycons in solution. The CD curves of all Pro-containing peptides show minima at about $\lambda \approx 195$–199 nm, but only for the glycopeptides (**11**, **13**, **15**) maxima at about $\lambda \approx 218$–221 nm (Figure 1). The typical features in CD spectra of peptides with PPII helical conformation are the negative CD effect between 195 and 210 nm (depending on the proline content) and the slightly positive CD effect between 210 and 220 nm. All spectra hint toward a threefold left-handed helix[28] similar to a polyproline type structure (PPII)[29] or a conformational mixture with random coil.[30] As expected, the tertiary amide chromophores of Pro give rise to a red-shift of the minimum compared to glycopeptides without any Pro residues in their sequence.[15,18] The secondary amino group and the five-membered ring of the Pro residues restrict the torsion angles ϕ to $-65° \pm 15°$ supporting the formation of a PPII helical structure.[29]

The glycosylated peptides **11**, **13** and **15** contain a different number of Pro residues in different positions and exhibit variable red-shifts of the CD minima. The AFGP analogue



Scheme 4. Synthetic AFGP analogues obtained containing monosaccharide-substituted Thr together with monosaccharide-substituted Hyp residues in different positions (**17**, **19**) and the corresponding aglycons (**18**, **20**).

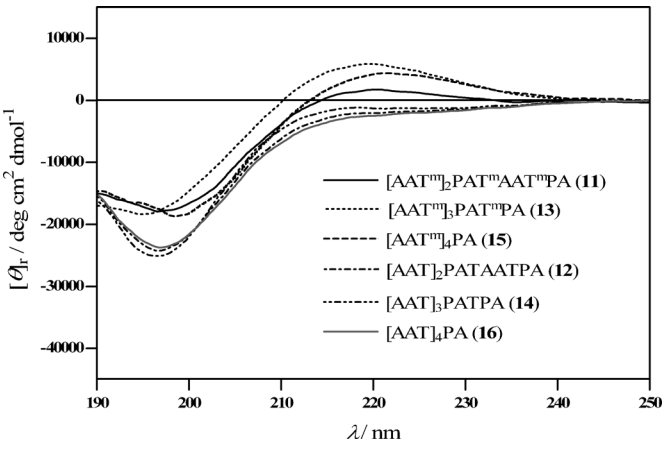
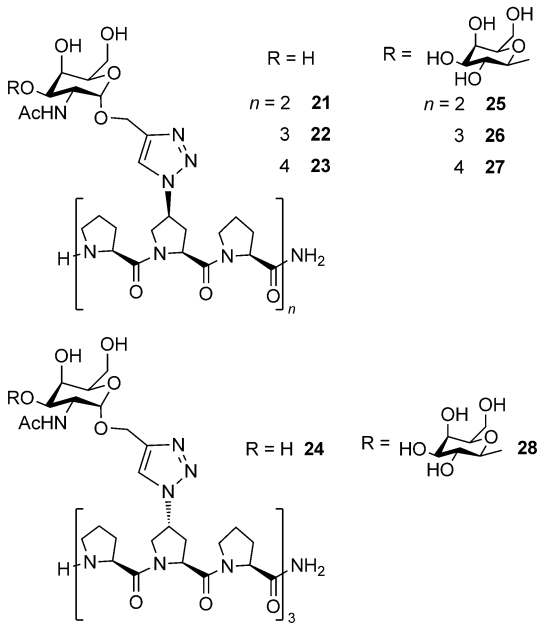


Figure 1. CD spectra of the monosaccharide-substituted Pro-containing AFGP analogues (**11**, **13**, **15**) and their corresponding aglycons (**12**, **14**, **16**) at a concentration of 0.2 mg mL^{-1} in water at 20 °C (m = GalNAc).



Scheme 5. Synthetic AFGP analogues with triazole-linked mono- and disaccharide moieties (**21**–**28**).

containing only one Pro residue (**15**) shows the least red-shift with a negative band at $\lambda \approx 195$ nm and a positive band at $\lambda \approx 218$ nm. The isomeric glycopeptides containing two Pro residues in sequence positions 7 and 13 (**11**) or 10 and 13 (**13**) give rise to similar, but slightly changed CD curves; this indicates that the position of the Pro residues has an influence on the conformation. Peptide **13** comprises a Pro pair in positions i and $i+3$ and adopts a PPII helix. This gives rise to CD curves with a shift to higher wavelength.[31] Ala is also known to have a high propensity for a PPII helix.[32] Interestingly, it was shown by ATR-FT-IR studies that the α-helical content of AFGP increased at the ice/water interface upon freezing of a supercooled solution film at −15 °C.[33]

Aglycons **12**, **14**, and **16** give rise to CD spectra with a slightly more intense negative band in comparison to the glycosylated counterparts, but lack a positive CD effect between 210 and 230 nm. Similar differences in CD between glycosylated and unglycosylated peptides were already described by Peltier et al.[17] Aglycons **12**, **14**, and **16** presumably adopt PPII helical conformation to a lesser extent; obviously, glycosylation stabilizes the PPII helix.[29,34] This is particularly true for the aglycon exclusively containing Ala-Ala-Thr units, as reported earlier by us. The CD spectrum of the peptide revealed a characteristic β-strand or β-turn motif.[18] The carbohydrate moiety *N*-acetyl-D-galactosamine seems to have an important impact on conformational stability due to additional interactions, such as hydrogen bonds and the presence of water pockets/bridges established between the monosaccharide and the peptide backbone.[35] Interestingly, no difference in the position of the negative band, that is, no red-shift is observed among the aglycons

with increasing Pro content (**12**, **14**, **16**). All three curves show a negative CD effect at about $\lambda \approx 196$–197 nm.

A CD signature with a minimum closely below $\lambda \approx 200$ nm and a maximum at approximately 218 nm is usually associated with random-coil structures.[30] Temperature-dependent CD experiments were accomplished in water between 0 °C and +80 °C to further probe the existence of a PPII helix. Tiffany and Krimm proposed a conformational connection between a PPII helix and a random coil. In temperature-dependent CD experiments of polypeptides and collagen at least locally ordered extended helix structure was found.[36] This was confirmed by experimental data by Dukor and Keiderling, who compared vibrational CD with electronic CD of polyproline peptides. It was concluded that the random coil structure has a large fraction of helical regions similar to the PPII conformation.[37] The CD spectra recorded at different temperatures (Figures S16–S21 in the Supporting Information) exhibit one isodichroic point at about $\lambda \approx 207$–208 nm for both the Pro-containing glycopeptides (**11**, **13**, **15**) and the corresponding aglycons (**12**, **14**, **16**). This hints towards an equilibrium between at least two conformers, presumably between a PPII helix and a random coil.[15,18] CD difference spectra (e.g., Figure S34 in the Supporting Information) in which the spectrum recorded at the lowest temperature (0 °C) is subtracted from the spectrum recorded at the highest temperature (+80 °C) might be indicative of β-structure-like motifs being present at higher temperature.[38] The PPII helix and β-strand conformers are very close in the alanine dipeptide ϕ/ψ energy map.[39] These results can be interpreted with a change in conformation from a PPII helix to an assumedly β-turn-like-structure, accompanied by random-coil contributions.[15,38a,40]

Comparison of the synthetic Pro-containing peptide **11** with the highly similar **AFGP8**, isolated from natural sources illustrates the conformational similarity according to CD (Figure 2).[15] **AFGP8** and **11** share the number of amino acids and the sequence, especially with respect to the Pro residues, but differ in the carbohydrate moiety at Thr (**AFGP8**: β-D-galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine; **11**: α-*N*-acetyl-D-galactosamine). Although both peptides are derivatized with different carbohydrate moieties, both of them give rise to nearly identical CD spectra. The additional carbohydrate moieties do not seem to further influence the conformation according to the CD spectra.

The influence of regular incorporation of proline residues on AFGP conformation was further probed by comparison of the CD spectra of the prototype monosaccharide-substituted AFGP $[AAT^m]_4AA$ (**29**) with the Pro-containing glycopeptide $[PAT^m]_4PA$ (**30**).[18] The former clearly shows the prevalence of a PPII helix with the highest magnitude in the minimum and maximum, whereas the CD spectrum of the latter exhibits a red-shifted, less intense minimum and does not show a maximum at all. The high Pro content dislocates the CD curve to a higher wavelength due to the tertiary amide chromophore.

Fibrous collagen with the repetitive sequence (Gly-Pro-Hyp) represents another prominent protein known to adopt PPII helical conformation.[30] Therefore, collagen–AFGP hybrid peptides **17** and **19** glycosylated with α-*N*-acetyl-D-galactosamine at the hydroxyproline residues were synthesized. Glycopeptide **17** exhibits a negative CD effect at $\lambda \approx 199$ nm. The minimum is significantly more red-shifted than that of peptide **19** with a negative band at about $\lambda \approx 195$ nm (Figure 3). The position of the negative CD effect of **19** is comparable with glycopeptide **15**, which has one Pro residue. The CD spectra of the corresponding aglycons **18** and **20** are similar to the aglycons **12**, **14**, and **16**. They show a more intense negative CD than the glycosylated peptides, **17** and **19**, but a less intense positive band at $\lambda \approx 220$ nm, indicating a PPII helix albeit with lower propensity than the glycopeptides (Figure 3). Nevertheless, both aglycons exhibit a PPII-like conformation, regardless of the number of the Gly-Pro-Hyp units, with a minimum at about $\lambda \approx 198$ nm and a marginal visible red-shift in comparison to each other.

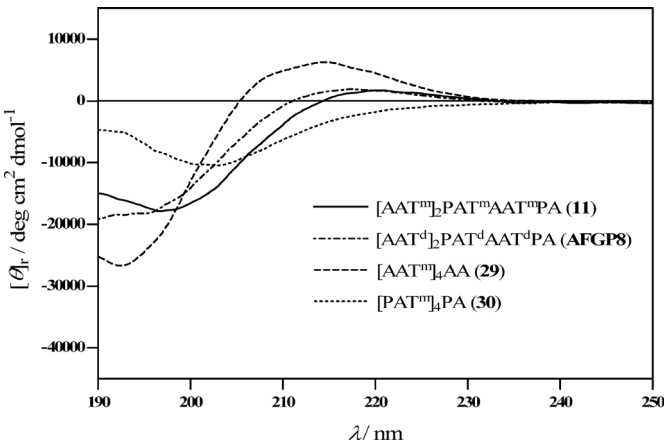


Figure 2. CD spectra of the monosaccharide-substituted Pro-containing AFGP analogue (**11**), the corresponding natural **AFGP8** and AFGP analogues $[AAT(GalNAc)]_4AA$ (**29**) and $[PAT(GalNAc)]_4AA$ (**30**) in water at 20 °C (m = GalNAc; d = Gal-GalNAc).

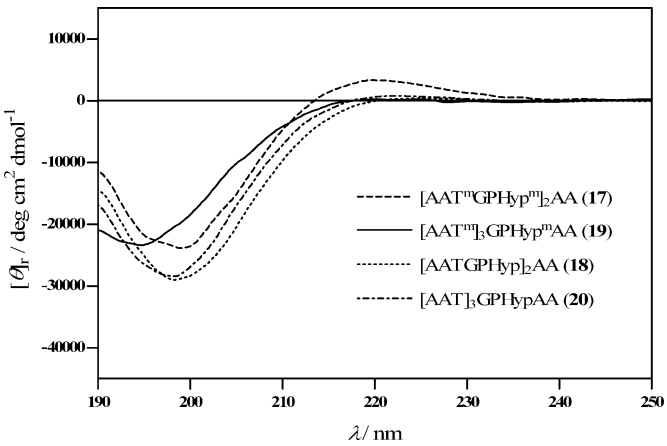


Figure 3. CD spectra of the monosaccharide-substituted peptides consisting of Gly-Pro-Hyp(GalNAc) tripeptide units (**17**, **19**) and their corresponding aglycons (**18**, **20**) with a concentration of 0.2 mg mL^{-1} in water at 20 °C (m = GalNAc).

As mentioned before for the Pro-containing peptides, the CD of the glycopeptides **17** and **19** shows a significant difference in the position of the negative band, whereas the CD curves of the corresponding aglycons **18** and **20** are only slightly changed.

The temperature-dependent CD experiments recorded in the range from 0 °C to 80 °C (Figures S22–S25 in the Supporting Information) also show isodichroic points between 205 and 209 nm for the collagen–AFGP hybrid peptides **17**–**20**, indicating an equilibrium between two conformations. Again, the aglycons are characterized by higher propensity for random coil than the glycopeptides. The difference spectra obtained by subtracting the CD spectrum recorded at 0 °C from the one recorded at 80 °C indicate a β-turn or β-structure-like motif. The Gly-Pro-Hyp containing peptides adopt a PPII structure in a mixture with random coil at low temperatures. With increasing temperature the overall structure shows a higher contribution of unordered conformation accompanied by β-turn-like conformations.[15,38a,39]

The oligoprolines **21**–**24** containing the α-*N*-acetyl-D-galactosamine motifs at every third residue, connected across a triazole moiety, represent a further modification. The CD spectra show the presence of a PPII helical structure, albeit with distinct differences regarding the intensity of the minima and maxima. The molar ellipticity per residue increases as expected with the number of amino acid residues. Moreover, the configuration of the triazolylproline (Tzp) also has an influence. Peptides **21**, **22** and **23** all contain 2*S*,4*S* configured Tzp residues. Peptides **21** and **22** reveal a negative band at about $\lambda \approx 204$ and 205 nm and positive maxima at $\lambda \approx 223$ and 224 nm, respectively. Peptide **23** with the longest chain length shows the highest intensity in negative CD effect and in the red-shift with a negative band at $\lambda \approx 206$ nm and a positive band at $\lambda \approx 224$ nm (Figure 4).[41] The intensity of the negative band and the red-shift increase with the number of repeating units in the peptide; this is in accordance with an increasing stabilization of the PPII helix.[42] Peptide **24** with 2*S*,4*R* configured triazolylproline residues gave a slightly different CD spectrum with a lower intensity of the negative band but a higher one of the positive band, accompanied by a distinct red-shift (Figure 4).

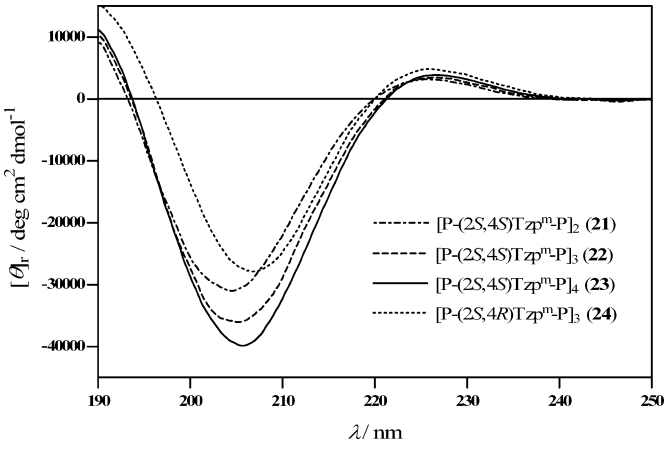


Figure 4. CD spectra of the monosaccharide-substituted oligoprolines (**21**–**24**) at a concentration of 0.2 mg mL^{-1} in water at 20 °C (m = GalNAc).

The related oligoprolines **25**–**28** carrying the disaccharide β-D-galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine connected across a triazole moiety at every third residue, give rise to CD spectra very similar to **21**–**24**. The PPII helical conformation is indicated by a minimum at $\lambda \approx 203$–206 nm and a positive band at $\lambda \approx 223$–227 nm (Figure 5). The additional

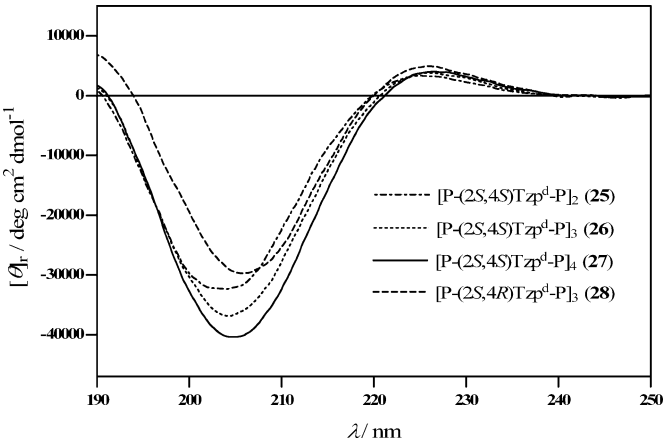


Figure 5. CD spectra of the disaccharide-substituted oligoprolines (**25**–**28**) at a concentration of 0.2 mg mL^{-1} in water at 20 °C (d = Gal-GalNAc).

β-D-galactosyl moiety does not seem to have significant influence on the overall peptide conformation as it was observed for the previously discussed AFGP. In almost the same manner, the minima and maxima are red-shifted and the intensity of the minimum increases with the length of the glycopeptide **25**–**27**. The 2*S*,4*R* configured triazolylproline containing peptide **28**, like analogue **24**, gives rise to a CD spectrum conformation with the highest red-shift in comparison to the 2*S*,4*S* configured peptides but with the lowest intensity of the minimum at $\lambda \approx 206$ nm.

Temperature-dependent CD spectra in the temperature range from 0 °C to +80 °C were recorded to obtain further insight into the conformational features of the AFGP analogues (Figures S26–S33 in the Supporting Information). The CD spectra accumulated for all peptides of this type (**21**–**28**) exhibit two isodichroic points at $\lambda \approx 192$–193 and 212–214 nm, respectively. The isodichroic point at shorter wavelength often is not reported because of problems with the absorbance of the sample below 195 nm. The CD spectra decrease in intensity with higher temperature, as mentioned above, for the Pro-containing AFGP analogues.[15,36,37] However, the pronounced CD effects suggest a higher stability of the PPII helix than was observed for the Pro-containing peptides (**11**–**16**).[43] The intensity changes with temperature by only about 20 %. Especially the peptides with the 2*S*,4*R* configured triazolylproline residues (**24**, **28**) exhibit somewhat lower temperature dependence; this is indicative of a higher conformational stability. This could

arise from the higher preference of (2*S*,4*R*)-Tzp compared to (2*S*,4*S*)-Tzp for a *trans* amide bond conformation which is required for the PPII conformation.[26a]

In summary, the Pro-containing peptides **11**–**16** and the collagen motif-containing peptides (**17**–**20**), upon heating undergo a structural transition from a mixture of a PPII and random coil potentially to a mixture of a β-structure and random coil, whereas the oligoprolines carrying either a mono- or a disaccharide (**21**–**28**) basically retain their PPII conformation upon heating. In the first two types of peptides the PPII helical structure is presumably disrupted with short fractions of unordered peptide at low temperature, whereas at high temperature the conformational mixture consist of β-structures with short random coil structures.[36a] The most stable PPII conformation is found in the oligoprolines (**21**–**28**) as indicated by the more intensive bands. This higher stability most probably arises by the higher proline content of these peptides and the amidated *C* terminus.[44] In turn, the AFGP analogues **11**, **13**, **15**, **17**, and **19** adopt a PPII helix to a larger extent in comparison to their agylcons; this is probably explicable with additionally formed interactions by the *N*-acetyl group of the carbohydrate moieties, but also by the formation of water pockets/bridges between the carbohydrate and peptide moieties.[15,36,39c] More Pro residues present in the sequence do most likely favor a PPII helical conformation at the expense of an unordered structure;[30] this is mostly apparent in the slight red-shift of the CD curves on the one hand and in the high magnitude in positive CD effect in the higher wavelength region at about $\lambda \approx 220$ nm.[41c]

The activity and efficiency of antifreeze agents can be tested in ice recrystallization experiments.[3c,45–47] The antifreeze activity of the synthesized peptides was investigated in a previously described ice recrystallization inhibition assay, which allows for the separation of the antifreeze effect from other factors that influence recrystallization, such as the ice volume fraction.[47] In this assay all peptides are dissolved in 45 wt % sucrose solutions and the recrystallization kinetics is determined at −8 °C. The recrystallization rate constant, k, is determined according to the Lifshitz–Slyozov–Wagner (LSW) theory by analyzing the cubic mean crystal radius, r^3, as a function of the recrystallization time, t: $r^3 = r_0^3 + kt$, where r_0 is the cubic mean radius at time t = 0 min. Several experimental parameters that influence the rate constant, k, can be neglected when all experiments are performed with the same basic solute (sucrose), at the same temperature (−8 °C), and at small ice volume fractions. In that case the observed rate constants in each experiment all converge to the theoretical diffusion-limited LSW rate constant at zero volume fractions in sucrose solutions at −8 °C. Diffusion-limited implies that the diffusion of water molecules between the ice crystals is the slowest and, hence, rate-limiting process that is responsible for the observed ice recrystallization rate. We also take into account the effects of the nonzero ice volume fraction.[46] The resulting rate constant, k_{l0}, is then dependent upon the presence, the efficiency, and the concentration of ice-binding agents (Figure 6 a).

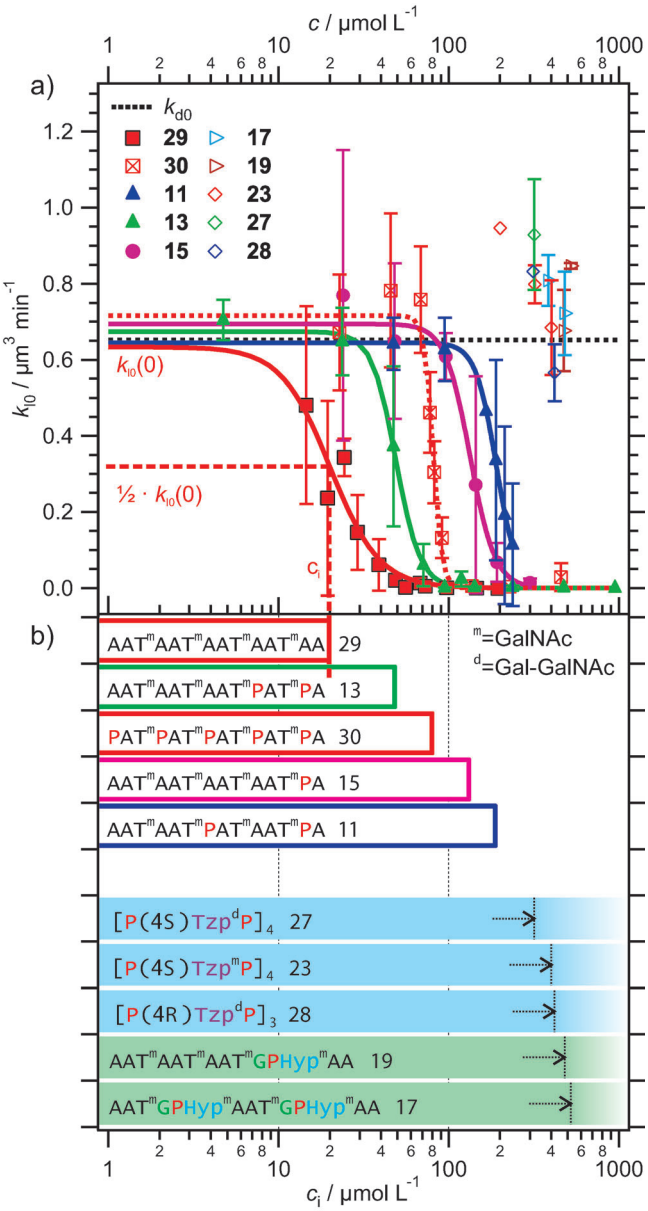


Figure 6. a) Recrystallization rate constant, k_{l0}, as a function of the peptide concentration, c. Shown are the mean values of typically 3–5 individual experiments represented by symbols. The corresponding standard errors are indicated by error bars. Active peptides show a decrease of the rate constant, k_{l0}, down to negligible values. When k_{l0} becomes half of the diffusion-limited value of pure sucrose solutions the corresponding concentration is defined at the inhibitor concentration, c_i, due to the ice-binding ability of antifreeze agents. b) Inhibitory efficiency represented by the inhibitor concentration, c_i. For peptides that were inactive in the tested concentration range arrows indicate the highest investigated concentrations.

When the ice surface coverage of ice-binding agents becomes sufficiently large, the limiting process for recrystallization changes from water diffusion to liquid-to-ice transfer. This changeover can be used to determine the peptide-specific inhibition concentration, c_i, which we define as that concentration at which k_{l0} has decreased to half of the diffu-

sion-limited value. This definition of c_i is illustrated in Figure 6a in the case of $[AAT^m]_4AA$ (**29**).

Figure 6b shows the resulting c_i values of selected peptides from this study, and also for comparison, those of previously investigated peptides.[18] At a c_i value of 20 μmol L^{-1} (41 μg mL^{-1}) the peptide $[AAT^m]_4AA$ (**29**) without any Pro is the most efficient ice recrystallization inhibitor within this series. The introduction of Pro for Ala does lead to a decrease in the inhibition efficiency regardless of the position or the number of exchanges, even though the antifreeze activity is retained. However, the Gly-Pro-Hyp containing peptides (**17**, **19**) and the polyproline peptides (**23**, **27**, **28**) do not show any antifreeze activity in the investigated concentration range.

Our previous investigations did not reveal any significant difference in the efficiency of the natural **AFGP8** ($[AAT^d]_2PAT^dAAT^dPA$), and its synthetic analogue $[AAT^d]_4AA$, which does not contain any Pro.[15] The associated c_i values are 0.086 μmol L^{-1} (0.22 μg mL^{-1}) and 0.085 μmol L^{-1} (0.23 μg mL^{-1}), respectively. In contrast, the c_i values of the corresponding monosaccharide AFGP analogues are 190 μmol L^{-1} (400 μg mL^{-1}) for the Pro-containing peptide **11** and 20 μmol L^{-1} (41 μg mL^{-1}) for $[AAT^m]_4AA$ (**29**). Thus, in this case the introduction of Pro at positions 7 and 13 leads to a recrystallization inhibitor that is less efficient by about one order of magnitude. These comparisons imply that the less efficient monosaccharide AFGP analogues are much more sensitive to backbone mutations than the natural disaccharide AFGP, that is, they reveal stronger effects on the recrystallization inhibition efficiencies. Similar structural differences are not observable as the addition of β-D-galactosyl moieties does not alter the CD spectra significantly. The change from $[AAT^m]_4AA$ to the $[Pro^7,Pro^{13}]$ derivative is similar to that from $[AAT^d]_4AA$ to **AFGP8**.

Interestingly, peptide **11** is the antifreeze agent with the lowest efficiency in this series. The c_i value of peptide **13** is 49 μmol L^{-1} (100 μg mL^{-1}). This peptide contains the same amount of Pro as peptide **11**, but at positions 10 and 13. Peptide **15**, which contains Pro only at position 13, is not more efficient than peptides **11** and **13**, which contain two Pro residues. The c_i value is 130 μmol L^{-1} (280 μg mL^{-1}). It seems that Pro introduction is less disturbing if it is positioned in neighboring tripeptide units. With 81 μmol L^{-1} (c_i = 180 μg mL^{-1}), $[PAT^m]_4PA$ (**30**) is less efficient than the $[Pro^{10},Pro^{13}]$ derivative **13**, but both are more efficient than peptides **11** and **15**. This result is in agreement with our previous statement that irregular substitutions of Pro for Ala may prevent the formation of a periodic, active conformation.[18] This may be due to non-periodic turn formations around the Pro residues.

In contrast to the hitherto discussed Pro-containing AFGP analogues the oligoprolines **23**, **27**, and **28** are totally inactive in the investigated concentration range. The highest concentrations that were tested are in the range of 400 μmol L^{-1} (1000 μg mL^{-1}). The observed inactivity may result from the linkage between the backbone and the carbohydrate moieties, the triazole unit. Previously investigated peptoids that contain similar groups did not show any activity either.[16] However, it has to be mentioned that there are several structural differences between these analogues and the natural AFGP. Hence, at present it is not possible to verify this hypothesis, but we can conclude that a periodic amino acid sequence and a PPII helical structure are not criteria that are sufficient for antifreeze activity. The CD curves of the oligoprolines **21**–**28** and the collagen analogues **17** and **19** all indicate the existence of a PPII helix. Nevertheless, all these peptides do not inhibit ice recrystallization up to concentrations of 1000 μg mL^{-1}. In the case of the Gly-Pro-Hyp containing peptides the main reason might be the substitution of Thr by Hyp. It was shown by Tachibana et al. that removal of the Thr γ-methyl group (substitution of Thr by Ser) suffices to lead to a significant reduction in antifreeze activity.[11] It could also be possible that the amino acid substitution contributes to a reduced recrystallization inhibition efficiency, and that the sum of these reductions leads to a non-efficient agent.

Conclusion

Monosaccharide- and disaccharide-substituted AFGP analogues were synthesized that contained amino acid substitutions (Ala→Pro). The influence of the saccharide portion (mono- vs. disaccharide) and of the Thr→Hyp exchange was investigated. Furthermore, glycosylated $(Pro\text{-}Tzp\text{-}Pro)_n$-peptides were assembled giving triazole-linked oligoprolines furnished either with a mono- or disaccharide. The conformation of these glycopeptides was examined by using CD and compared with their corresponding aglycons. In microphysical antifreeze assays the activity of all glycopeptides was compared.

Direct comparison of the monosaccharide- with the disaccharide-substituted AFGP analogues did not reveal any change in conformation but in antifreeze activity.[15,18] The replacement of Ala by Pro residues in the peptide backbone of **AFGP8** did not significantly lower the activity relative to Pro-free AFGP. In contrast, the incorporation of Pro in monosaccharide-substituted AFGP analogues led to decreased antifreeze effectiveness. The carbohydrate moiety overrides the influence of Pro residues present in the sequence. The influence of the disaccharide moiety in **AFGP8** on antifreeze activity is so pronounced that the mutation in the sequence does not affect the ability to inhibit ice crystal growth. The monosaccharide-substituted AFGP analogues with Pro mutations display lower activity than Pro-free glycopeptides.

Although the collagen motif-containing peptides (**17**, **19**) assumably adopt PPII helical conformation with similar propensity as the Pro-containing AFGP analogues (**11**, **13**, **15**), the ability to inhibit ice crystal growth was found only for the latter. High stability in PPII helical conformation obviously is not the only precondition for an effective antifreeze agent. The oligoprolines **21**–**28** lack antifreeze activity, presumably because of the larger triazole spacer between the

carbohydrate moiety and peptide backbone. AFGP seem to be able to structure the surrounding water in the first hydration shell[36b] and to perturb the hydration dynamics.[6] The carbohydrate residues in AFGP are assumed to be aligned on one side of the PPII helix. However, this alignment does not suffice for antifreeze activity, as shown here for example, for Gal-GalNAc derivatized PPII helical analogues.

Experimental Section

Experimental procedures and the full characterization of all intermediates and the final products are described in the Supporting Information.

Microphysical ice recrystallization analysis: The antifreeze activity was determined based on a previously described method in which the inhibitory effect of antifreezes on ice recrystallization is quantified. Briefly, the peptides (TFA salts) were dissolved in sucrose (45 wt%) solution at various concentrations; aliquots of these solutions (2 μL) were placed between two glass cover slides with a film thickness of about 10–20 μm, and the resulting samples were positioned on a temperature-controlled silver block inside a cold stage (Linkam MDBCS 196) mounted on an optical microscope (Olympus BX51) in bright field transmission mode. The samples were cooled to −50 °C at a rate of 20 °C min^{-1}, reheated to −8 °C at a rate of 10 °C min^{-1}, and annealed at this temperature for 2 h. The recrystallization of the resulting polycrystalline ice was recorded by taking images with a digital video camera in 12 s time intervals while the images were analyzed simultaneously by using a LabVIEW virtual instrument. The ice recrystallization rate was determined by using LSW theory and then used to obtain the inhibitor-related concentration, c_i, at which the recrystallization limiting process turns over from water diffusion to liquid-to-ice transfer due to the presence of ice-binding antifreezes.[46]

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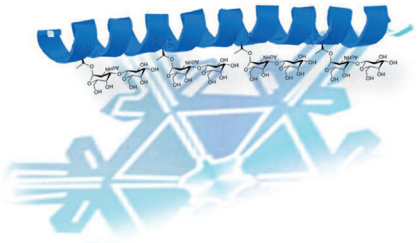
Glycopeptides



Out of the cold: The antifreeze activity of AFGP (antifreeze glycoprotein, see figure) analogues strongly depends on the amino acid composition. Synthetic, near-native compounds (e.g., with proline replacing alanine) retain the effect, whereas others exhibit reduced or abolished influence on ice recrystallization, despite having high propensity to adopt polyproline II helical conformation, as proposed for the native AFGP.