Peptide Ligations

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Total Synthesis of Homogeneous Antifreeze Glycopeptides and Glycoproteins**

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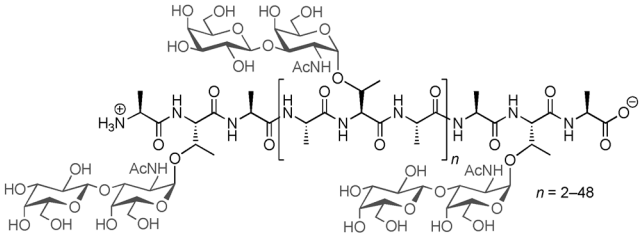
Antifreeze glycoproteins (AFGPs) are a class of natural products found in deep sea teleost fish in Arctic and Antarctic waters. The physiological role of these biomolecules is to protect against cryoinjury in environments with subzero temperatures by preventing the growth of ice crystals in vivo.[1] Structurally, AFGPs are polymeric, mucin-type glycoproteins that consist of a single glycotripeptide repeat (Ala-Thr-Ala/Pro) in which each secondary hydroxy group on threonine is linked to the disaccharide β-D-galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine (Scheme 1).[2] AFGPs range in molecular weight from approximately 2.6 kDa (4 repeat units) to 33.7 kDa (50 repeat units).



Scheme 1. Structure of native antifreeze glycoproteins (AFGPs).

The mechanism of action of AFGPs is proposed to occur through an adsorption-inhibition process in which the AFGP irreversibly binds to the surface of ice crystals, thus resulting in a localized freezing-point depression (through the Kelvin effect).[3] The difference between the depressed freezing point and the melting point of the ice crystal is termed thermal hysteresis (TH). The binding of AFGPs to ice results in a characteristic change in morphology from spherical to hexagonal-bipyramidal-shaped crystals.[3] AFGPs are also potent inhibitors of ice recrystallization, whereby they prevent the enthalpically driven growth of ice crystals, an activity known as ice recrystallization inhibition (IRI) activity.

The unique physicochemical properties of AFGPs have led to considerable interest in these molecules for applications in material science and medicine, including their use as cryoprotective agents for cells, tissues, and organs.[4] Such applications necessitate access to significant quantities of AFGPs in pure form, however, isolation and purification from natural sources is both labor-intensive and costly. An attractive alternative is the production of these biomolecules by chemical synthesis. To date, synthetic approaches have been limited to the preparation of small glycopeptides ($\leq$ 14 amino acids in length) by using solid-phase peptide synthesis (SPPS).[5] However, a landmark study by Nishimura and co-workers enabled access to larger AFGPs (ca. 6–21 amino acids in length), bearing native and non-native glycans, through polymerization of tripeptide glycopeptides and subsequent extensive chromatographic purification of the polydisperse oligomers.[6] Importantly, this study facilitated the delineation of a number of structural features now known to be critical for antifreeze activity. To facilitate more in-depth structure–activity studies and to provide material with potential biomedical and industrial applications, it is essential to develop a convergent synthetic strategy that enables the efficient synthesis of a range of homogeneous and native AFGPs.[7] Towards this goal, we were interested in exploiting a native chemical ligation[8]–desulfurization approach[9] for the first convergent synthesis of native, homogeneous AFGPs ranging in size from 1.2 to 19.5 kDa. Specifically, we envisioned an iterative synthetic strategy based on three key reactions: 1) native chemical ligation between a glycopeptide bearing an N-terminal thiazolidine (Thz) residue and a C-terminal thioester moiety and a glycopeptide containing an N-terminal cysteine (Cys) residue, 2) conversion of the N-terminal Thz moiety to an N-terminal Cys residue, and 3) desulfurization of the Cys-containing glycopeptides to afford native and homogeneous AFGPs. Furthermore, unlike in previous studies, where only TH activity has been assessed, in the present study we were interested in evaluating both the TH and IRI activities of these synthetic homogeneous AFGPs.

Our synthetic strategy commenced with the synthesis of the key dodecaglycopeptide **1** bearing an N-terminal Thz residue by using Fmoc-SPPS strategy starting from 2-chlorotrityl chloride resin (**2**) preloaded with Fmoc-Ala-OH (see the

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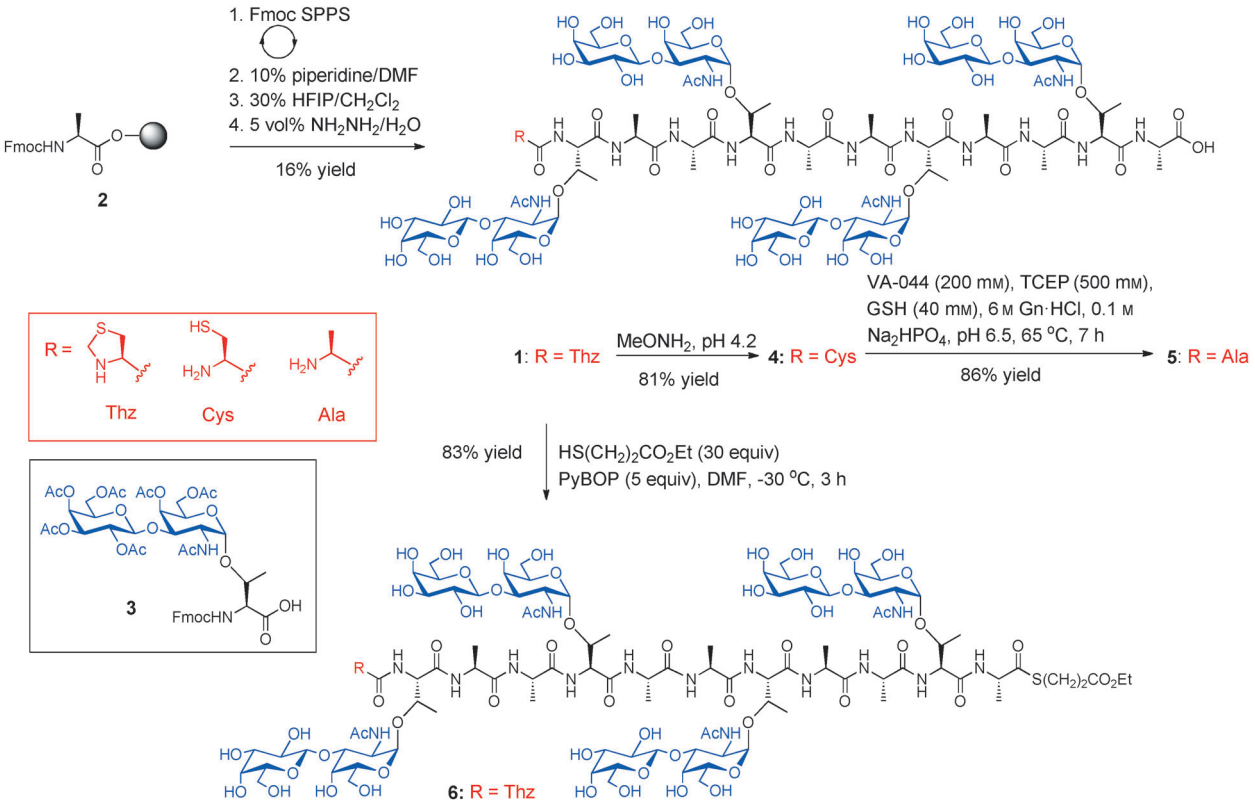
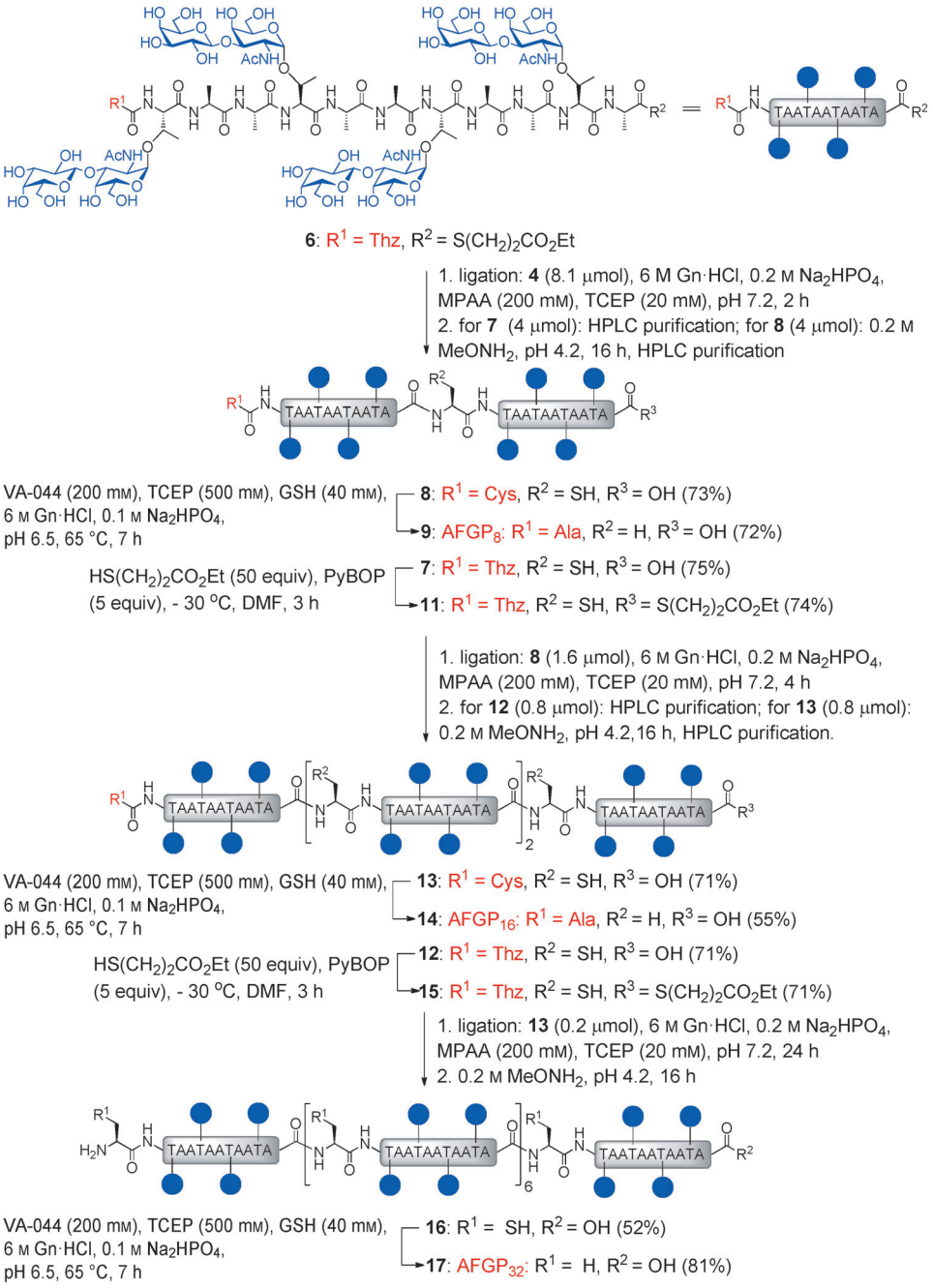


Scheme 2. Solid-phase synthesis of key dodecaglycopeptide **1**, $AFGP_4$ (**5**), and dodecaglycopeptide thioester **6**. One cycle of Fmoc SPPS includes the following steps: a) deprotection: 10% piperidine/DMF; b) coupling: Fmoc-Ala-OH or Fmoc-Thz-OH (4 equiv), PyBOP (4 equiv), NMM (8 equiv), DMF; for glycosylamino acid **3** (1.2 equiv), HATU (1.2 equiv), DIPEA (2.4 equiv), DMF; and c) capping: 9:1 v/v pyridine/Ac_2O. The yields given in the scheme are for the isolated products. Fmoc = 9-fluorenylmethoxycarbonyl, PyBOP = (1H-benzotriazol-1-yloxy)tris(pyrrolidino)-phosphonium hexafluorophosphate, NMM = *N*-methylmorpholine, HATU = O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetra-methyluronium hexafluoro-phosphate, DIPEA = diisopropylethylamine, HFIP = 1,1,1,3,3,3-hexafluoro-2-propanol, Gn·HCl = guanidine hydrochloride, GSH = reduced gluta-thione.

Supporting Information and Scheme 2). After iterative assembly by Fmoc SPPS (which included the incorporation of preformed glycosylamino acid **3**), cleavage from the resin, and hydrazinolysis of the acetate protecting groups on the glycans,[10] **1** was isolated in 16% yield (based on original resin loading, see the Supporting Information for synthetic details). We reasoned that dodecaglycopeptide **1** could serve both as a precursor for the synthesis of the native antifreeze glycopeptide $AFGP_4$ and as a key intermediate for the preparation of the coupling partners that are necessary for the convergent assembly of larger oligomers through native chemical ligation. To gain access to the native $AFGP_4$ analogue and to demonstrate the feasibility of our synthetic approach, glycopeptide **1** was treated with a methoxyamine hydrochloride solution (0.2 M, final pH 4.2) to effect the conversion of the N-terminal Thz residue to glycopeptide **4**, now bearing an N-terminal Cys residue, which was isolated in 81% yield after HPLC purification. We then investigated conditions for desulfurization of the cysteinyl thiol of **4** to provide the native $AFGP_4$ (**5**). Here, we chose to use a modified version of the metal-free desulfurization procedure reported by Danishefsky and co-workers, which employs 2,2′-azobis-[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) as a radical initiator.[11] Reaction of **4** with VA-044 in the presence of tris(2-carboxyethyl)phosphine (TCEP) and reduced glutathione (GSH) at 65 °C[12] afforded $AFGP_4$ (**5**) in 86% yield after HPLC purification (see the Supporting Information and Scheme 2).

In addition to cysteine-containing glycopeptide **4**, access to C-terminal glycopeptide thioester **6** was also necessary for the proposed native chemical ligation assembly. As such, dodecaglycopeptide **1** was subjected to thioesterification reaction conditions described by Kajihara et al. (ethyl-3-mercaptopropionate (30 equiv), PyBOP (5 equiv) in DMF at −30 °C for three hours)[13] to provide the corresponding glycopeptide thioester **6** in 83% yield without any detectable epimerization of the C-terminal alanine residue (see the Supporting Information).[13]

After having successfully prepared glycopeptide **4** and glycopeptide thioester **6**, we examined the assembly of a larger AFGP oligomer, namely the 24-amino acid $AFGP_8$, by using the proposed native chemical ligation–desulfuriza-tion strategy. To this end, C-terminal glycopeptide thioester **6** was reacted with glycopeptide **4** in the presence of 4-mercaptophenylacetic acid (MPAA)[14] as a thiol additive (200 mM) and TCEP (20 mM) in 6 M Gn·HCl/0.2 M Na_2HPO_4 buffer at pH 7.2 and 25 °C (see the Supporting Information and Scheme 3). The ligation proceeded efficiently and reached completion within two hours (determined by LC–MS analysis). At this stage the reaction mixture was split to



Scheme 3. Synthesis of homogeneous AFGP oligomers $AFGP_8$ (**9**), $AFGP_{16}$ (**14**), and $AFGP_{32}$ (**17**) through an iterative native chemical ligation–desulfurization strategy. A = alanine, T = threonine.

allow divergent access to $AFGP_8$ and the two coupling partners necessary for a subsequent ligation reaction. Direct HPLC purification of a portion of the crude ligation mixture (50%) provided the 24-amino acid glycopeptide **7**, possessing an N-terminal Thz moiety, in 75% yield. Direct treatment of the remaining portion of the ligation reaction with a solution of methoxyamine hydrochloride (0.2 M, final pH 4.2) led to conversion of the ligation product into glycopeptide **8**, bearing an N-terminal Cys residue, which was isolated in 73% yield after HPLC purification. Metal-free desulfurization of glycopeptide **8** by using the conditions described for $AFGP_4$ (**5**) provided the native $AFGP_8$ (**9**) in 72% yield after HPLC purification. Glycopeptide **8** was also reacted with dodecaglycopeptide thioester **6** by using the same native chemical ligation–desulfurization sequence described for the synthesis of $AFGP_8$ (**9**) to provide the 7.3 kDa 36-amino acid glycopeptide $AFGP_{12}$ (**10**; synthesis not shown, see the Supporting Information for synthetic details). After having successfully employed a protocol for the combination of native chemical ligation and Thz demasking to obtain glycopeptide **8**, we focused on the preparation of the 24-amino acid glycopeptide thioester **11** to serve as a coupling partner for the assembly of a 48-residue AFGP through native chemical ligation. This was achieved by treatment of **7** with a large excess of ethyl-3-mercaptopropionate (50 equiv) which provided the desired glycopeptide thioester **11** in 74% yield.

With 24-amino acid glycopeptide **8** and glycopeptide thioester **11** now in hand, the two fragments were next subjected to the native chemical ligation conditions employed above. On this occasion the reaction required four hours to reach completion (as determined by LC–MS analysis). A portion (50%) of the ligation mixture was directly purified by HPLC using a C4 column to afford glycopeptide **12** in 71% yield. The remaining ligation reaction mixture was subjected to the in situ Thz-demasking conditions to liberate glycopeptide **13**, bearing an N-terminal Cys residue, in 71% yield after HPLC purification using a C4 column. Metal-free desulfurization of glycopeptide **13** then provided the native 9.8 kDa 48-residue glycopeptide $AFGP_{16}$ (**14**) in 55% yield.

After successful preparation of the 48-amino acid $AFGP_{16}$ (**14**), we carried out one further ligation to access $AFGP_{32}$. Prior to the ligation event, the requisite C-terminal glycopeptide thioester **15** was prepared from **12** by using similar conditions as described for glycopeptide thioesters **6** and **11**, and **15** was isolated in good yield (71%). The 48-amino acid coupling partners **13** and **15** were next subjected to the

reaction sequence of native chemical ligation, Thz demasking, and desulfurization. The ligation reaction proceeded smoothly and, upon completion, treatment of the crude reaction mixture with methoxyamine hydrochloride provided the 96-amino acid glycoprotein **16**, which was isolated in 52% yield after purification by HPLC using a C4 column. Finally, complete desulfurization of the eight sulfhydryl groups in **16** furnished the 19.5 kDa $AFGP_{32}$ glycoprotein **17** in excellent yield (81%) and purity after purification by HPLC using a C4 column.

With a range of native, synthetic AFGPs now in hand, we next examined the effect of glycopeptide/glycoprotein size on antifreeze activity. Specifically, the synthetic AFGPs were examined for thermal hysteresis (TH) activity by using nanoliter osmometry[15] and were assessed for ice recrystallization inhibition (IRI) activity by using a "splat cooling" assay (see the Supporting Information).[16] Synthetic AFGPs **9**, **10**, **14**, and **17** all displayed TH activity (Figure 1A) and dynamic ice shaping to produce hexagonal-bipyramidal ice crystals, thereby indicating that AFGP oligomers **5**, **9**, **10**, **14**, and **17** were binding to ice (Figure 1B,e–h). The same set of

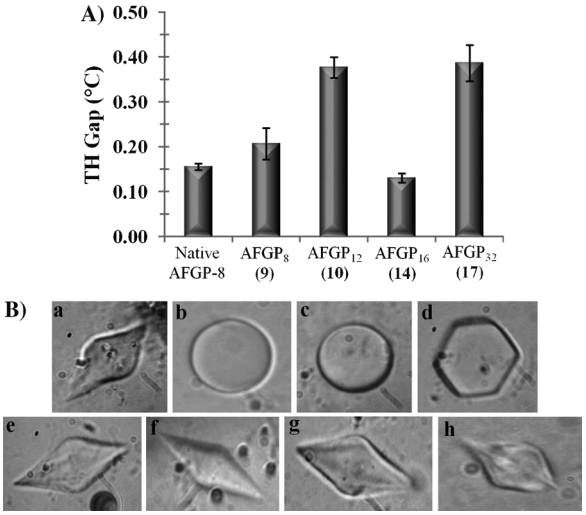


Figure 1. A) Thermal hysteresis (TH) activity of homogeneous synthetic AFGPs and isolated AFGP-8 at 10 mg mL^{-1}. B) Ice crystal morphology in the presence of: a) native AFGP-8; b) $AFGP_2$ (**18**); c) unglycosylated polypeptide $(ATA)_4$ (**19**; 5 mg mL^{-1}); d) $AFGP_4$ (**5**); e) $AFGP_8$ (**9**); f) $AFGP_{12}$ (**10**); g) $AFGP_{16}$ (**14**); h) $AFGP_{32}$ (**17**) at 10 mg mL^{-1}.

synthetic AFGPs also exhibited potent IRI activity at concentrations as low as 55 nM (Figure 2). The 24-amino acid $AFGP_8$ (**9**) possessed a significantly greater TH gap compared with AFGP-8 (a mixed fraction of isolated low-molecular-weight AFGPs) but similar TH activity to a reported AFGP with seven tripeptide repeats.[6] Interestingly, increasing the length of the synthetic AFGP in **10** ($AFGP_{12}$) led to a significant increase in both TH and IRI activities (Figures 1A and 2). However, further increasing the length of the glycopeptide in $AFGP_{16}$ (**14**) led to diminished TH and IRI activities. Specifically, the TH gap of **14** was approximately half of that exhibited by $AFGP_8$ (**9**; Figure 1A) but still exhibited similar IRI activity. The potent TH

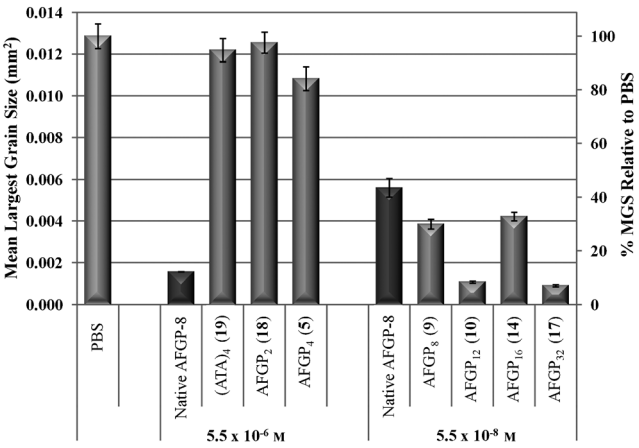


Figure 2. Ice recrystallization inhibition (IRI) activity of isolated AFGP-8, unglycosylated polypeptide $(ATA)_4$ (**19**), $AFGP_2$ (**18**), $AFGP_4$ (**5**), $AFGP_8$ (**9**), $AFGP_{12}$ (**10**), $AFGP_{16}$ (**14**), $AFGP_{32}$ (**17**) at the concentrations given in the figure. The %MGS (mean grain size) of ice crystals relative to the phosphate buffered saline (PBS) positive control is shown for all compounds.

and IRI activities displayed by $AFGP_{12}$ (**10**) is restored in the largest AFGP synthesized in this study, $AFGP_{32}$ (**17**). Specifically, this 19.5 kDa glycoprotein possessed the greatest TH gap (0.39°C) and IRI activity (at 55 nM) of all the compounds synthesized. Although $AFGP_4$ (**5**) produced hexagonal ice crystals (Figure 1B,d), it did not exhibit a measureable TH gap at 10 mg mL^{-1} or IRI activity at 5.5 μM (Figure 2). This was a surprising result given that this synthetic construct[6,17] and the isolated mixed glycopeptide fraction AFGP-8,[18] have been shown to exhibit a TH gap in prior investigations. Hexaglycopeptide $AFGP_2$ (**18**) and unglycosylated peptide $(ATA)_4$ (**19**; see the Supporting Information for synthesis) did not exhibit thermal hysteresis or ice-structuring ability (Figure 1B,b,c), nor did they possess IRI activity (Figure 2), thus highlighting the importance of glycopeptide length and the necessity of the disaccharide moiety for antifreeze activity as has been highlighted previously.[6] To our knowledge, the present study represents the first investigation of both TH and IRI activities of homogenous synthetic AFGPs with larger chain lengths than seven repeating tripeptide units[6] and has revealed a number of previously unknown structure–activity relationships, which should prove useful for future design of synthetic antifreeze molecules.

Despite there being some debate about the precise conformation of AFGPs in solution,[18,19] several reports have suggested that the molecules adopt the secondary structure of a type II polyproline (PPII) helix in solution, as indicated by CD and NMR spectroscopic studies.[18–20] We were therefore interested in probing the temperature dependent (−5 to 85°C) secondary structure of the synthetic homogeneous AFGPs in water by using far-UV CD spectroscopy to study correlations between secondary structure and antifreeze activity. At lower temperatures the CD spectra of all AFGPs ($AFGP_2$ (**18**), $AFGP_4$ (**5**), $AFGP_8$ (**9**), $AFGP_{12}$ (**10**), $AFGP_{16}$ (**14**), and $AFGP_{32}$ (**17**)) showed clear PPII helical structural features: a weak positive maximum at

a wavelength of approximately 215 nm and a stronger negative minimum below 200 nm (see the Supporting Information).[21] In contrast to $AFGP_4$ (**5**), the unglycosylated analogue $(ATA)_4$ (**19**) has a spectrum that is characteristic of β-sheet structure (maximum at ca. 195 and minimum at ca. 220 nm). Notably, the main structural features of all AFGPs diminished with increasing temperature, thereby suggesting a loss of structure in favor of random-coil conformation (see the Supporting Information).[22] The spectral features of a PPII helix were most pronounced for $AFGP_4$ (**5**), which did not exhibit antifreeze activity in this study. The spectra for the smaller $AFGP_2$ (**17**) gave approximately 80% of the $AFGP_4$ (**5**) signal intensity, and the longer AFGPs showed a general trend of diminishing signal intensity with increasing size. Whilst heterogeneous, high-molecular-weight AFGPs that are derived from natural sources are thought to be highly flexible and to lack both short- and long-range order,[19a] we have shown for the first time, that PPII helical structure is retained in larger AFGPs as demonstrated for the 19.5 kDa $AFGP_{32}$ glycoprotein **17**. We note, however, that the secondary structure is not uniform in the analogues with different lengths. Potential differences in the overall structure of these synthetic AFGPs warrant further investigation and will be the focus of future research in our laboratories.

In summary, we have successfully employed a convergent native chemical ligation–desulfurization strategy for the preparation of homogeneous AFGPs ranging in size from 1.2 to 19.5 kDa. The synthetic strategy enabled the assembly of discretely sized AFGP oligomers previously unattainable by conventional synthetic routes. We have established, for the first time, important structure–activity relationships of homogeneous, high-molecular-weight AFGPs. In particular, we have determined the TH and IRI activities of a native 19.5 kDa AFGP which represents, to our knowledge, the highest-molecular-weight AFGP accessed in homogeneous form and one of the largest native glycoproteins prepared by total chemical synthesis to date.

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