Antifreeze Glycoproteins—Preventing the Growth of Ice

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1. Introduction

Biological antifreezes constitute a diverse class of proteins found in arctic and antarctic fish, as well as in amphibians, trees, plants, and insects. These compounds are unique in that they have the ability to inhibit the growth of ice and consequently are essential for the survival of organisms inhabiting environments where sub-zero temperatures are routinely encountered. This is an unusual ability attributed *only* to biological antifreezes.

There are two types of biological antifreezes, the antifreeze proteins (AFPs) and the antifreeze glycoproteins (AFGPs).[1] Antifreeze proteins are divided into four subtypes (types 1 – 4) each possessing a very different primary, secondary, and tertiary structure. In contrast, AFGPs are subject to considerably less structural variation. A typical AFGP is composed of a repeating tripeptide unit (threonyl – alanyl – alanyl) in which the secondary hydroxy group of the threonine residue is glycosylated with the disaccharide β-D-galactosyl-(1,3)-α-D-*N*-acetylgalactosamine (Figure 1). Eight distinct AFGP subtypes exist; glycoproteins of 20 – 33 kDa are referred to as AFGPs 1 – 4 and those of less than 20 kDa constitute AFGPs 5 – 8. The lower molecular weight

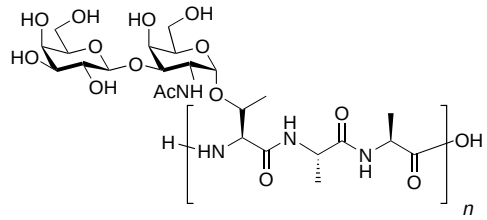

Figure 1. *Chemical structure of a typical antifreeze glycoprotein (AFGP);* n = *4 – 55.*

glycoproteins (AFGP 7 and 8) occasionally have the L-threonine residue substituted with L-arginine and one or both L-alanine residues substituted with L-proline.[2a,b] In addition to inhibiting the growth of ice, AFGP has been shown to protect cells from hyperthermic damage.[3a–c]

The ability to inhibit the growth of ice has potential medical, industrial, and commercial applications. Unfortunately, many of these applications have not been fully realized. One reason for this is that the isolation and purification of AFGPs is a laborious and costly process often resulting in mixtures, making characterization difficult.[4] Additional reasons include the fact that the AFGP mechanism of action is not understood at the molecular level and that the nature of the protein – ice interface remains in question.[5]

2. Mechanism of action

During the last decade, there has been great interest in elucidating the mechanism by which biological antifreezes bind to ice and inhibit its growth.[6a] On a macroscopic level, the AFGP (or AFP) binds to the surface of a growing ice crystal.[6b,c] Both direct and indirect experimental methods have confirmed this adsorption.[7a–c] At this stage, growth occurs on ice surfaces between adjacent AFGP molecules; however, these ice fronts grow with a large radius of curvature (Figure 2). Since the energetic cost of adding a water molecule to this convex surface is high, a non-equilibrium freezing point depression is observed while the melting point remains constant. This is known as the Kelvin effect, and the difference between melting and freezing points is defined as thermal hystersis (TH).

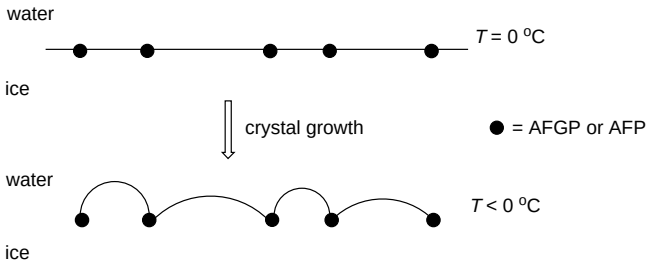

Figure 2. *Schematic representation of the adsorption – inhibition process. See text for details.*

An understanding of how these molecules inhibit ice crystal growth at the molecular level remains a source of intense debate.[8a,b] Researchers have long proposed that the binding of AFGPs to the ice surface likely involves hydrogen bonding between the polar groups of the saccharide residue (the hydroxy

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groups) and the ice surface. However, studies have demonstrated that the number of potential hydrogen bonds between the antifreeze molecule and the ice surface appears to be insufficient to explain the observed tight binding of AFGPs to ice.[9] Modeling studies have looked at all possible binding configurations, and in the best case only two hydroxy groups per disaccharide are in a position to form hydrogen bonds with the ice surface. As shown in Figure 3A, each hydroxy group forms

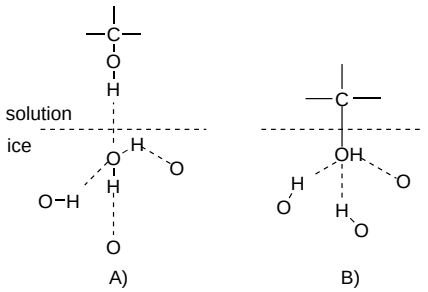


Figure 3. *Plausible hydrophilic interactions between an AFGP and the ice surface. See text for details.*

only one hydrogen bond with the ice surface. In AFGP 8 (with four glycosylated tripeptide units), this would allow for only eight hydrogen bonds with the ice surface. Consequently, it is difficult to explain how adsorption of AFGP 8 onto the ice surface is irreversible. In an attempt to rationalize the irreversible binding of AFGP 8 to an ice surface through only eight hydrogen bonds, Knight et al.[9] have proposed an alternate model. In this model, the hydroxy groups of the disaccharide are actually incorporated into the ice lattice as illustrated in Figure 3B. In this fashion, each hydroxy group is able to form three hydrogen bonds within the ice lattice. Assuming that in each disaccharide only two hydroxy groups are able to interact with the ice surface, this allows AFGP 8 a total of twenty-four hydrogen bonds to the ice surface instead of eight and may explain why adsorption is irreversible.

On a more fundamental level, researchers have been divided over the importance of hydrogen bonding and its role in the mechanism of action. While it has been proposed that the hydrophilic interactions between polar residues and the water molecules on the ice surface are extremely important,[10] other researchers have invoked the idea that entropic and enthalpic contributions from hydrophobic residues are crucial in the binding of an AFP or AFGP to an ice surface.[8a,b] Despite the fact that significant entropic contributions are likely to be gained upon exclusion of water from the protein and ice surfaces, a definitive mechanism invoking hydrophobic and/or hydrophilic interactions—with emphasis on the role they play in adsorption of the antifreeze to the ice surface—has failed to emerge.

In a separate approach, the cooperative binding of antifreeze proteins as well as the role of side chain flexibility have been investigated.[11a,b] However, further complications have arisen with the discovery that different antifreeze proteins bind to separate faces or surfaces of an ice crystal.[12] It is not surprising then that a unified hypothesis centered on the molecular mechanism of action describing how biological antifreezes inhibit the growth of ice crystals has not emerged.

Another consistent problem with elucidating the molecular mechanism of action for AFGPs (and AFPs) is that the ice–water interface has not been well characterized. In fact, the interface itself is probably not an abrupt transition as typically represented in the static models since the most recent evidence shows the loss of organized ice structure at the interface as being fairly gradual, occurring over approximately ten ångstroms.[5] This is a problem, especially when attempting to "map" possible interactions between AFGP and the ice surface. Since dynamic models of the ice–water interface have not been developed, static models continue to be used largely because the absorption event has been shown to be irreversible in nature.

3. Early structure–function studies

Extensive structure–activity-relationship (SAR) studies have been conducted on AFGPs and AFPs. Much of the prior SAR work for AFGPs was performed on a native AFGP isolated from a cold-adapted antarctic fish, *Trematomus borchgrevinki*.[13a–e] Through a systematic series of chemical modifications as well as enzymatic and chemical degradations, certain basic structural requirements have been identified as having a critical role in the ability of AFGPs to inhibit ice crystal growth.

There exists a definite relationship between the length of an AFGP and the level of its activity. As mentioned previously (see Section 1), AFGPs are made up of repeating tripeptide units; the number of repeating units may be as small as four or as large as fifty-five. Small glycopeptides obtained by extensive subtilisin hydrolysis of an active AFGP were reported to have no detectable antifreeze activity.[13d] In these studies, the largest glycopeptide fragment tested was a pentapeptide consisting of Ala-Ala-Thr-Ala-Ala. It was later shown that even hexa- and heptapeptides possessing two disaccharide units were inactive. This trend is reflected by the fact that AFGP 8 (having four glycosylated tripeptide units) retains only 30% of the activity observed with AFGP 1.

The oligosaccharide moiety has been shown to be crucial to activity and while extensive chemical and enzymatic modifications have been performed, a detailed discussion of these results is beyond the scope of this Minireview and thus will only be highlighted here. The β-elimination of the disaccharide promoted by treatment with 0.5 N NaOH at room temperature for 24 h resulted in cleavage of the base-sensitive glycosidic bond and a complete loss of antifreeze activity. While this implies that the carbohydrate moiety is essential for activity, it also suggests that the amino acid side chain may be important since significant racemization was observed. Removal of more than 60% of the galactosyl residues by periodate oxidation also resulted in a total loss of antifreeze activity. Acetylation of the hydroxy groups on the disaccharide produced similar results, but antifreeze activity was completely restored upon deacetylation with hydroxylamine at pH 9.5. Interestingly, oxidation of the C6 hydroxy groups of galactose and galactosamine residues by treatment

with galactose oxidase had little effect on activity (the fully oxidized AFGP derivative retained approximately 70% activity).

While the earliest work examining structure – activity relationships of native AFGPs dealt with issues relating to primary structure, a large number of studies on the secondary and higher order structures of AFGPs have been carried out in the last two decades. Two techniques have been utilized to accomplish this: circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy.

Early CD measurements indicated an extended random-coil structure but subsequent studies by Franks and Morris[14] suggested an ordered conformation similar to a left-handed α-helical structure but with substantially different molecular geometry. Vacuum-ultraviolet CD studies by Bush et al.[15] have implied a threefold left-handed helical conformation of the polypeptide backbone. By using a model disaccharide, Bush et al. also demonstrated that contributions from the sugar moiety to the CD spectrum appeared to be negligible.

Investigations with NMR spectroscopy have yielded more detailed information about the conformations of AFGPs. Bush and Feeney[16] have performed many variable-temperature ^{1}H- and ^{13}C-NMR studies, as well as NOE (nuclear Overhauser effect) experiments. Based upon these results and data from CD measurements, they have concluded that low molecular weight AFGPs adopt a rod-shaped structure similar to the left-handed threefold polyproline type-II helix at low temperatures, whereas at higher temperatures the structure becomes more like a flexible coil. Higher molecular weight fractions (AFGPs 1 – 4) seem to be flexible rods with significant segmental mobility and thus are not regarded as ordered structures.

Given the importance of the saccharide residues for AFGP activity, several studies have attempted to examine the oligosaccharide conformation relative to the peptide backbone. Franks and Morris[14] have proposed a planar conformation for the disaccharide residue in which the hydrophilic groups are exposed to the aqueous solvent and the hydrophobic groups are facing the polypeptide backbone. Mimura et al.[17a] propose the existence of an intramolecular hydrogen bond between the carbonyl group oxygen atom of the threonine residue and the NHAc group of the *N*-acetyl-β-D-galactosamine residue of the disaccharide to stabilize the carbohydrate structure against the polypeptide backbone. With such a conformation, the polypeptide chain adopts a left-handed helix having three residues per turn. Consequently, the saccharide residues are aligned such that they reside on only *one* side of the helix.

Rao and Bush[17b] have used correlated spectroscopy (COSY) and NOE techniques to assign the proton NMR signals of AFGP 8 and have concluded that the observed difference in activities of AFGP 8 and AFGPs 1 – 4 are due to a difference in overall length of peptide rather than a difference in conformation. Based upon coupling constant data and semi-empirical molecular modeling calculations, they proposed that the threefold left-handed helix is definitely one of *several* minimum-energy conformations. Thus, while the model of Rao and Bush[17b] is a reasonable one, it does not necessarily reflect the absolute conformation of AFGPs.

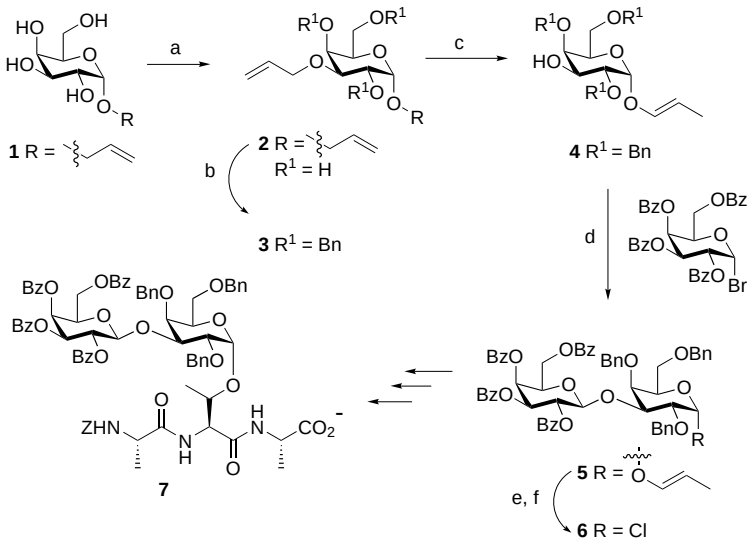
More recently, Lane et al.[18] assigned the ^{1}H- and ^{13}C-NMR spectra of a 14-residue antifreeze glycopeptide from antarctic cod. ^{13}C-NMR relaxation data indicated motional anisotropy of a linear peptide undergoing significant segmental motion. While molecular modeling studies failed to produce evidence of long-range order, portions of the structure resembled an extended polyproline helix. These results are consistent with earlier studies.

Despite the valuable insights gained by many of these studies, the molecular mechanism of action of AFGPs still remains to be elucidated. While the detailed mechanism of AFGPs may be different from other biological antifreezes, it is clear that the carbohydrate moiety is essential to AFGP activity. Since other biological antifreezes tend to possess very rigid solution structures, it is not known if the inherent flexibility of AFGPs is important to the mechanism. Direct physical observation of how these molecules are positioned on the ice lattice may facilitate elucidation of the mechanism of action.

4. Synthesis of AFGPs and AFGP analogues

An alternative approach to studying the molecular mechanism of action involves synthesizing AFGPs and AFGP analogues; this is an attractive alternative to the isolation and purification of an AFGP. However, despite the monumental advances in the synthesis of complex oligosaccharides and glycopeptides[19a–m] the preparation of such systems still remains a formidable synthetic challenge. Two reasons for this are the need to employ orthogonal protecting-group strategies and the fact that the bond between the anomeric carbon atom and the oxygen atom is unstable under strongly acidic and/or basic conditions. During the last ten years only five synthetic strategies affording AFGPs or AFGP analogues have been reported. These strategies center on a stepwise elongation of the peptide chain (using solution or solid-phase techniques) with the appropriate glycosylated amino acid derivatives.

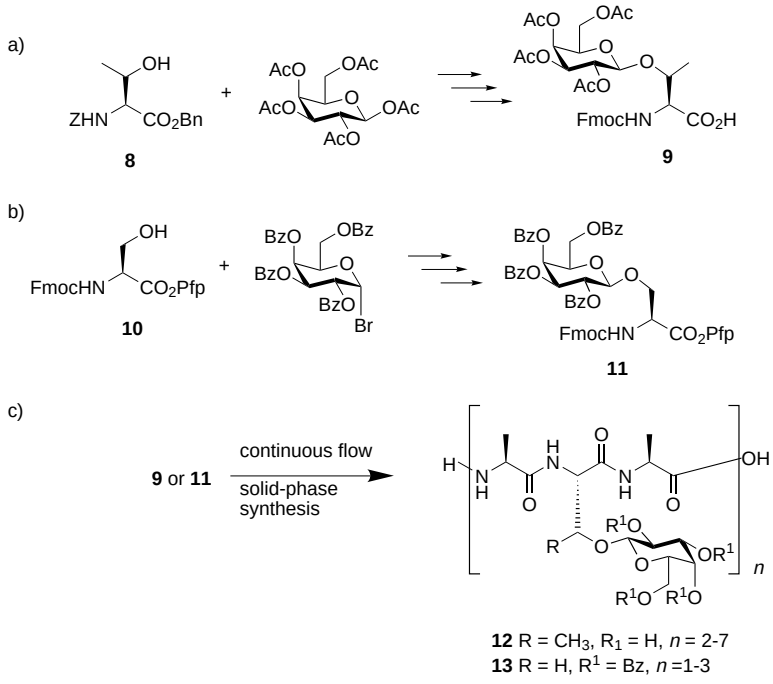
In 1988, Anderson et al. synthesized a structural analogue of the glycosylated tripeptide core unit native to AFGPs.[20] As illustrated in Scheme 1, the synthesis is convergent and afforded an analogue of the repeating tripeptide unit in which β-D-galactosyl-(1,3)-α-D-*N*-acetylgalactosamine was substituted with β-D-galactosyl-(1,3)-α-D-galactose. The key step involved reaction of the unprotected galactoside **1** with dibutyltin oxide and subsequent selective alkylation at the C3 hydroxy group to afford **2**.[21] The threefold benzylated saccharide **3** then underwent concomitant unmasking of the C3 hydroxy group and isomerization to the propenyl glycoside **4**. Reaction of glycosyl donor **4** with α-bromogalactoside in a glycosylation catalyzed by silver triflate (triflate = trifluoromethane sulfonate) furnished the disaccharide **5** that was subsequently converted into the respective chloride **6** by treatment with the Vilsmeier reagent. A second silver triflate mediated glycosylation reaction between **6** and the protected alanyl-threonyl-alanyl tripeptide, which had been prepared by using typical peptide synthesis protocols, proceeded smoothly to give glycopeptide **7** in 60% yield (of isolated product).



Scheme 1. *Synthesis of a structural analogue of the glycosylated tripeptide core unit of AFGPs. a) $(Bu_3Sn)_2O$, allyl bromide; b) NaH, BnBr; c)* tert*-BuOK; d) AgOTf, tetramethyl urea, 2,6-dimethylpyridine; e) $HgCl_2$, HgO; f) $[(CH_3)_2N{=}CHCl]^+Cl^-$, CH_2Cl_2. Bn = benzyl; Bz = benzoyl; Z = benzyloxycarbonyl.*

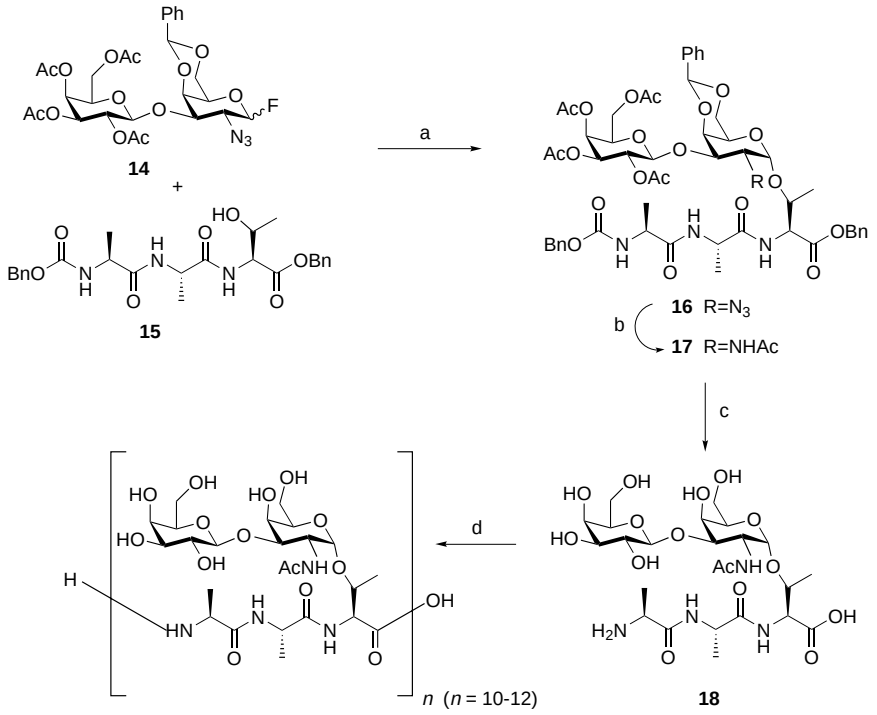
In a separate approach, Filira et al.[22] prepared AFGP analogues consisting of two to seven glycosylated tripeptide units by using continuous-flow solid-phase synthesis (SPS). This example was the first to demonstrate the utility of solid-phase synthesis in the preparation of glycopolymers. The approach is a linear one in which the AFGP analogue is assembled in a stepwise fashion from suitably protected L-alanine and glycoconjugate **9** on a Pepsyn KA solid-phase resin (Scheme 2 c). Building block **9** was prepared by reaction of the protected threonine **8** with commercially available β-D-galactose pentaacetate mediated by boron trifluoride diethyl etherate (Scheme 2 a). This procedure produced a glycoconjugate having the β configuration at the anomeric center. Interconversion and manipulation of protecting groups was accomplished by using standard procedures. Stepwise assembly of the peptide framework, cleavage of the glycopeptide from the resin, and removal of protecting groups afforded AFGP derivatives **12** after purification by reversed-phase HPLC (Scheme 2 c). In these structures, the β linkage was designed to mimic the glycosidic bond connecting the terminal nonreducing galactose unit in the native AFGP structure.

A similar solid-phase approach was reported by Meldal et al. to prepare AFGP analogues in which the native AFGP disaccharide unit is replaced with a β-D-galactoside and the threonine residue in the alanyl–threonyl–alanyl polypeptide is substituted with serine.[23] An attractive feature of this approach is the use of pentafluorophenyl (Pfp) esters. As in the approach of Filira et al., the synthesis is linear, employing Macrosorb SPR-250 resin, appropriately protected L-alanine residues, and the glycosylated L-serine derivative **11**, which was produced by reaction of the protected serine **10** with tetrabenzoyl-α-D-galactopyranosyl bromide (Scheme 2 b) in the presence of silver triflate. AFGP analogues **13** ranging in length from one to three tripeptide units were prepared (Scheme 2 c). The Pfp ester **10** is stable under the glycosylation conditions and is also reactive enough to be utilized directly in solid-phase synthesis. This is an effective way to ameliorate difficulties with the synthesis since C-terminal protecting groups need not be exchanged. Prior to this approach, other active esters (*N*-hydroxysuccinamidyl, 4-nitrophenyl, etc.) were employed for protection of the amino acid C terminus during the glycosylation. However, these groups are generally not reactive enough to be employed in solid-phase chemistry and thus must be removed after glycosylation; furthermore, hydroxybenzotriazole esters or symmetrical anhydrides must be generated in situ at the cost of increased steps and lower overall yield of product.



Scheme 2. *Preparation of building blocks (a, b) for the solid-phase synthesis of AFGP analogues (c). Fmoc = fluoren-9-ylmethoxycarbonyl; Pfp = pentafluorophenyl.*

In 1996, Nishimura and Tsuda published the first synthesis of a native AFGP.[24] The approach, outlined in Scheme 3, utilizes a DPPA-catalyzed polymerization of the fully deprotected glycosylated tripeptide unit **18** (DPPA = diphenylphosphoryl azide). The polymerization precursor was assembled from disaccharide intermediate **14** and tripeptide **15** by a cyclopentadienylzirconium dichloride/silver perchlorate mediated coupling.[25] Standard functional group manipulation of glycopeptide **16** afforded polymerization precursor **18** after complete deprotection. Gel permeation chromatographic analysis revealed a relatively uniform molecular weight between 6000–7300 Da, corresponding to 10–12



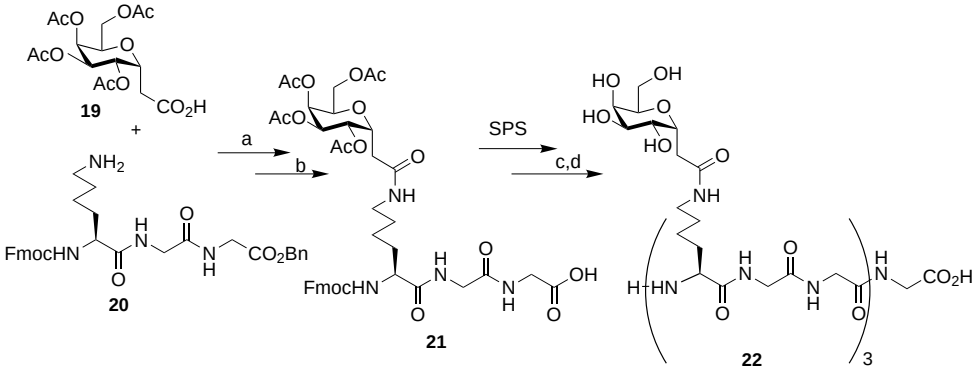
Scheme 3. *Synthesis of a native AFGP by DPPA-catalyzed polymerization of a tripeptide unit. a) $[(C_5H_5)_2ZrCl_2]$, $AgClO_4$, CH_2Cl_2, molecular sieves; b) $NiCl_2 \cdot 6H_2O$, $B(OH)_3$, $NaBH_4$, EtOH, then Ac_2O (excess); c) NaOMe, THF/MeOH, then Pd/C, H_2, MeOH; d) $Ph_2P(O)N_3$ (DPPA), Et_3N, DMSO.*

tripeptide units. The C-terminal activation of the unprotected tripeptide by DPPA is likely the first step in this reaction. Remarkably, no by-products arising from potential side reactions were detected. This example constitutes the *only* chemical synthesis of native AFGP.

An attractive alternative to the preparation of *O*-linked glycopeptides is the preparation of glycopeptide mimetics. Such compounds have superior chemical and biological stabilities and lend themselves to a more facile synthesis. There are many classes of glycopeptide mimetics (i.e. *C*-linked, *S*-linked, and glycopeptoids) and a comprehensive review highlighting this area of research has been published recently.[26] Concurrent with this theme, the synthesis of *C*-linked analogues of low molecular weight AFGP analogues by using conventional solid-phase chemistry was recently published by Ben et al.[27] As in some of the previous approaches, the native disaccharide was substituted with a monosaccharide, specifically a *C*-linked α-D-galactoside. This *C*-linked saccharide **19** was linked to the L-lysine – glycinyl – glycinyl tripeptide **20** (Scheme 4) through an amide bond. This particular tripeptide was chosen for two reasons. Firstly, the L-lysine amide bond motif is structurally similar to the L-arginine residue frequently substituted for L-threonine in native AFGPs 7 – 8.[2a,b] While lysine is not typically found in AFGPs, recent results have shown that an (L-lysine – L-alanine)$_n$ polypeptide displays thermal hysteresis.[28] Secondly, to avoid potential problems with racemization during the solid-phase synthesis, achiral glycine residues were utilized instead of L-alanine residues. This is the first example where a complex glycoconjugate of approximately 1.5 kDa was synthesized by using conventional solid-phase synthesis. The glycosylated tripeptide **21** was prepared in a convergent fashion by a CDI-mediated coupling between the *C*-linked galactose derivative **19** and tripeptide **20** followed by hydrogenolysis of the benzyl ester (CDI = 1,1-carbonyldiimidazole). Iterative couplings of **21** on a commercially available Wang resin yielded glycopeptide **22** in 55% yield after acid-catalyzed cleavage from the resin and removal of the acetate protecting groups. Purification was accomplished by using reversed-phase HPLC. This *C*-linked glycoconjugate is stable under a variety of chemical and biological conditions, and these are attractive qualities for glycoconjugates that may have potential in vivo applications as cryoprotectants, antiadhesives, synthetic vaccines, or biological probes.

5. Future directions

While preliminary structure – function studies have been conducted with native AFGPs, little insight into ice-binding affinity and specificity at the molecular level has resulted. Consequently, structurally diverse analogues of AFGPs are urgently required. The synthetic approaches described here are ideally suited to produce such AFGP analogues in order to elucidate the molecular mechanism of action for AFGPs. With this knowledge,



Scheme 4. *Synthesis of a C-linked low molecular weight AFGP analogue. a) CDI, DIPEA, CH_2Cl_2; b) H_2/Pd, EtOH; c) TFA/CH_2Cl_2; d) $NaOCH_3/CH_3OH$. SPS = solid-phase synthesis.*

the rational design of low molecular weight synthetic antifreezes may be feasible.

The list of potential applications for such compounds is lengthy. At the commercial level, such compounds could be used as additives in frozen foods.[29a] Natural or synthetic antifreezes could improve the qualities of foods that are eaten while frozen by inhibiting recrystallization and thus ensuring a smooth texture. In addition, antifreezes could be added to foods that are only frozen for preservation and in this way will prevent cellular damage and improve texture and taste. While these may seem unlikely applications, biological antifreezes are already present in many foods routinely consumed as part of the human diet.[30] Alternatively, the ability of these compounds to interact with cell membranes suggests a range of medical applications that center upon cryo- or hyperthermic preservation of cells and tissues.[1c, 3a–c] Recent studies have demonstrated that depending upon the choice of antifreeze and concentration they may either protect or damage cells. The latter has been exploited in new noninvasive cryosurgical techniques for the treatment of cancer.[29b,c]

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