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Synthesis and evaluation of linear CuAAC-oligomerized antifreeze neo-glycopeptides†

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Antifreeze glycoproteins (AFGPs) are important naturally occurring biological antifreezes that lower the freezing point of a solution, thereby preventing uncontrolled ice growth. These compounds also inhibit ice recrystallization. Described in this paper is a synthetic antifreeze glycopeptide-based polymer synthesized from an azide/alkyne glycopeptide building block by partial reduction of the azide and subsequent copper catalyzed azide alkyne cycloaddition (CuAAC) polymerization to obtain linear oligomers. To compare the activity with native AFGPs, a linear dodecapeptide (oligomer with four repeating units) was synthesized and isolated which had a comparable length to AFGP-8, the lowest molecular mass glycoprotein AFGP found in nature. In terms of ice recrystallization inhibition (IRI) activity, the triazole-based oligomers displayed only modest IRI activity compared with AFGP-8 and a previously described carbon-linked AFGP analogue. However, CD spectroscopy showed that the triazole-based tetramer possessed a similar secondary structure to the related amide based carbon-linked AFGP tetramer based on AFGP-8.

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Introduction

Antifreeze glycoproteins (AFGPs) are naturally occurring antifreeze compounds found in several deep sea teleost fish. These proteins play an important role in the survival of these organisms living in sub-zero temperatures.[1] In contrast to antifreeze proteins (AFPs) that show a relative diversity in their structures,[2] the structure of AFGPs is relatively conserved. A key feature of these proteins is that they are mostly composed of repeating tripeptide units (Fig. 1).[3,4]

The tripeptide repeat consists of two L-alanine residues and one L-threonine which is glycosylated with β-Gal-(1,3)α-GalNAc. AFGPs occur in different lengths and are named based on their size or molecular mass. The shortest fragment, named AFGP-8, consists of 4 tripeptide repeats, while the longest fragment, AFGP-1, contains approximately 50 repeats.[3]

AFGPs exhibit two distinct "antifreeze" properties. One is a lowering of the freezing point by the antifreeze compound, while the melting point remains unchanged. The difference between melting and freezing points is known as thermal hysteresis (TH).[5] This effect can lower the freezing point of a biological fluid enough to allow for survival in Arctic environments. The other "antifreeze" effect of the AFGPs is the ability to inhibit ice recrystallization. Specifically, this is the ability to prevent the enthalpy driven process of large ice crystals growing larger at the expense of small ice crystals.[6,7] Large ice crystals are highly damaging to cells and tissues, and as such the prevention of the formation of these large crystals is a good strategy in the cryopreservation of biological materials (cells, tissues, *etc.*).[8] Previously tested antifreeze glycoprotein analogues have shown clinical potential by rivalling the efficacy of the often-used cryoprotectant DMSO in the preservation of embryonic liver cells, when used at a lower concentration.[8] Using larger molecular weight oligomers or more potent analogues could further increase the potential of AFGPs in the cryoprotection of sensitive tissues and cells.

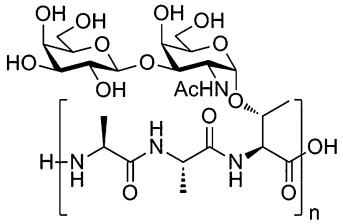

Fig. 1 General structure of AFGPs with the repeating structure H–(Ala-Ala-Thr(β-D-Gal-(1,3)α-D-GalNAc))$_n$–OH.

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† Electronic supplementary information (ESI) available: Full experimental details, ^{1}H and ^{13}C NMR spectra of all synthesized intermediates plus ^{1}H NMR and ESI data of the final compounds. See DOI: 10.1039/c4md00013g

It is thought that the interaction of the sugar hydroxyl moieties with the water–ice interface is responsible for the antifreeze properties of AFGPs. However, model AFGPs have recently been investigated, showing that the most active compound in terms of IRI activity showed a distinct folding, thereby creating a hydrophobic pocket that may be responsible for its specific water–ice interface interactions.[9]

The use of antifreeze glycoproteins and their analogues for cryopreservation applications has been limited by the low availability of these compounds from natural sources. Consequently, effort has been undertaken to obtain these compounds synthetically. The total synthesis of natural AFGPs has been described in the literature,[10–12] and while elegant, it is not a viable large scale synthesis of antifreeze compounds on a cost-effective basis. Therefore, previous work has sought to simplify the structure of native AFGP-8 while maintaining the antifreeze activity, in particular the ability to inhibit ice recrystallization. To date, the best examples of this are the carbon-linked antifreeze glycoprotein analogues[13] (C-AFGPs), *e.g.* OGG-Gal analogue **1** shown in Fig. 2. Interestingly, these studies have demonstrated that glycopeptides containing the monosaccharide galactose are very effective ice recrystallization inhibitors,[14] despite the fact that the monosaccharide is less structurally complex than the disaccharide moiety found in native AFGPs.

One of the most easily accessible structures was found to be a galactose derived carboxylic acid conjugated *via* the side chain of an ornithine residue instead of a threonine residue (**1**, Fig. 2).[15] Furthermore, it was found that replacing the two alanine residues in the natural AFGP by glycine did not result in loss of IRI activity.

This compound, which is already more synthetically accessible than natural AFGP, was chosen as the starting point for creating a glycopeptide monomer that can be polymerized into longer compounds that resemble natural AFGPs and synthetic C-linked AFGPs.

The idea of treating the tripeptide repeat as a monomer has been explored before, using peptide coupling reagents as polymerization agents.[16] To simplify the polymerization and increase the compatibility with more functional groups, the orthogonal CuAAC reaction was employed in our approach (Fig. 3). The 1,2,3-triazole ring has previously been used as a nonconventional bioisostere of an amide bond[17–19] and incorporated as an amide

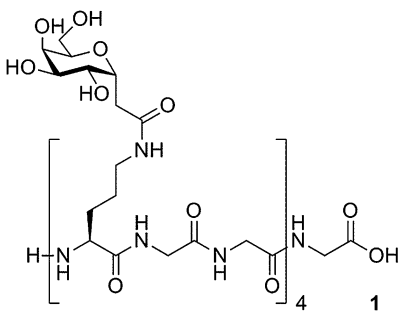


Fig. 2 Structure of the galactose based ornithine-Gly-Gly AFGP **1** by Ben *et al.*[8,13,15]

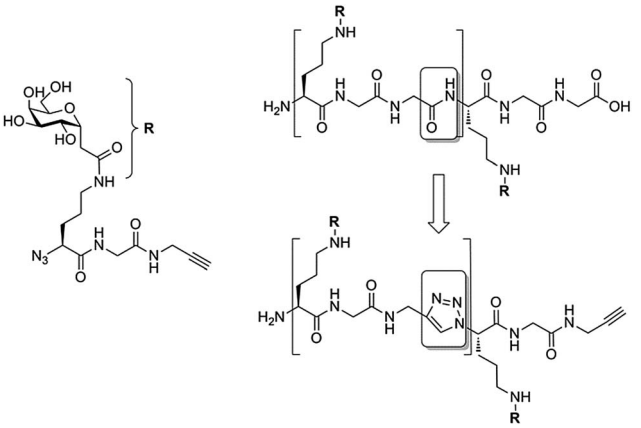


Fig. 3 (Left) The proposed structure of the glycopeptide monomer. (Right) Comparison between the published AFGP mimic by Ben *et al.* (top) and the resulting polymer obtained from the proposed monomer.

isostere into various structure-containing peptides such as in α-helical peptides,[20] small cyclic peptides[21] and β-sheet-forming peptides.[22] Moreover, the strategy of incorporating azide and alkyne termini proved feasible in the synthesis of Aβ peptide-based oligomers[23] and click chemistry was used successfully in an approach to biodegradable polymeric systems.[24–26]

Unfortunately, this method usually generates a significant amount of cyclic molecules due to intramolecular cycloaddition. Although cyclic glycoproteins have been synthesized before and are a potentially interesting group of molecules,[27] for direct comparison it was decided to focus on smaller linear oligomers to better compare the IRI activity of the CuAAC oligomerized antifreeze glycoproteins to native antifreeze glycoproteins.

While the CuAAC approach has been utilized previously to link carbohydrate moieties to polypeptide backbones of various AFGP analogues, this is the first instance where it has been used to replace the amide bonds in the polypeptide backbone of an AFGP (analogue).[28–30]

One of the key steps in generating linear oligomers is blocking one of the reactive azide or alkyne endgroups of a (growing) oligomer chain (see Fig. 4). In the case of azide/alkyne monomers this can be accomplished either by having a fraction of the monomers protected on the alkyne, for example by the TIPS protecting group, or by reduction of a fraction of the azide to the amine. Both are potentially feasible strategies, but in the end the latter was the method of choice, as reduction of an azide to an amine can be performed with phosphine reducing agents that are compatible with CuAAC click chemistry.[31] Since some of the starting azide/alkyne monomers can be converted into amino/alkyne monomers in the same pot by addition of a certain amount of phosphine, the two discrete starting monomers do not need to be synthesized separately, which is very advantageous.

The main aim of this research was to evaluate the design of the resulting oligomers by investigating whether the triazole moiety does not reduce IRI activity and whether the amide bonds in the polypeptide backbone of these analogues are not an essential structural feature of IRI activity. Therefore a

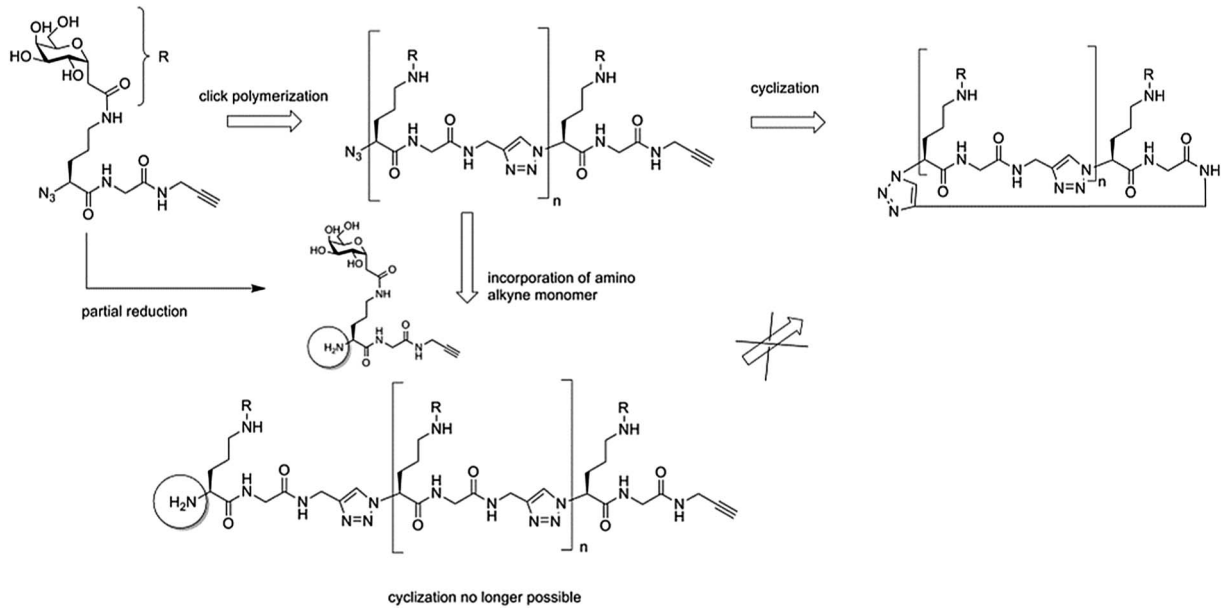


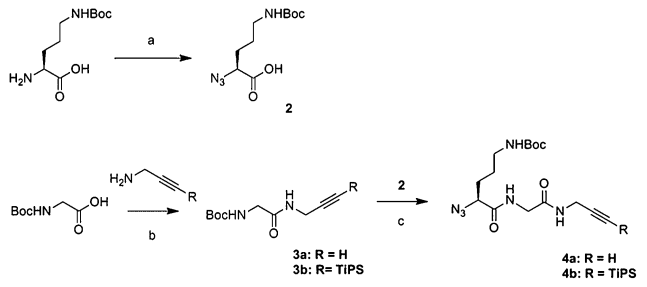
Fig. 4 Schematic overview of obtaining AFGPs by linear polymerization of an azido–alkyne monomer using CuAAC chemistry.

synthetic oligomer with a similar length to natural AFGP-8 was synthesized.[32] This was accomplished by tuning the amount of phosphine to monomer ratio to 3 : 1. The resulting distribution of linear oligomers can then, when the hydroxyl groups of the carbohydrate are protected, be separated by HPLC. Separation was possible because every oligomer has one positive charge and a hydrophobic C-glycosidic moiety; the physical properties of short oligomers are likely to be more dominated by the positive charge whereas longer oligomers become more apolar due to the amount of hydrophobic moieties per molecule. The oligomers were deprotected and the isolated tetramer was compared with the previously described C-AFGP **1** (shown in Fig. 2) by evaluation of TH and IRI activity and determination of the secondary structure by CD spectroscopy.

Results and discussion

Synthesis of the azido–alkyne glycopeptide monomer

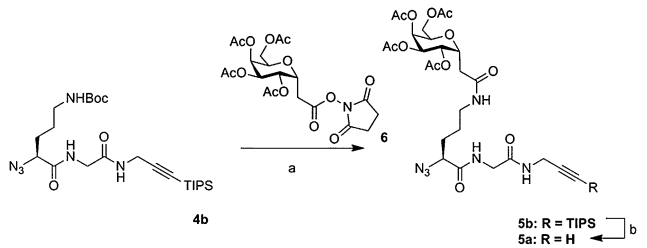
The azido/alkyne tripeptide backbone **4a** required for the synthesis of the glycopeptide monomer was synthesized from two building blocks (Scheme 1).



Scheme 1 (a) Imidazole-1-sulfonyl-azide hydrochloride, $CuSO_4$, $MeOH \cdot H_2O$, 98%; (b) BOP, DiPEA, CH_2Cl_2, 82%[a], 99%[b]; (c) (1) TFA, CH_2Cl_2 and (2) **2**, BOP, DiPEA, CH_2Cl_2, 55%[a], 75%[b].

First, commercially available Boc-sidechain protected ornithine was transformed by diazotransfer into the corresponding α-azido compound **2** using imidazolium sulfonyl azide, in a slightly modified procedure.[33] Compound **2** was purified by crystallization as the dicyclohexylamine salt from diethyl ether in excellent yield. Next, commercially available Boc-glycine was coupled to propargylamine using the BOP reagent. The resulting dipeptide **3a** could be purified by crystallization in good yield (82%). Precursor **4a** was assembled from these two building blocks by removal of the Boc protecting group of **3a** followed by BOP coupling with **2** and was obtained in an acceptable yield (55%). Solid compound **4a** could be stored at 4 °C for 2 weeks without any significant auto-polymerization, but prolonged storage or at room temperature led to the formation of a mixture of oligomeric impurities. For smaller scale reactions this stability of compound **4a** was satisfactory; however, it was insufficient for synthesizing larger stocks. Thus, the triisopropylsilyl (TIPS) protected alkyne **4b** was also synthesized. The TIPS protecting group is one of the more stable silyl protecting groups and is tolerant to treatment with trifluoroacetic acid.[34]

Synthesis of TIPS protected azido/alkyne tripeptide **4b** was similar to the synthesis of the unprotected peptide **4a**, but uses TIPS protected propargylamine instead of propargylamine.[35] Since the intermediate **3b** and final product **4b** were oils, purification was carried out using silica chromatography. The resulting protected azide/alkyne tripeptide could be stored for months at room temperature without significant oligomerization or degradation. In view of its increased stability as compared with precursor **4a**, **4b** was chosen as the starting material for the synthesis of glycopeptide monomer **5a**. Glycosylated TIPS-protected monomer **5b** was synthesized by Boc-deprotection of **4b** using TFA, followed by a coupling with the *N*-hydroxysuccinimide ester of the galactosylic acid **6** to obtain compound **5b** in good yield (Scheme 2).



Scheme 2 (a) (1) TFA/CH_2Cl_2 and (2) **6**, DiPEA, CH_2Cl_2 84% and (b) TBAF, phenol, THF, 90%.

Deprotection of the TIPS–alkyne **5b** was first attempted with tetrabutylammonium fluoride trihydrate (TBAF) in THF. Equimolar or slightly higher amounts of TBAF gave sluggish reactions. To prevent deacetylation of the C-glycoside moiety when a larger excess of TBAF was used, acetic acid was added to reduce the basic conditions of the reaction.[36,37] Unfortunately, even when 5 equivalents of TBAF and acetic acid were used, the reaction was still sluggish, so presumably acetic acid was too strong an acid, thereby reducing the nucleophilicity of F^- considerably. To our delight, when instead an equimolar amount of the weaker acid phenol was added to TBAF, complete deprotection to **5a** was observed in a relatively short time (1–3 hours) without any detectable deacetylation of the carbohydrate moiety.

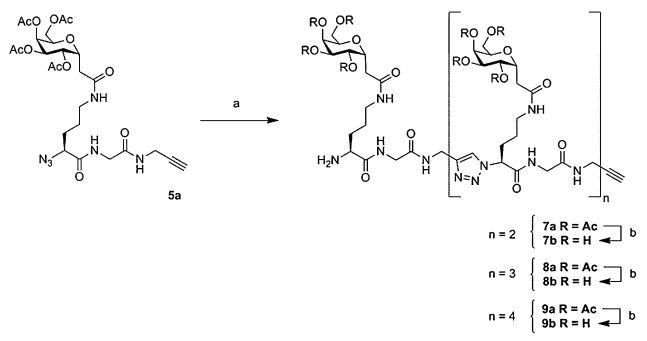
Linear CuAAC oligomerization of the azido–alkyne monomer

The linear oligomerization of monomer **5a** consists of two steps in a one-pot procedure (Scheme 3). In the first step, a trisubstituted phosphine is added to a solution of the azido/alkyne monomer to reduce a fraction of the azide to amine. Next, to the resulting mixture of this amino/alkyne and azido/alkyne, copper(I) is introduced to start polymerization. By performing this reaction at high concentration, intermolecular cycloaddition may outcompete intramolecular cyclization, which would otherwise result in cyclic peptides. Initially triphenylphosphine was chosen as the phosphine to reduce a fraction of the azide of **5a** due to its widespread use in the Staudinger reaction.[38] Although the initial stage of the reduction of the azide to the phosphorus-aza-ylide was rapid, hydrolysis of the ylide to the amine was sluggish (data not shown).

Instead, a more reactive and less sterically hindered phosphine, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was chosen, which has also the advantage that its aza–ylide should be more prone to hydrolysis due to the more polar nature of the phosphorus substituents. Indeed, it was observed that upon addition of TCEP, nitrogen gas evolved immediately, and after 2 hours at room temperature, both the reaction and hydrolysis of the ylide appeared to be complete as was shown by HPLC and ESI-MS analyses.

This reaction mixture was used directly for the CuAAC polymerization: copper(II)sulfate was added followed by the reducing agent sodium ascorbate. It was allowed to polymerize for two hours at room temperature, after which the reaction mixture was diluted and acidified. HPLC analysis showed a distinct distribution of peaks with little of the starting materials present (Fig. 5, top).

As expected, the smaller linear oligomers are more polar and eluted first and the higher molecular weight oligomers eluted later. The degree of polymerization could be directed by adding different amounts of TCEP. Small amounts of mono-acetyl deprotected species were observed eluting slightly before the major peaks. Cyclic peptides however, apart from a small amount cyclic dimer, were not observed as major compounds. Separation of the main oligomer peaks of **5a** (**7a**, **8a** and **9a**) by preparative HPLC proved feasible and yielded these smaller oligomers (three to five tripeptide repeats) in good purity (Fig. 5, bottom). The identity of these compounds was confirmed by MALDI-TOF. Small amounts of impurities eluting slightly



Scheme 3 (a) (1) TCEP (0.25 eq.), DiPEA, DMF/H_2O and (2) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, H_2O/DMF and (b) NaOMe in MeOH.

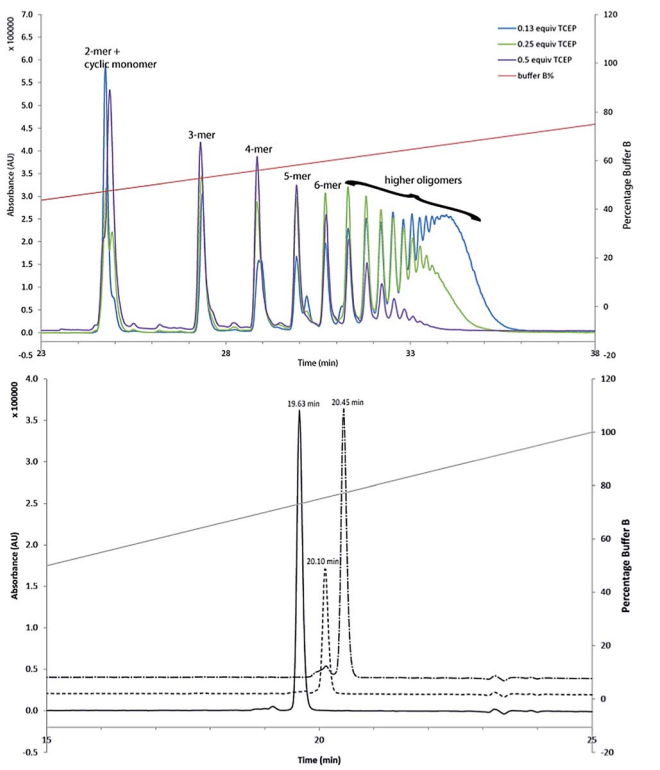


Fig. 5 (Top) HPLC trace of the crude polymeric mixtures of **5a** with three different TCEP amounts, using a 48 min gradient. (Bottom) HPLC traces of the purified 0.25 equiv. TCEP polymerization with **7a** (continuous), **8a** (dotted) and **9a** (semi continuous) on a 20 min gradient, offset on the *Y*-axis.

earlier than the oligomers could be attributed by MS to acetyl hydrolysis during the purification and subsequent lyophilisation. The higher oligomers (>7 tripeptide units) were collected together as a mixed oligomeric fraction.

Removal of the acetyl groups was performed with sodium methoxide in methanol, which caused precipitation of the deprotected oligomers. Water was later added to solubilize the suspension and cation exchange resin was used to neutralize the reaction mixture. After filtration of the resin and lyophilization, the oligomers **7b**, **8b** and **9b** were obtained in sufficient amounts for testing of antifreeze activity.

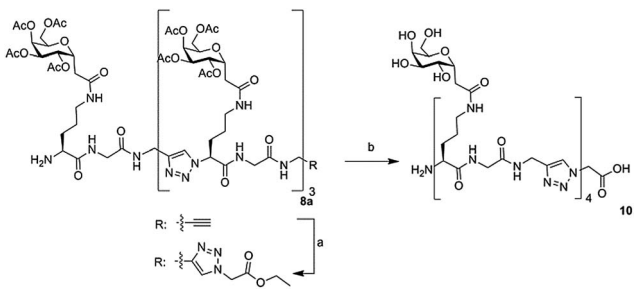
C-terminal variation of the synthetic AFGPs

The synthesized triazole-linked oligomers have a C-terminal alkyne moiety, compared to a C-terminal acid functionality of the natural AFGP-8 and earlier described OGG-Gal **1** (Fig. 1 and 2, respectively). Although oligomers **7b–9b** possess a free N-terminal amine, the C-terminus is an alkyne, whereas both the natural and earlier developed AFGPs are zwitterions by having in addition to the amine also a C-terminal carboxylic acid. Therefore the role of the zwitterionic nature of the AFGP on the structure of these triazole-linked oligomers was also studied. Thus, purified tetramer **8a**, containing a C-terminal alkyne was coupled to ethyl azidoacetate by CuAAC, which after deprotection yielded the structural triazole analogue of OGG-Gal, **10** (Scheme 4).

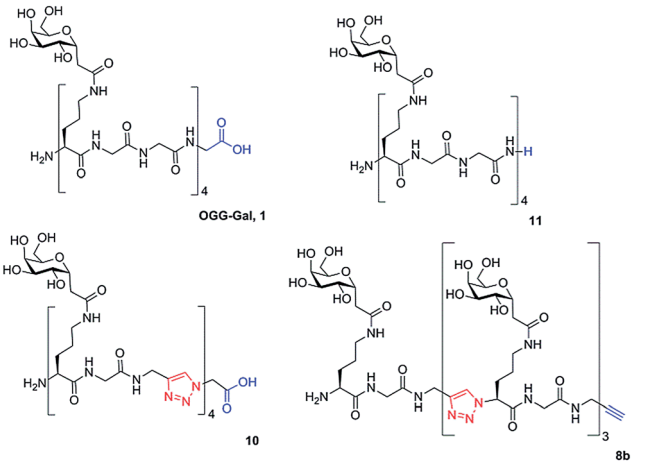
Similarly, a C-terminal variation of OGG-Gal **1** was synthesized wherein the C-terminal glycine with a free carboxylic acid moiety was omitted, and a carboxamide moiety was introduced, leading to **11**, which corresponds to the structure of the triazole alkyne tetramer **8b**. This was accomplished by solid phase synthesis on a Rink-linker instead of the preloaded glycine–Wang resin as was used for the synthesis of OGG-Gal **1**. The resulting four compounds, that is the C-terminal acids **1** and **10** and non-charged C-terminal amides **8b** and **11** of both the peptidic and triazole tetramers of the synthetic AFGP are depicted in Scheme 5.

Evaluation of the antifreeze activity of the oligomers

All glycopeptides were assessed for thermal hysteresis (TH) activity using nanoliter osmometry[39] and ice recrystallization inhibition (IRI) activity using a "splat cooling" assay as was described earlier.[40,41] No TH activity or dynamic ice shaping capabilities were observed for these oligomers, as the water



Scheme 4 (a) Ethyl azidoacetate, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, H_2O/THF and (b) NaOMe in MeOH.



Scheme 5 (Top left) Reference compound OGG-Gal (**1**), (top right) carboxamide terminated version of reference compound OGG-Gal (**11**), (bottom left) triazole linked mimic of reference OGG-Gal (**10**), and (bottom right) tetramer **8b**, which is a triazole linked mimic of compound **11**.

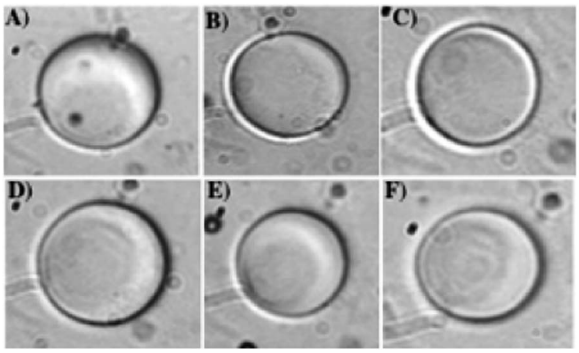


Fig. 6 Ice crystal habit in the presence of (A) triazole tetramer, C-terminal alkyne **8b**; (B) triazole tetramer, C-terminal acid **10**; (C) peptide tetramer, C-terminal carboxamide **11**; (D) triazole trimer, C-terminal alkyne **7b**; (E) triazole pentamer, C-terminal alkyne **9b**; (F) triazole polymeric fraction ($n = 6–17$). All compounds were assayed in water at 10 mg mL^{-1}.

froze in spherical form (center of the images), indicating that similar to the reference compound OGG-Gal **1** these compounds do not interact with the ice lattice (Fig. 6).[15]

The IRI activity of the glycopeptides in Scheme 5 is shown in Fig. 7. The triazole containing oligomer **8b** was only moderately IRI active at 5.5 mM and was substantially less active than the related C-AFGP OGG-Gal **1**, even at a 1000-fold higher concentration. OGG-Gal **1** exhibits equipotent IRI activity to native AFGP-8 at a significantly lower concentration of 5.5 μM. These results indicate that the replacement of the peptide bonds with triazole linkages and loss of the C-terminal negative charge in the polypeptide backbone of OGG-Gal **1** is detrimental and greatly reduces the potent IRI activity of this C-AFGP analogue. From the data presented in Fig. 7, it is also clear that the C-terminus also plays a dominant role in the IRI activity. Compound **11**, which contains a C-terminal carboxamide and is slightly shorter than the C-terminus of compound OGG-Gal **1**, displayed a similar reduction

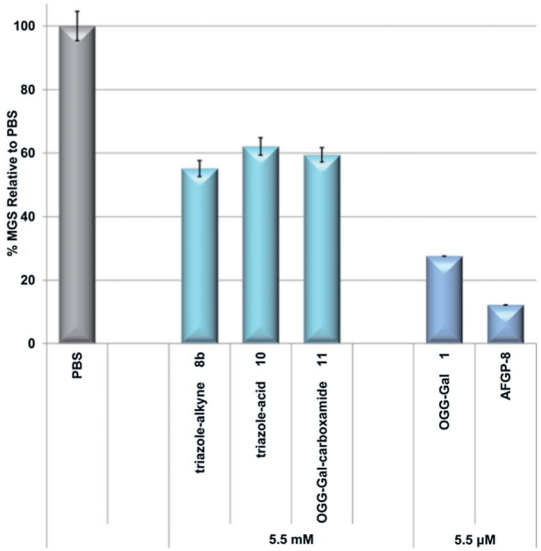


Fig. 7 Ice recrystallization inhibition (IRI) activity of oligomers **8b**, **10**, and **11** at 5.5 mM, and OGG-Gal **1** and AFGP-8 at 5.5 μM in PBS. The % MGS (mean grain size) relative to a PBS positive control is shown for all compounds.

in IRI activity to the triazole with an alkyne terminus **8b**. Therefore we investigated compound **10**, which is a better mimic of reference compound **1**, since it also contains the C-terminal glycine-like moiety. Unfortunately, this modification did not restore the IRI activity back to a similar level to compound **1**, and it still displayed a similar reduced IRI activity to compound **8b**, suggesting that the presence of the triazole rings in AFGP-mimics **8b** and **10** is responsible for the majority of IRI activity loss. The higher oligomeric fraction of **8b** ($n > 6$) yielded no significant increase in IRI activity over the tetramer **8b** (data not shown), suggesting that the limited size alone was not the reason for lower activity of the triazole-containing oligomers.

CD spectroscopy

To investigate whether this loss of IRI activity was caused by a change in the conformation of the backbone of the triazole containing AFGP, CD spectroscopy was performed. In view of the environment in which these peptides are normally active, measurements were performed in water at 4 °C.[11] Interestingly, in spite of the differences in IRI activity, the CD spectra of the four AFGP mimics **8a**, **10**, **11** and **1** were very similar (Fig. 8).

Strikingly, the peptide OGG-Gal **1** and the amidated mimic **11** showed a nearly identical CD spectrum, even though these compounds differed by 3 orders of magnitude in IRI activity. Similarly, the difference in the CD spectrum between the two triazole linked AFGPs **8b** and **10** is small. From this, it can be concluded that the negative charge on the C-terminus does not influence the secondary structure of the peptide to a large degree. This is in contrast to the observed effect of the C-terminus on the IRI activity, especially in the case of the mimics **1** and **11**. On the whole, the conformational effect of the triazole on the structure of the AFGPs seemed limited; nevertheless a shift of the maximum $[\theta]$ was observed from 212 nm in

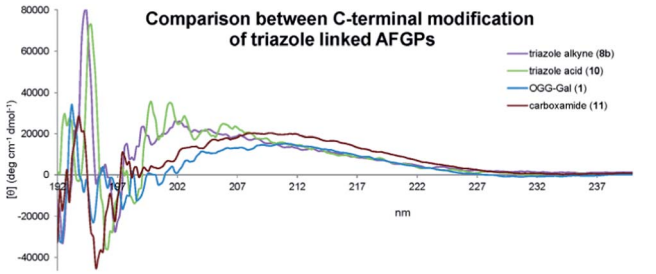


Fig. 8 Far-UV CD spectrogram comparisons: compound **1** (blue) and **11** (red); compound **10** (green) and **8b** (purple).

the case of mimics **1** and **11** to 202 nm in the case of **8b** and **10**. Calculation of the secondary structure element contributions to the CD spectrum was performed using the CDPro package.[9,42] There was no strong consensus among the three software tools, suggesting difficulty in matching the measurements to the datasets. The SELCON3 tool, which gave the most consistent results, showed similar secondary structures for compounds **8b**, **10** and **11**, while the more active compound **1** displayed a slightly less unordered structure and helical contributions and slightly more sheet-, turn- and PP2 structural contributions than the other three compounds as analysed by comparison with two datasets of standard proteins. Although further different conformational organizations of these peptides on the ice interface upon freezing cannot be excluded, a large structural difference when substituting amide bonds by triazole moieties is the loss of a hydrogen bond donor per substituted amide bond. Potentially this loss leads to a different degree of hydration and/or different interactions of the backbone with the carbohydrate part of the antifreeze glycopeptide.

Conclusions

The synthesis of the simplified azide/alkyne glycopeptide building block described here was found to be efficient and simple. The use of the TIPS protecting group allowed long term storage and its removal was very facile.

The oligomerization approach with the *in situ* generation of a small amount of terminating monomer worked conveniently and separation of the individual length oligomers by HPLC was feasible. While such separation might not be necessary or even ultimately desirable for use as antifreeze, for the purpose of this study the focus was a comparison with natural AFGP-8 and earlier made analogues. When a strictly defined length of the oligomers is not required, the deacetylated derivative of **5a** could in principle be used as a monomer for the polymerization, eliminating the need for the more laborious deactylation of the resulting polymers.

Comparison of **8b** with natural AFGP-8, or the previously published OGG-Gal **1**, showed modest IRI activity of the C-terminal alkyne triazole analogue **8b**. Interestingly, the C-terminus plays a dominant role in the IRI activity of C-AFGPs, and removal of the C-terminal acid in the reference compound OGG-Gal **1** caused a 1000 fold reduction in IRI activity, similar to that observed in the alkyne-triazole compound **8b**. The introduction of a C-terminal charge on the triazole analogue **8b** to give structure **10** unfortunately did not

restore the activity. Although the secondary structure of mimics **8b** and **10** resembled the structure of the parent AFGPs **1** and **11** as assayed with CD spectroscopy, small differences in the solution conformation, molecular structure or physical interaction during the organization on the water–ice interfaces can apparently lead to a different IRI activity that is hard to predict based on CD measurements or structural features.[43]

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Notes and references

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