Biomimetic Glycopeptides for Biomineralisation

CH401 Research Project

Caroline Moore

Dr Matthew Gibson
Acknowledgements

Primarily, I would like to thank Dr Matthew Gibson for providing me with an enjoyable and challenging project and for his invaluable support, guidance, enthusiasm and optimism throughout the year. I would also like to thank Robert Deller for teaching me how to undertake the RI measurements, Dr Florence Gayet for her assistance in the laboratory and Daniel Philips for the helpful discussions and his always cheerful company.
Abstract

The mechanism of action and structure-activity relationships of most biological antifreezes, including the antifreeze glycoproteins, are poorly understood. This is due primarily to a lack of native antifreezes for testing which has driven research into developing synthetic analogues of these medically important compounds. The antifreeze glycoproteins are complex structures consisting of (Ala-Ala-Thr)$_n$ peptides with β-D-galactosyl-(1-3)-α-D-N-acetylgalactosamine carbohydrate moieties on each threonine residue, proving to be difficult, time consuming and very low yielding to access synthetically. There is no minimum consensus sequence which is necessary to maintain antifreeze activity, nor are the individual roles of the carbohydrate and peptide understood. This work has used solid phase peptide synthesis to efficiently synthesise a glycine-glycine-allylglycine peptide scaffold, onto which several hydroxylated groups were attached, using highly efficient thiol-ene ‘click’ chemistry. Both optimisation of the process, through the choice of resin and radical initiator, and a purification technique have been discussed.

Four hydroxy functional thiols (thioglycerol, mercaptoethanol, thioglucose and thiogalactose) were added to the glycine-glycine-allylglycine scaffold, to produce a variety of functional peptides for antifreeze activity analysis. Specifically, the ability of each compound to inhibit the recrystallisation of ice was evaluated. Ice recrystallisation is hypothesised to be the key cause in the failure of biological cryoprotectants. Quantitative assessment of recrystallisation inhibition activity was obtained using a modified ‘splat’ assay. The results demonstrated that the inclusion of a carbohydrate onto the peptide promotes recrystallisation inhibition activity, with glucose being slightly more active than galactose, and that the effects are concentration dependant. Thioglycerol-modified peptides showed no activity, indicating the number and stereochemistry of the hydroxyl groups are critical to activity. These promising results, and the synthetic process developed in the work, will greatly aid further investigation into the structure-activity relationships and mode of action of AFGPs and their analogues, which may lead to improved cryoprotection strategies.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AFGP</td>
<td>Antifreeze glycoprotein</td>
</tr>
<tr>
<td>AFP</td>
<td>Antifreeze protein</td>
</tr>
<tr>
<td>AIBN</td>
<td>Azobisisobutyronitrile (here used for 4,4’-Azobis(4-cyanovaleric acid))</td>
</tr>
<tr>
<td>Allylgly</td>
<td>Allylglycine</td>
</tr>
<tr>
<td>Boc</td>
<td>Di-tert-butyl dicarbonate</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>DIPCDI</td>
<td>N,N'-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DPAP</td>
<td>2,2-Dimethoxy-2-phenylacetophenone</td>
</tr>
<tr>
<td>FMOC</td>
<td>Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>HoBt</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>MS</td>
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</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Oxyma</td>
<td>Ethyl cyanoglyoxylate-2-oxime</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>RI</td>
<td>Recrystallisation inhibition</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TH</td>
<td>Thermal hysteresis</td>
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</table>
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1. Introduction

1.1. Native Antifreeze (Glyco)Proteins

Biological antifreezes possess the ability to interact with, modify and inhibit the growth of ice crystals. These compounds are found naturally in the blood of Arctic and Antarctic fish species, allowing them to survive either continuous or sporadic subzero temperatures (Arctic surface temperatures ~ -1.9 ºC), which would otherwise lead to cryo-injury or death. Termed antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs), depending on the nature of the protein, they lower the equilibrium freezing point of the blood in a non-colligative manner, through inhibiting the growth of any ice crystals which may enter the bloodstream. These polar species also have significantly raised solute levels, which in addition to the AF(G)Ps, allow life to flourish in the harsh marine environment.

The structures of the AFPs found in marine species were found to be quite diverse, requiring subdivision into Marine AFP I, II, III and IV categories. Each subcategory has well-defined structural features, as summarised in Table 1. In addition, AFPs have been discovered in insects and plants, although those found in insects show no resemblance to those in plants and marine life. On the other hand, the AFGPs found in species such as the Antarctic notothenioids and northern cods were found to be highly conserved. They consist predominantly of a three amino acid repeat unit (Ala-Ala-Thr)_, with a β-D-galactosyl-(1-3)-α-D-N-acetylgalactosamine complex disaccharide attached to the hydroxyl oxygen of each threonine (Figure 1). Sequence variations are occasionally observed in smaller AFGPs, typically a few (Ala-Ala-Thr) residues are replaced with (Pro-Ala-Thr). The peptide sequence varies between four and 50 amino acids, with the longer chains exhibiting increased antifreeze activity.

Work into understanding the mechanism of action of the AFGPs is still ongoing and not fully understood, whereas the understanding of structure-activity relationships of the AFPs is far more advanced. AFPs tend to adopt a rigid conformation and many crystal structures have been reported, additionally de novo design of new peptides has been achieved. AFGPs, on the other hand, are less well understood and have a more flexible conformation; no crystal structures have yet been reported. There are also a large number of isoforms present in blood plasma leading to difficulties in obtaining sufficient pure
material for experimentation, highlighting the need for facile synthetic routes to structural analogues for both fundamental studies and for application (see below).

<table>
<thead>
<tr>
<th>Marine AFP Type</th>
<th>Species</th>
<th>Structural Features</th>
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<tbody>
<tr>
<td>I</td>
<td>Righteye flounders, sculpins</td>
<td>Single α-helix</td>
</tr>
<tr>
<td>II</td>
<td>Sea raven, rainbow smelt, Atlantic herring</td>
<td>Globular</td>
</tr>
<tr>
<td>III</td>
<td>Eel pouts</td>
<td>Globular with one flattened surface</td>
</tr>
<tr>
<td>IV</td>
<td>Longhorn sculpin</td>
<td>Antiparallel helix bundle</td>
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</table>

Table 1: Overview of marine antifreeze protein characteristics

![Figure 1: Structure of a native AFGP molecule](image)

1.2. Measurement of Antifreeze Properties

Antifreeze activity can be classed under three main categories: thermal hysteresis (TH), dynamic ice shaping (crystal habit modification) and ice recrystallisation inhibition (RI). Thermal hysteresis, also referred to as freezing point depression, is the most commonly referenced characteristic of AF(G)Ps and occurs whenever a solute is added to a solvent. This colligative effect causes a lowering of the equilibrium freezing/ melting point proportional to the concentration of solute. When a AF(G)P is added to a solution, the thermal hysteresis effect observed is higher than would be predicted from only the colligative effect. A freezing point lower than the melting point is observed, implying the effect is kinetic, not thermodynamic. The AF(G)Ps are believed to cause this effect by adsorbing onto specific faces of ice crystals, typically the prism faces, and therefore preventing crystal growth, predominantly along the α-axis. Once the prism faces have been surrounded with AF(G)Ps the growth cannot be continued until the temperature is further decreased to a new hysteresis freezing point. If the temperature further decreases
below this point crystal growth proceeds rapidly, along the c-axis, resulting in needle shaped bipyramidal crystals.\textsuperscript{15} Thermal hysteresis is typically measured by use of a nanolitre osmometer to observe the growth of a single crystal;\textsuperscript{16} it is however important to note that not all ice crystal modifying molecules exhibit this property.\textsuperscript{1}

The second property, dynamic ice crystal shaping or crystal habit modification, describes how the morphology of an ice crystal is affected by an antifreeze active compound. This macroscopic effect occurs as a result of changes to the crystal habit on a molecular level. If the compound exhibits a thermal hysteresis gap, then the morphology is generally determined within this range. It is observed by either monitoring the growth of a single crystal, as for thermal hysteresis, or by formation of pits during ice growth\textsuperscript{14} or hemispherical sketching.\textsuperscript{17}

The final key measurement of activity is recrystallisation inhibition; the ability of an AF(G)P to inhibit the rate of growth of an ice crystal. During ice recrystallisation, larger crystals grow at the expense of smaller ones. This Oswald ripening process minimises the total surface energy but may be slowed or stopped by an AF(G)P.\textsuperscript{2} Recrystallisation inhibition is typically quantitatively\textsuperscript{18} assessed using a ‘splat’ method,\textsuperscript{19} during which the antifreeze active compound in a salt solution is dropped onto a glass slide and allowed to anneal at -6 °C for 30 minutes. The crystals can then be visualised; conservation of the initial crystal size distribution indicates recrystallisation inhibition.

Conformation of AFGPs in the presence of ice is still to be elucidated, however it is generally accepted that they contain a hydrophilic face, with the exposed hydroxyl groups of the sugars, and a hydrophobic face, resulting from the exposed methyl groups of the amino acid backbone and the N-acetyl groups.\textsuperscript{1} The strong binding of the AFGP to ice is proposed to be a result of many hydrogen bonds forming between the sugar hydroxyl groups and the ice lattice.\textsuperscript{19} The difficulties in studying these AF(G)Ps arise from an inability to obtain large quantities of the molecules. Extraction from fish blood is a long and expensive process,\textsuperscript{12} preventing the development of medicinal and industrial applications, and driving research into the synthesis of natural AF(G)Ps and their mimics.

AFGPs exhibiting thermal hysteresis/ice shaping have been shown to cause significant cellular damage when the temperature is below the thermal hysteric gap, due to the
formation of needle-like crystals, and are therefore unsuitable for cryopreservation. Thus it is necessary to produce AFGP mimics which only exhibit the desirable ice recrystallisation inhibition without thermal hysteresis, termed “custom-tailored antifreeze activity” by Ben et al.

### 1.3. Potential Uses of Antifreeze (Glyco)Proteins

The ability of AF(G)P to control and prevent ice crystal growth and recrystallisation makes them desirable for medical applications where low-temperature preservation is required. They could provide cryopreservation, acting to protect cells from the membrane damage usually caused by ice crystal growth and thus revolutionise the transport and storage of transplant organs. Currently three distinct modes of cell death occur when the cell is exposed to temperatures close to or below its freezing point: cell rupture, necrosis and apoptosis. Cell rupture is most the most common issue in cryopreservation and is being targeted by AF(G)Ps and mimics. Additionally, AF(G)Ps have the potential to become more common in the food industry, already being used in ice-cream, their properties could reduce food damage during the freezing, storage and defrosting processes and improve product textures.

### 1.4. Synthesis of Antifreeze GlycoProtein Mimics

There has been significant research into the synthesis of native AFGPs, which are structurally simpler than AFPs, from which two main techniques have been developed: polymerisation of the building blocks in solution and using glycosylated amino-acids in solid phase peptide synthesis. The problems of high cost and low yield exist throughout the work into the synthesis of native and modified AFGPs, driving research into alternatives. Structure-property relationship research by Tachibana et al. has shown that a tripeptide alone can exhibit antifreeze activity and no increase is seen when the chain length is increased to five. Also, converting from the native α-peptide-sugar link to the β alternative, whilst simplifying the synthesis, removed the thermal hysteresis activity. Notably, replacement of the complex native disaccharide with N-acetyl galactose allowed retention of almost all activity. The work is summarised in Table 2; it is important to note that no recrystallisation inhibition measurements were taken. Work by Ben et al. has shown that even when the AFGP structure is dramatically modified and thermal hysteresis activity lost, an excellent level of recrystallisation inhibition activity remains. It is concluded that the spacer length of 0-2 between the backbone and carbohydrate was
essential to allow the carbohydrate to fold back on the peptide, produce a hydrophobic pocket and provide recrystallisation inhibition. Additionally, for recrystallisation inhibition a high degree of carbohydrate solvation is required and charged functional groups are not tolerated. \(^2\)

In addition to the synthesis of simpler native AFGP analogues, antifreeze synthetic polymers could provide the requirements for recrystallisation inhibition activity, however not necessarily for thermal hysteresis. \(^2\) The use of synthetic polymers allows highly tuneable chemical compositions, architectures and molecular weights. It is also possible to produce short peptide sequence hybrid materials using controlled radical polymerisation, \(^3\) yet few examples of functional polymer AF(G)P mimics exist.

![Chemical structure diagram]

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Table 2: Structure-activity relationships for modified AFGP molecules

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1.5. Solid Phase Peptide Synthesis (SPPS)

The use of cross-linked polystyrene resin beads for organic synthesis was developed by Merrifield in 1963<sup>31</sup> and has since been extended to peptide synthesis, Figure 2. The process involves use of insoluble, porous, solid bead resins and allows immobilisation of the reactant; in this case the growing peptide chain. There are significant advantages over solution phase synthesis: (i) For each reaction only addition of reagents, filtering and washing is required and the process can be automated, (ii) Purification steps are eliminated and only resin-washing is needed, (iii) The reaction can be driven forward by use of high concentrations of reagents and (iv) Parallel synthesis are straight forward. In SPPS linkers are used to form the bonds between the resin and peptide chain, holding it securely until cleavage, usually using anhydrous hydrogen fluoride or trifluoroacetic acid. Linkers are chosen to remain stable during both synthesis and cleavage; they should remain on the
resin when the product is isolated. They can be classified into (i) Integral which are formed, at least in part, from the solid core and (ii) Nonintegral or grafted which are separate to the core.\textsuperscript{32} For SPPS either acid labile (e.g. Wang\textsuperscript{33} or RINK\textsuperscript{34}) or super acid-sensitive (e.g. SASRIN\textsuperscript{35}) are typically used.

The addition of peptides onto the chain proceeds from the free $N$-terminus, in the opposite direction to biological peptide synthesis. The amino acids to be coupled require $N$-terminus protection, in the form of Fmoc or Boc groups and must be deprotected, to expose the $\alpha$-amino group and couple with the incoming amino acid. The Fmoc technique uses piperidine or morpholine for deprotection\textsuperscript{36} and Boc removal is achieved with TFA.\textsuperscript{37} SPPS is both reliable and efficient for constructing peptides of up to 40-50 amino acids, however, beyond this length problems of truncated sequences, side products and epimerisation are common.\textsuperscript{38}

![Schematic of solid phase peptide synthesis](image)

**Figure 2: Schematic of solid phase peptide synthesis**
1.6. Click Chemistry

‘Click chemistry’, a technique introduced by Sharpless et al in 2001,\(^3\) is characterised by highly efficient, stereospecific synthetic techniques producing inoffensive biproducts.\(^4\) The most common examples of ‘click chemistry’ involve Huisgen 1,3-dipolar cycloaddition of azides and alkynes\(^5\) and Diels-Alder reactions.\(^6\) Glycopeptides have been synthesised through the reaction of peptides with ‘clickable’ alkyne side chains and sugar azides.\(^7\) The work of Haddleton has demonstrated copper-catalyzed living radical polymerisation and alkyne-azide cycloaddition ‘click’ to produce neoglicopolymers.\(^8\) Despite safety concerns due to their potentially explosive character, there have been many recent advances in azide chemistry, particularly regarding reactions without the need for, potentially toxic, metal ions.\(^9\)

Glycopeptide synthesis could also be achieved using a ‘thiol-click’ radical addition of RSH onto vinyl double bonds.\(^7\) Performed under feasible, mild conditions and proceeding to quantitative, regioselective (anti-Markovnikov) yield within a day,\(^9\) these reactions are highly efficient. Thiol-click reactions are currently used in polymerisation and post-polymerisation modification. Reactions can be carried out by exposure to UV-light in an inert atmosphere, with the advantage of relatively short reaction times.\(^9\)
2. Aims

AFGPs are complex structures consisting of (Ala-Ala-Thr), peptides with β-D-galactosyl-(1-3)-α-D-N-acetylgalactosamine sugar moieties and therefore their mimics are complicated and time consuming to synthesise. This has resulted in a lack of fundamental research into their structural-property relationships and understanding of their function. This work aims to use SPPS to develop an efficient synthesis of alkene containing tripeptides. These tripeptides can function as a scaffold onto which a variety of thiol groups can be added, using highly efficient ‘click’ chemistry. The resulting library of compounds can then be tested for antifreeze (specifically RI) activity and structure-function information can be collected.
3. Results and Discussion

3.1. Wang Resin

Initially a preloaded Fmoc-glycine-Wang resin (1), Figure 3, was chosen to facilitate the solid phase peptide synthesis. This work involved the use of a disposable sample tube fitted with a filter and sealable tap to allow efficient synthesis of short peptides. The resin, too large to pass through the filter, was swelled in the tube. Reagents could then be added, left to react as necessary, and drained and rinsed easily. Cleavage could also occur within the tube. Once cleaved from the resin the peptides could pass through the filter, be precipitated into ether and isolated. Further refinement of the technique involved a nitrogen agitation system.

![Figure 3: Preloaded Fmoc-gly-Wang resin](image)

Wang resin was chosen to synthesise the peptides, using standard solid phase synthesis protocols. DIPCDI (N,N'-diisopropylcarbodiimide) and oxyma (ethyl cyanoglyoxylate-2-oxime) (Figure 4) were chosen as the coupling reagents due to their efficiency and non-explosive nature, unlike HoBt (hydroxybenzotriazole) which is often used for carbodiimide mediated peptide coupling. The mechanism for the coupling is shown in Figure 5. Peptides of glycine-glycine-glycine (5) and glycine-glycine-allylglycine (9) were synthesised through sequential deprotection and coupling steps. The desired peptides were cleaved from the resin using TFA and precipitated into diethyl ether. Additionally tetramers (6) and pentamers (7) of glycine were synthesised.
Results and Discussion

Figure 4: Synthesis of the glycine peptides
Results and Discussion

Figure 5: The carbodiimide mediated coupling of glycine (10) to the resin (2). The carboxylic acid of the amino acid is deprotonated by and then reacts with the DIPCDI to give structure 11. This can then produce the desired peptide (13) directly or via compound 12.

The results of the MS analysis for these compounds remain ambiguous, with no evidence of the expected products. It is possible that dimerisation or cyclisation could be occurring due to the presence of an amine and carboxylic acid at opposite ends of the molecule. Reaction of these two functional groups would lead to elimination of water and formation of an amide bond, Figure 6. The peptide cleavage is achieved using acid, this can therefore promote the acid catalysed amide bond formation reaction. MS experimentation on the peptides proved difficult due to both poor solubility and ionisation. To overcome this problem 1% formic acid was added to the samples to protonate the peptides and increase their ionisation ability. The products were not seen by MS.
Figure 6: Formation of an amide bond occurring intermolecularly to produce a dimer. This is also possible intramolecularly to give a cyclised product

Due to solubility problems with the peptides it was not possible to obtain NMR data and it was therefore not possible to confirm the structures of the products. The decision was made to refine the experimental procedure by changing the type of resin used and thus prevent possible cyclisation or dimerisation reactions. From this point onwards a Rink amide resin was used.

3.2. Rink Amide

Rink amide resins, Figure 7, differ from Wang resins in that cleavage produces a C-terminal amide. Thus the peptide contains one amine and one amide functional group, which cannot react together, avoiding the problems seen with the Wang resin, Figure 8.

Figure 7: Preloaded Fmoc-gly-Rink amide resin
Results and Discussion

Figure 8: Comparison of Wang resin (2) and Rink amide (18) cleavage to produce C-terminal carboxylic acids (10) and C-terminal amides (22)

Tripeptides of glycine-glycine-allylglycine (Figure 9) were synthesised using the Rink amide resin, following the procedure as was used for the Wang resin, Figure 4. This tripeptide scaffold was analysed by MS, with the product clearly seen as an [M+H]$^+$ and [M+Na]$^+$ peak at m/z = 229.2 and 251.1 respectively (Figure 10). Despite the low solubility of the product in common NMR solvents, the structure was confirmed, with the presence of the glycine and allylglycine peaks clearly seen (Figure 11).

Figure 9: Glycine-glycine-allylglycine tripeptide
Figure 10: MS of gly-gly-allygly

Figure 11: $^1$H NMR of gly-gly-allygly. Residual solvent peaks are indicated (DMF and diethyl ether)
3.2.1. Thiol-ene Click Radical Addition with AIBN

With the successful synthesis of the glycine-glycine-allylglycine tripeptide scaffold complete, the chemical modification to produce AFGP analogues could begin. The alkene bond of the allylglycine residue allows thiol functionalisation in a highly efficient manner, using a radical source such as AIBN (azobisisobutyronitrile, 24). As seen in Figure 12, the AIBN splits homolytically, due to thermal decomposition, generating two radical species. These radicals can then abstract a hydrogen atom from a thiol species (25) to generate a thiol radical (26). The thiol radical then adds to the alkene bond in 23; addition occurs at the least hindered end to facilitate formation of the more stable secondary radical species 27. This addition product can then abstract a hydrogen from the thiol (25) producing the thiol radical 26 and the desired product 28. As with all radical chemistry it was critical to exclude oxygen to prevent the oxygen diradical from removing the initiator species.

![Radical thiol addition to the allylglycine residue](image)

Thioglycerol was chosen as a model thiol to test these reactions, Figure 13. The aim of this work was to conduct the radical addition with the peptide still on the resin and hence maintain the advantages of solid phase synthesis, such as (i) Ease of reagent removal through filtering and washing; (ii) Elimination of purification steps; (iii) The reaction can be driven forward by use of high concentrations of reagents; (iv) Parallel syntheses are
straight forward. Following the radical addition, the peptide (33) was cleaved and isolated as before, with the product detected by MS as a [M+H]$^+$ peak at m/z = 337.1 and by NMR, Figure 14. By comparing the two spectra it is clear that the size of the vinyl peaks has decreased and new peaks corresponding to the protons on the thiol R group have appeared.

Figure 13: Thiol radical addition of thioglycerol on the resin
The radical addition was also carried out on a cleaved peptide, Figure 15, to compare how the presence of the resin, linker and Fmoc protecting group affect the extent of thiol addition. The percentage conversion of the alkene to thiol was calculated by analysing the ratio of the unreacted alkene peaks to the thiol peaks, as seen in Figure 14.

**Figure 14:** $^1$H NMR spectra of the gly-gly-allylgly peptide before and after addition of the thiol showing the appearance of the product CH$_2$ (green)

**Figure 15:** Radical thiol addition of thioglycerol off the resin
3.2.2. Thiol-ene Click Radical Addition with DPAP

Due to the low conversions obtained with the thermal-initiator (AIBN, 24) it was decided to switch to a photo-initiator. This has the advantage of not requiring any heating and the reaction has been reported to complete in only two hours in comparison to the seven hours needed for AIBN. The photo-initiator chosen was 2,2-Dimethoxy-2-phenylacetophenone (DPAP, 34), and it was used in 50 mol% compared to thioglycerol. The photo-decomposition of DPAP, Figure 16, generates radical species 35 and 36 which can then initiate the thiol (25) and the mechanism can proceed as seen in Figure 12. After degassing, the reaction was placed under an ultraviolet lamp for two hours, then the peptide cleaved as usual. As with the AIBN addition, the reaction was carried out on and off the resin for comparison and the percentage conversion determined from the NMR spectra, summarised in Table 4. The product (33) was seen by MS as an [M+H]+ peak at m/z = 337.1 and by 1H NMR. The conversion was seen to be higher than for the AIBN mediated addition on the resin, showing this to be a more efficient technique.

<table>
<thead>
<tr>
<th></th>
<th>Conversion from alkene to thioglycerol product / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIBN Radical Addition on the Resin</td>
<td>44.2</td>
</tr>
<tr>
<td>AIBN Radical Addition off the Resin</td>
<td>58.1</td>
</tr>
</tbody>
</table>

Table 3: Comparison of the percentage conversion of the alkene both on and off the resin with AIBN

<table>
<thead>
<tr>
<th></th>
<th>Conversion from alkene to thioglycerol product / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPAP Radical Addition on the Resin</td>
<td>69.2</td>
</tr>
<tr>
<td>DPAP Radical Addition off the Resin</td>
<td>70.2</td>
</tr>
</tbody>
</table>

Table 4: Comparison of the percentage conversion of the alkene both on and off the resin with DPAP
3.2.3. Investigating the Addition of other Thiols

With the addition of thioglycerol successfully achieved both on and off the resin, the addition of other thiols was investigated. Mercaptoethanol was chosen as another readily available thiol and the addition carried out on the resin, as in Figure 13, but with DPAP in place of AIBN. The conversion is seen in Table 5. The product, seen in Figure 17, proved to be very insoluble and was not detected by MS. The structure was confirmed by $^1$H NMR and the percentage conversion was seen to be very similar to that of the thioglycerol product.

![Figure 17: Mercaptoethanol and thiol addition product](image)

<table>
<thead>
<tr>
<th>Conversion from alkene to mercaptoethanol product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPAP Radical Addition on the Resin: 25.4</td>
</tr>
</tbody>
</table>

Table 5: Conversion of the alkene on the resin with DPAP

The overall aim of this work is to obtain structural-property relationships between peptide structure and antifreeze activity, to better understand AFGP function. Therefore, along with thioglycerol and mercaptoethanol, two thio-functional carbohydrates were also chosen to react with the peptide. These were thioglucose, Figure 18, and thiogalactose, Figure 19, which are both used as the sodium salt and thus require protonation prior to the radical addition reaction. Previous work by Tachibana had shown that peptides of alanine exhibit antifreeze activity but only when bonded to a carbohydrate. The potential of galactose to exhibit activity was discussed and it was stated that, ‘the galactose residue may enhance the binding of an AFGP with the ice surface’. Additionally, work by Ben has show the activity of triazole derived galactose-peptides. However, the exact role of the carbohydrate, its stereochemistry and size (di/ tri saccharides) is not known.
Both thioglucose and thiogalactose were successfully reacted with the peptide and the products seen by MS (m/z = 425.2 [M+H]) and $^1$H NMR, Figure 20. Six of the sugar protons are clearly seen as a two multiplets at 3.15-3.56 ppm, whilst the ring proton nearest the sulphur is seen as a doublet at 4.45 ppm. The conversions were calculated and can be seen in Table 6.

![Chemical structures](image1)

**Figure 18:** 1-Thio-beta-D-glucose sodium salt and thiol addition product

![Chemical structures](image2)

**Figure 19:** 1-Thio-beta-D-galactose sodium salt and thiol addition product

<table>
<thead>
<tr>
<th>Conversion from alkene to thio-sugar product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioglucose DPAP Radical Addition on the Resin 65.0</td>
</tr>
<tr>
<td>Thiogalactose DPAP Radical Addition on the Resin 62.0</td>
</tr>
</tbody>
</table>

**Table 6:** Conversion of the alkene on the resin with DPAP
3.2.4. Product Purification

During the process of freeze drying the thio-peptide products, many solubility problems were encountered. Once the majority of the solid had dissolved a small amount of white precipitate still remained, even after heating and sonnicating the solution. The solubility of the products increases with the thiol addition, due to the increase in hydrogen-bondable hydroxyl groups, so the unreacted tripeptide is left as the insoluble product. It was decided that this could be used as a purification technique. The thio-peptide products were dissolved in water, heated gently and sonnicated to ensure the maximum solubility of the desired thio-peptides. Centrifugation allowed removal of only the soluble product, which could then be freeze dried, leaving the insoluble unreacted tripeptide. This process was applied to the thioglycerol (33) and mercaptoethanol (38) products and the NMR spectra, Figure 21, were recorded to show that the conversion had increased (Table 7). Post purification, it can be seen that the integral of the $^1$H allyl peak has decreased from 11.18 to 3.07, whilst the comparison peak has been kept constant, showing a 30 % increase in purity. Following this procedure all peptides were greater than 60 % pure, which was sufficient to function as a screening tool for AFGP properties.
Figure 21: $^1$H NMR of thioglycerol showing how purification increases the conversion, cropped for clarity. Peaks are not normalized; hence integral values should be used.

<table>
<thead>
<tr>
<th>Conversion from alkene to thiol product / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioglycerol product after purification</td>
</tr>
<tr>
<td>Mercaptoethanol product after purification</td>
</tr>
</tbody>
</table>

Table 7: Effect of purification on conversion of the alkene

3.3. Measuring Antifreeze Activity

The antifreeze activity, specifically the recrystallisation inhibition, of the thio-peptides was then investigated using a ‘splat’ method. It was hypothesised that the number of hydroxyl groups could influence the antifreeze activity, by affecting the extent at which they could interact with the ice-water interface. Therefore thioglycerol, with two hydroxyl groups, was tested alongside the carbohydrates which contain four. As already discussed, galactose has shown activity in previous work, but never with this peptide chain or linker, making it an interesting compound for screening. Also, as glucose differs from galactose only in the
stereochemistry at one carbon, it was predicted that it may also exhibit activity. The mercaptoethanol product proved insoluble in PBS so could not be tested.

A modified ‘splat’ assay was used to quantitatively evaluate the peptides’ RI activity. Briefly, a 10 μL droplet was dispensed from a microsyringe, at a height of 150 cm, onto a piece of polished aluminum which sat on solid CO₂ (-76 °C). Upon hitting a cover slip placed on the aluminum, a polycrystalline wafer was formed. The cover slip was quickly transferred to a cold stage set at -8 °C and the size of the ice crystals measured after 30 minutes to assess the degree of crystal growth. Smaller crystals indicate more RI active compounds. This assay is fast, reproducible and allows easy separation of nucleation and growth processes.

The images seen in Figure 22 show the difference in the size of the crystals with the peptides. In the first image, the largest grain size is bigger and the number of larger crystals present is greater. The second image shows the result for the thioglycerol product (33) and the image closely resembles that of PBS, showing little RI activity. In comparison, when the peptide-sugar compounds 40 and 42 are added, the largest grain size is smaller and there are more small crystals present, therefore they are exhibiting RI activity.

![Figure 22: Crystal size after 30 minutes for PBS, 40 mg mL⁻¹ thioglycerol addition product (33), 40 mg mL⁻¹ thioglucose addition product (40) and 40 mg mL⁻¹ thiogalactose addition product (42)](image)

Dilutions were then carried out, as detailed in Table 8, to rule out false positives, as RI activity is known to be concentration dependant. The images (Figure 23, Figure 24 and Figure 25) show these results. With the thioglycerol product, there is little difference between the images, indicating very little activity, as was expected. This could be due to the presence of only few hydroxyl groups for interaction with the water/ ice, but their stereochemistry and spacing may also be important. With the thioglucose peptide the abundance of smaller crystals is decreased when the concentration is halved. The same is
seen for the thiogalactose product and once the concentration is decreased down to 10 mg mL\(^{-1}\) the crystals begin to resemble those seen for the PBS control.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration 1</th>
<th>Concentration 2</th>
<th>Concentration 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioglycerol addition product (33)</td>
<td>40 mg mL(^{-1})</td>
<td>20 mg mL(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Thioglucose addition product (40)</td>
<td>40 mg mL(^{-1})</td>
<td>20 mg mL(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Thiogalactose addition product (42)</td>
<td>40 mg mL(^{-1})</td>
<td>20 mg mL(^{-1})</td>
<td>10 mg mL(^{-1})</td>
</tr>
</tbody>
</table>

Table 8: Compounds to be tested for RI activity

Figure 23: Thioglycerol addition product (33) at 40 mg mL\(^{-1}\) and 20 mg mL\(^{-1}\)

Figure 24: Thioglucose addition product (40) at 40 mg mL\(^{-1}\) and 20 mg mL\(^{-1}\)

Figure 25: Thiogalactose addition product (42) at 40 mg mL\(^{-1}\), 20 mg mL\(^{-1}\) and 10 mg mL\(^{-1}\)
In order to obtain numerical data from the images the mean largest grain size of the crystals, taken from three repeats, was calculated. For comparison, the results for two different molecular weight polymers of poly(ethylene glycol) (PEG) and a sample of poly(vinylalcohol) (PVA) have also been included. PEG is known to exhibit no antifreeze activity and at the other end of the scale PVA is active. The results are summarised in Figure 26, from which it is clear that the carbohydrate-functional peptides exhibit antifreeze properties in a concentration dependant manner, with the greatest activity shown by the thioglucose compound at 40 mg mL$^{-1}$. The results are highly reproducible, shown by the very small error bars.

Comparing the mean largest grain size for each sample to that obtained for the PBS control allows the trend to be more clearly seen. In Figure 27 the data is presented as a percentage of the size of the PBS crystals, which are denoted as a size of 100 %. The most active compound, PVA, has an size of 33 % with the most active of the new thio-peptides exhibiting half this activity with a reading of 66 %, a very promising result. We can conclude that a sugar group on the peptide increases the activity, with glucose showing slightly more activity than galactose.
Figure 27: Mean largest grain size as a percentage of the PBS control

These results have great value as a screening tool for the investigation of structural-activity relationships in AFGP mimics. It should be emphasized that these compounds were not designed to exhibit high antifreeze activity, but rather to form a scaffold to allow the investigation of a great variety of different thiols and peptide chain lengths, to assess their RI properties and begin to understand which structural features are necessary for activity to be seen. It is hoped that this approach will streamline the design-synthesis-testing feedback loop and lead to greater understanding of AFGP activity. For comparison, native AFGPs are typically 50 amino acids long,\(^2\) which is very difficult to achieve on a solid phase, giving low yields, requiring extensive purification and limiting the ability to obtain libraries of materials. This development of a facile synthesis of a tunable peptide backbone with demonstratable activity is therefore a significant new tool.
4. Conclusions

This work has investigated the use of Wang and Rink amide resins for the synthesis of tripeptides by SPPS, leading to the successful synthesis of gly-gly-allylgly on the Rink amide resin. The subsequent functionalisation of the peptides by a thiol-ene ‘click’ reaction has been achieved both on and off the solid support and with a thermal-initiator (AIBN) and photo-initiator (DPAP). In addition, a simple technique for product purification has been achieved. Both simple thiols (mercaptothanol and thioglycerol) and thiosugars (thioglucose and thiogalactose) have been added to produce a variety of thiopeptides for antifreeze activity analysis.

The antifreeze activity of the products, specifically the ice recrystallisation inhibition, was determined through quantitative ‘splat’ assays. RI was observed visually and numerically, through measurement of the mean largest grain size of the ice crystals, with the results compared to a PBS control. It was noted that the inclusion of a sugar on the peptide promotes RI activity, with glucose seen to be slightly more active than galactose, and that the effects are concentration dependant. A simple diol (thioglycerol) showed no activity. These promising results clearly demonstrate that short peptide sequences combined with highly efficient ‘on resin click-chemistry’ are a powerful tool to screen for structure-activity relationships in AFGPs. This will allow for further investigations and ultimately lead to a fundamental understanding, which should translate into viable cryoprotection additives.
5. Further Work

5.1. Generating a Library of Compounds to Investigate Structure-Activity Relationships

Moving on from the gly-gly-allylgly peptides used in this work, there are many opportunities to vary the structure and investigate the effect it has on RI activity:

- More glycine residues could be added to produce longer peptides, still terminating with an allylglycine for ‘click’ chemistry.
- A peptide containing more than one, or entirely, allylglycine could be synthesised. This would allow more than one thiol addition, either the same thiol on each amino acid or a variety could be used.
- A greater selection of thiols, particularly other sugars and dissacharides, could be added.
- The carbon chain length between the thiol and peptide could be increased.
- Peptides of other amino acids could be synthesised, in particular alanine and threonine would be interesting as these constitute the native AFGP structure.
- Evaluation of cryoprotective properties of peptides.

5.2. Thiol-ene Click Initiators

In this work a thermal-initiator (AIBN) and photo-initiator (DPAP) were successfully trialed; if more time was available a greater variety of radical sources could be investigated with the aim of increasing the conversion of the alkene bond to thiol product. Examples include alternative Azo-based initiators or benzoyl peroxide style initiators. Additionally, the reaction conditions could be modified with the same aim.
6. Experimental

6.1. General Experimental

All reagents and solvents were used as received from the supplier.

6.1.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

All NMR spectra were obtained using a Bruker DPX-300 (300 MHz) or DPX-400 (400 MHz) spectrometer using deuterated solvents obtained from Aldrich. Chemical shifts were recorded as δ values in parts per million (ppm) and referenced to the solvent used. In the assignment of the spectra, the following abbreviations are used:

<table>
<thead>
<tr>
<th>s</th>
<th>singlet</th>
</tr>
</thead>
<tbody>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant / Hz</td>
</tr>
</tbody>
</table>

6.1.2. Mass Spectrometry

MS data was obtained using a Bruker Esquire 2000 electrospray instrument. The molecular ion and mass fragments are quoted and assigned.

6.1.3. Ice Recrystallisation Inhibition Measurements

Recrystallisation inhibition measurements were recorded using an Otago Osmometers nanolitre osmometer set at -8.00 ºC and the ice crystals were visualised with an Olympus CX41 microscope fitted with 4x, 10x and 20x zoom lenses. The images were then captured using Canon EOS digital camera and Digital Photo Professional software. The crystal sizes were analysed using ImageJ software with the largest dimension of the largest crystal recorded.
6.2. Synthesis of Peptides on a Wang Resin

6.2.1. Synthesis of Gly-Gly-Gly

Wang-Gly Fmoc Resin (0.2001 g, 0.2 mmol of glycine) was swollen and filtered in DMF (3 x 2 mL). A DMF solution containing 20% v/v 4-methylpiperidine (2 mL) was added and the solution was agitated by nitrogen bubbling (10 min). After this time, the resin was washed with DMF (2 x 2 mL) then a pre-activated (10 min) solution of Fmoc-Glycine (0.178 g, 0.6 mmol), Oxyma (0.133 g, 0.9 mmol) and DIPCDI (0.14 mL, 0.9 mmol) in DMF (2 mL) was added. The solution was agitated by nitrogen bubbling (30 min) then washed with DMF (3 x 2 mL). The removal of the Fmoc group with 4-methylpiperidine and addition of activated glycine solution was repeated. Agitation of the peptide in 95% TFA/water (2 mL, 30 min) was followed by precipitation into diethyl ether (35 mL), resulting in a white solid. The resin was washed with further 95% TFA/water (2 x 2 mL) and the ether solution was then cooled in the fridge (1 hour) and the solid isolated and freeze dried (0.021 g, 0.106 mmol, 52%).

6.2.2. Synthesis of Gly-Gly-Gly-Gly

The synthesis proceeded as for gly-gly-gly (Figure 28) but with a third Fmoc removal and addition of a third pre-activated glycine solution prior to TFA/H₂O addition (0.019 g, 0.078 mmol, 39%).
6.2.3. Synthesis of Gly-Gly-Gly-Gly-Gly

Figure 30: Glycylglycylglycylglycylglycine

The synthesis proceeded as for gly-gly-gly (Figure 28) but with a third Fmoc removal and addition of a third pre-activated glycine solution, followed by a forth Fmoc removal and addition of a forth pre-activated glycine solution, both prior to TFA/H₂O addition (0.020 g, 0.067 mmol, 32%).

6.2.4. Synthesis of Gly-Gly-allylgly

Figure 31: N-(2-aminopent-4-enoyl)glycylglycine

The synthesis proceeded as for gly-gly-gly (Figure 28) but with the omission of the third glycine residue. Instead a pre-activated (10 min) solution of Fmoc-allylglycine (0.202g, 0.6 mmol), Oxyma (0.133g, 0.9 mmol) and DIPCDI (0.14 mL, 0.9 mmol) in DMF (2 mL) was added. The deprotection and cleavage then proceeded as before to isolate gly-gly-allylgly (0.016g, 0.07 mmol, 35%).
6.3. Synthesis of Peptides on a Rink Amide Resin

6.3.1. Synthesis of Rink Amide Gly-Gly-Allylgly

![Figure 32: Fmoc-N-(2-aminopent-4-enoyl)glycylglycinamide Rink Amide](image)

Rink amide-gly Fmoc resin (0.2002 g, 0.2 mmol of glycine) was swollen and filtered in DMF (3 x 2 mL). The addition of a glycine and allylglycine residue proceeded as for the Wang resin gly-gly-allylgly (Figure 31) product. This intermediate product (2.3868 g) was not analysed due to the presence of the resin.

6.3.2. Synthesis of Rink Amide Gly-Gly-Allylgly

![Figure 33: N-(2-aminopent-4-enoyl)glycylglycinamide](image)

Rink Amide-Gly Fmoc resin (0.2003 g, 0.2 mmol of glycine) was swollen and filtered in DMF (3 x 2 mL). The synthesis, deprotection and cleavage then proceeded as for gly-gly-allylgly (Figure 31) yielding product 23 (23.0 mg, 0.100 mmol, 50%).

**1H NMR** (D$_2$O) 300 MHz, δ ppm; 2.55 (2H, m, H$_4$), 3.81 (2H, s, H$_1$), 3.91 (1H, m, H$_{2a}$), 4.15 (1H, m, H$_3$), 4.31 (1H, m, H$_{2b}$), 5.18 (2H, m, H$_6$), 5.62 (1H, m, H$_5$)

**MS** (ESI) m/z = 229.2 [M+H]$^+$, 251.1 [M+Na]$^+$
6.4. Thiol-ene Click Radical Additions

6.4.1. Thioglycerol Addition

Method 1: AIBN off the Resin

To a solution of gly-gly-allylgly (Figure 33) (0.023 g, 0.100 mmol) in DMF (2 mL) was added 4,4′-Azobis(4-cyanovaleric acid) (0.100 mg, 0.357 μmol) and thioglycerol (0.1 mL, 1.156 mmol). The solution was degassed (N₂, 30 min) then heated in an oil bath (70°C, 8 hours). The solution was allowed to cool then precipitated into diethyl ether and the solid isolated and freeze dried (0.011 g, 0.033 mmol, 16 % with respect to Rink amide-gly Fmoc resin starting material).

Method 2: AIBN on the Resin

To a solution of Rink amide-gly-gly-allylgly (Figure 32) (2.387 g) in DMF (4 mL) was added 4,4′-Azobis(4-cyanovaleric acid) (0.800 mg, 1.071 μmol) and thioglycerol (0.1 mL, 1.156 mmol). The solution was degassed (N₂, 30 min) then heated in an oil bath (70°C, 8 hours). Agitation of the resulting peptide in 95% TFA/ water (2 mL, 30 min) was followed by precipitation into diethyl ether (35 mL) resulting in a white solid. The resin was washed with further 95% TFA/ water (2 x 2 mL), the ether solution was cooled in the fridge (1 hr) and the solid isolated and freeze dried (0.027 g, 0.080 mmol, 40 % with respect to Rink amide-gly Fmoc resin starting material).
Method 3: DPAP off the Resin
To a solution of gly-gly-allylgly (Figure 33) (0.022 g, 0.096 mmol) in DMF (2 mL) was added 2,2-Dimethoxy-2-phenylacetophenone (0.7405 mg, 2.89 mmol) and thioglycerol (0.5 mL, 5.78 mmol). The solution was degassed (N₂, 30 min) then placed under a UV lamp (320 nm wavelength, 2 hours). The solution was allowed to cool then precipitated into diethyl ether and the solid isolated and freeze dried (0.013 g, 0.039 mmol, 20% with respect to Rink amide-gly Fmoc resin starting material).

Method 4: DPAP on the Resin
To a solution of Rink amide-gly-gly-allylgly (Figure 32) (2.410 g) in DMF (4 mL) was added 2,2-Dimethoxy-2-phenylacetophenone (0.7405 mg, 2.89 mmol) and thioglycerol (0.5 mL, 5.78 mmol). The solution was degassed (N₂, 30 min) then placed under a UV lamp (320 nm wavelength, 2 hours). Agitation of the resulting peptide in 95% TFA/ water (2 mL, 30 min) was followed by precipitation into diethyl ether (35 mL), resulting in a white solid. The resin was washed with further 95% TFA/ water (2 x 2 mL) and the ether solution was then cooled in the fridge (1 hr) and the solid isolated and freeze dried (0.031 g, 0.09 mmol, 45% with respect to Rink amide-gly Fmoc resin starting material).

**1H NMR** (D₂O) 300 MHz, δppm: 1.60 (2H, m, H⁵), 1.79 (2H, m, H⁶), 2.55 (2H, m, H⁴), 3.35 (8H, m, H¹, H²a, H⁷, H⁸, H⁹), 3.55 (1H, m, H³), 3.85 (1H, m, H²b), 5.18 (2H, m, residual allyl), 5.62 (1H, m, residual allyl)

**MS** (ESI) m/z = 337.1 [M+H]+, 359.1 [M+Na]+

### 6.4.2. DPAP Mercaptoethanol Addition on the Resin

![Figure 35: 5-[(2-hydroxyethyl)thio]norvalylglycylglycinamide](image)
To a solution of Rink amide-gly-gly-allylgly (Figure 32) (2.331 g) in DMF (4 mL) was added 2,2-Dimethoxy-2-phenylacetophenone (0.7405 mg, 2.89 mmol) and thioglycerol (0.45 mL, 6.40 mmol). The solution was degassed (N₂, 30 min) then placed under a UV lamp (320 nm wavelength, 2 hours). Agitation of the resulting peptide in 95% TFA/ water (2 mL, 30 min) was followed by precipitation into diethyl ether (35 mL) resulting in a white solid. The resin was washed with further 95% TFA/ water (2 x 2 mL) and the ether solution was then cooled in the fridge (1 hr) and the solid isolated and freeze dried (0.032 g, 0.104 mmol, 52 % with respect to Rink amide-gly Fmoc resin starting material).

**1H NMR** (D₂O) 300 MHz, δppm; 1.58 (2H, m, H⁵), 1.89 (2H, m, H⁶), 2.55 (2H, m, H⁴), 3.52 (2H, m, H¹), 3.81 (5H, m, H²a, H⁷, H⁸), 3.92 (1H, m, H³), 4.11 (1H, m, H²b), 5.18 (2H, m, residual allyl), 5.62 (1H, m, residual allyl)

### 6.4.3. DPAP Thiogluucose Addition on the Resin

![Figure 36: 5-(b-D-glucopyranosylthio)norvalylglycylglycinamide](image)

Thio-beta-D-glucose sodium salt (0.501 g, 2.30 mmol) was protonated with a drop of concentrated hydrochloric acid and added to a solution of Rink amide-gly-gly-allylgly (Figure 32) (2.294 g) and 2,2-Dimethoxy-2-phenylacetophenone (0.7405 mg, 2.89 mmol) in DMF (4 mL). The reaction then proceed as for the mercaptoethanol addition (Figure 35) to produce the sugar-peptide (0.100 g, 0.024 mmol, 12 % with respect to Rink amide-gly Fmoc resin starting material).

**1H NMR** (D₂O) 300 MHz, δppm; 1.55 (2H, m, H⁵), 1.85 (2H, m, H⁶), 2.55 (2H, m, H⁴), 3.15-3.65 (6H, m, H⁸⁻¹²), 3.81 (2H, s, H¹), 3.91 (1H, m, H²a), 4.05 (1H, m, H³), 4.10 (1H, m, H²b), 4.45 (1H, d, J₇,₈ = 2.1 Hz, H⁷) 5.18 (2H, m, residual allyl), 5.62 (1H, m, residual allyl)
**6.4.4. DPAP Thiogalactose Addition on the Resin**

![Chemical Structure](image)

**Figure 37: 5-[(b-D-galactopyranosylthio)norvalylglycylglycinamide**

Thio-beta-D-galactose sodium salt (0.504 g, 2.31 mmol) was protonated with a drop of concentrated hydrochloric acid and added to a solution of Rink amide-gly-gly-allylgly (Figure 32) (2.281 g) and 2,2-Dimethoxy-2-phenylacetophenone (0.7405 mg, 2.89 mmol) in DMF (4 mL). The reaction then proceed as for the mercaptoethanol addition (Figure 35) to produce the sugar-peptide (0.100 g, 0.130 mmol, 65% with respect to Rink amide-gly Fmoc resin starting material).

**1H NMR** (D₂O) 300 MHz, δppm; 1.58 (2H, m, H⁵), 1.89 (2H, m, H⁶), 3.15-3.65 (6H, m, H⁸-12), 3.81 (2H, s, H¹), 3.91 (1H, m, H²b), 4.05 (1H, m, H³), 4.10 (1H, m, H²b), 4.45 (1H, d, J₇-₈ = 2.1 Hz, H⁷), 5.18 (2H, m, residual allyl), 5.62 (1H, m, residual allyl)

**MS (ESI) m/z = 425.2 [M+H]^+**

**6.5. Product Purification**

**6.5.1. DPAP Thioglycerol Addition Product**

The product from the DPAP thioglycerol addition on the Resin (Figure 34) (0.026 g, 0.077 mmol) was dissolved in water (15 mL) and warmed under sonication (45 °C, 30 minutes). Centrifugation allowed isolation of the solution only, which was freeze dried (0.023 g, 0.068 mmol, 88% with respect to impure thioglycerol addition product).
**1H NMR** (D₂O) 300 MHz, δppm; 1.53 (2H, m, H⁵), 1.79 (2H, m, H⁶), 2.57 (2H, m, H⁴), 3.35 (8H, m, H¹, H²a, H⁷, H⁸, H⁹), 3.51 (1H, m, H³), 3.85 (1H, m, H²b), 5.18 (2H, m, residual allyl), 5.62 (1H, m, residual allyl)

**MS** (ESI) m/z = 337.1 [M+H]^+, 359.1 [M+Na]^+

### 6.5.2. DPAP Mercaptoethanol Addition Product

This process was repeated with the DPAP mercaptoethanol addition product (Figure 35) (0.028 g, 0.091 mmol) to give 0.024 g of pure product (0.078 mmol, 86 % with respect to impure mercaptoethanol addition product).

**1H NMR** (D₂O) 300 MHz, δppm; 1.62 (2H, m, H⁵), 1.88 (2H, m, H⁶), 2.55 (2H, m, H⁴), 3.52 (2H, m, H¹), 3.81 (5H, m, H²a, H⁷, H⁸), 3.92 (1H, m, H³), 4.13 (1H, m, H²b), 5.18 (2H, m, residual allyl), 5.61 (1H, m, residual allyl)

### 6.6. Ice Recrystallisation Inhibition Measurements

The following solutions were made in PBS:

<table>
<thead>
<tr>
<th>Addition Product</th>
<th>Dilution 1</th>
<th>Dilution 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioglycerol addition product (33)</td>
<td>40 mg mL⁻¹</td>
<td>20 mg mL⁻¹</td>
</tr>
<tr>
<td>Thioglucose addition product (40)</td>
<td>40 mg mL⁻¹</td>
<td>20 mg mL⁻¹</td>
</tr>
<tr>
<td>Thiogalactose addition product (42)</td>
<td>40 mg mL⁻¹</td>
<td>20 mg mL⁻¹</td>
</tr>
</tbody>
</table>

**Table 9: Solutions to be tested for RI activity**

A microscope slide was cooled by placing it on a metal sheet beneath which laid solid carbon dioxide. Onto the slide 1 µL of the solution was dropped, from a syringe at a height of 1.5 m, creating the ‘splat’. The slide was then transferred to the microscope, connected to the nanolitre osmometer set to -8.00 ºC. The crystals were photographed immediately and then again after 30 minutes. This process was repeated at least three times for each solution and the images used to obtain the mean largest grain size.
### 7. Supporting Information

#### 7.1. Tabulated RI Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Mean largest grain size/ microns</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td>134.9</td>
<td>6.5</td>
</tr>
<tr>
<td>Thioglycerol addition product</td>
<td>40 mg/ mL</td>
<td>119.9</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>20 mg/ mL</td>
<td>119.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Thioglucose addition product</td>
<td>40 mg/ mL</td>
<td>89.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>20 mg/ mL</td>
<td>102.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Thiogalactose addition product</td>
<td>40 mg/ mL</td>
<td>99.3</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>20 mg/ mL</td>
<td>108.6</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>10 mg/ mL</td>
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<td>5.5</td>
</tr>
<tr>
<td>8 kDa PEG</td>
<td>10 mg/ mL</td>
<td>119(^{(a)})</td>
<td>12.9</td>
</tr>
<tr>
<td>100 kDa PEG</td>
<td>10 mg/ mL</td>
<td>115.5(^{(a)})</td>
<td>10.8</td>
</tr>
<tr>
<td>PVA</td>
<td>10 mg/ mL</td>
<td>44(^{(a)})</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Table 10: Mean largest grain size (a) Data points supplied by R. Deller, University of Warwick, obtained on the same microscope using the same experimental procedure.
8. References


2. Gibson, M. I., Slowing the growth of ice with synthetic macromolecules: beyond antifreeze(glyco) proteins. *Polymer Chemistry* 2010, 1 (8), 1141-1152.


