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Design, Synthesis and Characterisation of Antifreeze Protein Mimetic Polymers

9 Month Progress Report

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Abbreviations

AFGP	Antifreeze glycoprotein
AFP	Antifreeze protein
IRI	Ice recrystallisation inhibition/inhibitory
IR	Ice recrystallisation
PBS	Phosphate buffered saline (0.01M)
PPII	Polyproline (II) helix
Pro-NCA	Proline N-carboxyanhydride
QLL	Quasi Liquid Layer
ROMP	Ring opening metathesis polymerisation
SPLAT	IRI activity assay

Abstract

Antifreeze glycoproteins (AFGPs) have been shown in nature to exhibit remarkable effects on various aquatic species, which as a result of their ice recrystallisation inhibitory (IRI) effect on ice crystal growth, makes organisms such as the Atlantic cod capable of surviving in sub-zero temperatures. Synthetically challenging to prepare and difficult to isolate *ex natura*, synthetic analogues of AFGPs are ultimately required due to their prohibitive expense. As such, we report herein on the design, synthesis and testing of novel IRI active polymers, to better understand their function and translational applications to biomedicine and cryopreservation. In summary, several disparate polymeric candidates featuring facial amphiphilicity have been identified, and synthetic routes devised. Assessment of the eclectic range of macromolecules prepared to date has shown all to have reasonable to excellent IRI activity.

Introduction

Initially discovered in 1971⁰ in the blood serum of notothenioids inhabiting the icy waters of the Antarctic, Antifreeze glycoproteins have three principle effects on aqueous solutions; Non-colligative freezing point depression – a thermal hysteresis phenomenon whereby the freezing point is lowered beyond its equilibrium value whilst the melting point remains unchanged, dynamic ice shaping – which describes the ability of the protein to manipulate the morphology of a growing ice crystal, and ice recrystallisation inhibition (IRI) – whereby the rate of growth of a propagating ice crystal is slowed to the point of inhibition.^{1,2} Collectively, these properties of AFGPs act to prevent the formation and growth of extracellular ice *in vivo*,^{1,2} and have been identified as the agents which allow aquatic species to thrive in sub-zero environments. The ability to apply the fundamentals of this phenomenon to other biological systems is highly desirable, and would in principle have many beneficial applications in biomedicine and cryopreservation.

Blood stocks suffer from a short shelf life, and in conjunction with a shortage of donors, supplies are often stretched in some geographical areas, whilst discarded unused in others. These issues are particularly pronounced when ensuring sufficient blood supplies of rare blood groups, such as O Negative,³ which despite being the universal donor cannot be substituted for in an individual who is themselves O Negative.⁴ Cryopreservation of blood is therefore desirable but ultimately problematic as a result of extracellular ice formation during freezing, which leads to mechanical damage (puncturing) and subsequent lysis as a result of repeat freeze-thaw cycles, whereby larger ice crystals grow at the expense of smaller ones through slow recrystallisation.^{1,2,5,6,7} Equally, rapid thawing as a preventative technique is often difficult to achieve homogeneously. The formation of intracellular ice at very low temperatures (–40°C) is also a factor, particularly during rapid freezing, leading to cell lysis by the freezing and hence expansion of trapped intracellular water.⁸ Expansion related lysis is also noted with extracellular ice due to hypotonic related osmosis during thawing.⁸ AFGPs acting as cryopreservatives would seemingly have a suitable application here, however the prohibitive expense and synthetic difficulty associated with accessing AFGPs, in addition to their significant cytotoxicity outlines the need for biocompatible synthetic mimics.

Contemporary solutions to this problem have so far been unsatisfactory, the use of glycerol and dimethyl sulphoxide (DMSO) as cryopreservatives have their own associated faults and complications. Aside from being toxic (in the case of DMSO), these systems typically work on

the basis of transfusing unsustainably large quantities of the organic solvent, in a complicated multi-step process, which leads to the suppression of ice crystal formation.⁵

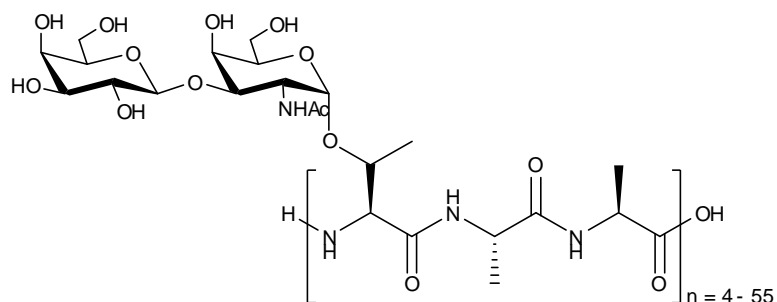


Figure 1 – ‘Native’ AFGP

Due to the lack of an X-Ray crystal structure, the secondary structure of AFGP has not been definitively and unambiguously proven. However, its intrinsic nature as a protein have allowed for solution phase NMR studies which have demonstrated its significant degree of flexibility; although circular dichroism analysis of AFGP has indicated (as depicted by **Figure 2**) that it adopts a polyproline type (II) helical structure (**Figure 3, 5.0 mM**), similar to homopolyproline.

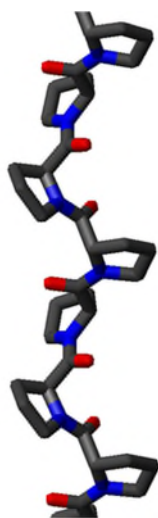


Figure 2 – PPII Helix

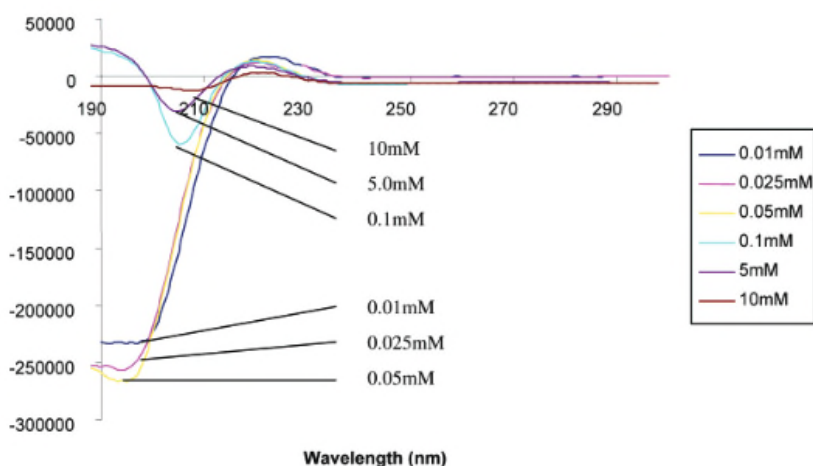


Figure 3 – CD Spectrum of AFGP 8 as function of [AFGP] at 21°C

Furthermore, whilst no definitive mechanism of action has been established for AFGP or any other species which demonstrates IRI activity,¹ various conflicting papers on the necessary criteria for inhibition have been published in recent years. It was previously understood that it was necessary for the polymer/protein to bind to the ice face, however contemporary conjecture suggests that IR inhibitors function by occupying the ‘bulk water’ space between two adjacent

ice crystals – the Quasi Liquid Layer (QLL).^{1,2,5} The effect of which is to disrupt the water ordering and impeding water molecule transfer between the ice crystal surfaces due to energetic unfavourability, thereby preventing growth. In association with this, it is currently hypothesised that the amphipathic PPII helix of AFGP is essential for its activity.

As stated, the background of understanding of the field is varied and conflicting. Knight *et. al.* first reported in 1995 that three synthetic polymers; polyvinyl alcohol (PVA), poly-L-histidine and poly-L-hydroxyproline all showed measureable IRI activity,^{1,2,9} essentially establishing the field and demonstrating that mimics of AFGP maybe practically possible. Cameron *et. al.* later reported that upon testing and comparison of an eclectic range of water soluble polymers containing peptide or vinyl derived backbones, it was clear that only those species which contained side chain located hydroxyl groups demonstrated any significant antifreeze activity, whilst those featuring glyco-functionalities (containing 5 hydroxyls) showed a reduced but nonetheless meaningful IRI effect.¹⁰ This work ultimately concluded that it is likely the distance of the hydroxyl group from the backbone and not the density of the groups that contributes towards the IRI activity. Interestingly, this paper also cites the IRI activities of poly-L-hydroxyproline once again (which according to Ben *et. al.*, is known to adopt a similar secondary structure as native AFGP)² and poly-L-lysine, highlighting the potential for further study. These polymers maybe easily synthesised from *N*-carboxyanhydrides (NCAs) derived from the relevant amino acid, which provides an efficient and economical route for the preparation of large polypeptide chains of high molecular weight, in good to high yield. Furthermore the lack of racemisation or stereochemical ‘scrambling’ is an added benefit, in addition to the simplicity of the reagents (triphosgene and the Boc-protected amino acid) and reaction conditions (often triethylamine in EtOAc at 50°C for 3 hours). However, NCAs are prone to side reactions due to chain transfer, meaning precise control over molecular weight can be difficult to achieve.^{11,12}

PVA has since been recognised as the preminent ‘first generation’ ice recrystallisation inhibitor, able to replicate the effects of AFGP despite having an acutely dissimilar secondary structure, and unrelated chemical and physical properties.^{1,2} Bioincision reports that the polypeptide backbone and carbohydrate functionality previously thought essential for IRI activity is now no longer held true, and that PVA of Mw ~ 80 kDa is just as effective.⁶ Indeed, compared to poly-L-hydroxyproline (which possesses similar activity to PVA), PVA is unstructured in solution, implying that the nature of the secondary structure may not be an absolute requirement for IRI activity.^{1,2}

The Ben group have also reported several diverse AFGP analogues with appreciable IRI activity, including triazole functionalised polymers, and a α -D-galactosyl conjugated to the side chain of ϵ -Lysine in a tripeptide repeat unit of Lys-Gly-Gly.² It is of interest to note that whilst the shorter backbones of only one repeat unit failed to induce a significant IRI effect, the longer chain lengths of 6 or 9 repeat units showed substantial IRI activity. A later variant featuring the same α -D-galactosyl conjugated to four serine repeat units showed IRI activity comparable to native AFGP without the undesirable Thermal Hysteresis or Dynamic Ice Shaping effects.² Ultimately, the Ben group have shown how minor structural adjustments can cause major changes in IRI activity, even when the backbone is considered to be more tolerant to modification. Additionally, Ben *et. al.* has also shown that the extent of hydration and therefore the nature of the sugar residue (galactose being more hydrated than glucose, for example) plays a crucial role in conferring IRI activity.²

More recently, Gibson *et. al.* has however suggested that amphiphilicity may be a common vein in AFGP and its analogues, in that the presentation of an ordered hydrophilic/hydrophobic domain by the polymer/protein may be deemed essential for IRI activity, by disrupting the aforementioned Water/Ice interface.^{1,5} Gibson *et. al.* later reported the simple synthesis of a new and potent IRI active polyampholyte, which may be easily removed post-thaw and provides for up to 60 – 80% red blood cell recovery, whilst decreasing ice recrystallisation by half.^{5,13} The 1:1 charge distribution of this polymer is further supported by previous work by Gibson, which highlights the need for an even charge distribution in polyampholytes for the species to be IRI active.¹⁴

Despite the interest associated with peptide derived polymers, alternative classes of macromolecules should not be ignored and may also yield novel IRI active candidates. It has been noted that many antimicrobial polymers also feature the presentation of a hydrophilic and a hydrophobic domain, which is deemed to be essential for their activity. Specifically, Tew *et. al.* has done momentous work in the field of synthetic mimics of antimicrobial peptides (SMAMPS)¹⁷, which contain both a hydrophilic and a hydrophobic domain are prepared through synthetic organic methods followed by polymerisation, exploiting Ring Opening Metathesis Polymerisation (ROMP) techniques. Primarily, ROMP is a high yielding, economical, and efficient polymerisation method offering excellent control of PDIs, chain lengths, and molecular weight.^{15,16} It has been shown by Tew *et. al.* that it can be used to produce a diverse array of neutral, charged, and amphiphilic polymers derived from amino acids, sugars, and norbornene rings for antimicrobial applications.^{15,17} As a result, the potential

to apply the chemistry of ROMP to the synthesis of new amphiphilic IR Inhibitors is of significant interest. It is clear that should this amphipathic structural requirement also be essential to IRI, that these systems should be further investigated to ascertain whether they have any relevant application.

Aims & Objectives

- To assess the activity of synthetic and commercial poly-L-proline as an ice recrystallisation inhibitor, and to compare to poly-L-lysine, poly-L-glutamic acid, and poly-L-hydroxyproline, which have been previously reported in the literature.
- To develop a multi-step synthetic route to a class of novel amphiphilic polymeric macromolecules (*via* ROMP), of which I am to prepare several variants of differing molecular weights and functionalities, for assessment with regards to their ice recrystallisation inhibitory properties.
- Utilising the above methodology, to also prepare ‘disrupted’ systems with impaired hydrophobicity to ascertain IRI effect and aid assessment of whether amphiphilicity is essential for IRI activity.
- Based on the observation that many amphiphilic antimicrobial polymers are also IRI active, to begin to assess the potential IRI activity of a range of antimicrobial peptides which possess amphiphilic structures, with the intention to apply the same species or polymeric derivatives thereof to IRI applications.

Deviations in Aims & Objectives since Term 1 Report

Whilst the IRI activities of poly-L-proline (both prepared and commercial) were assessed, it was ultimately not necessary to synthesise and SPLAT test poly-L-lysine or derivatives thereof, due to sufficient data pre-existing in the literature. Additionally, synthetic routes towards poly-L-proline were only partially successful, and due to the complexity associated with such a task, were pulled back in the interests of time. As such, no attempts were made to synthesise pure poly-L-hydroxyproline for comparison and determination of its ‘true’ IRI activity. Instead, activity of proline was compared to literature studies of poly-L-hydroxyproline and was determined to be much more active.

Milestones

Term 1 Report:

- **Literature survey:** Complete a survey of previous work in the synthesis of NCAs and ROMP monomers their polymerisations, and the role of macromolecular additives in the inhibition of ice recrystallisation. – **Achieved**
- **Synthesise L-Lys-Boc NCA:** Polymerise and compare *via* SPLAT to poly-L-proline – **Not Achieved**
- **Synthesis of three ROMP monomers:** Prepare at least three amphiphilic Norbornene/Imide–Sugar monomers, each with a varying sugar functional group (glucose, galactose, and N-acetyl-galactosamine initially). Polymerise to assess IRI activity *via* SPLAT *after* completion of the next step (below). – **Achieved**
- **Trial ROMP and Kinetic Studies:** Carry out a test of the ROMP reaction with a similar analogue to the synthesised monomers – the Norbornene/Imide starting material – to ensure reaction feasibility. Further conduct a kinetic study *via* ¹H NMR on Ozric of the ROMP reaction, again using the Norbornene/Imide starting material, to ascertain the order of the reaction. – **Achieved**
- **Optimise ROMP monomer synthesis:** Trial different sugars ‘pedants’. Investigate the use of bromo-sugars (Gabriel synthesis) to yield the product and avoid Mitsunobu reaction (undesirable Ph₃PO production). Investigate post-polymerisation click reactions – e.g. with azido sugars and an alkyne ‘tether’ on the norbornene/imide. – **Achieved**
- **To present and discuss my work in front of a scientific audience:** RSC Midlands Young Researcher Meeting on Nanomaterials 2015, or at one of the weekly Group Meetings. – **Achieved**
- **To first present my work in poster form at a conference:** Warwick 2016 – **Pending**

Results & Discussion

The synthesis and assessment of two different and unique classes of IRI active macromolecules are outlined here, and so are discussed separately.

I. Polyproline Syntheses

The similarity between the supposed PPII secondary structures of AFGP and that of polyproline is a point of interest. As poly(hydroxyl)proline has been noted as a potent IRI inhibitor, likely with a related PPII structure, it is not unreasonable to determine that polyproline its self may actually be IRI active without the presence of hydroxyl groups. If correct, this would facilitate the synthesis of and provide a new, versatile and easy to access IRI active polymer.

Attempts to prepare poly-L-proline for IRI activity assessment were first made through methods involving the synthesis of *N*-carboxyanhydrides (NCA) of proline.

The ‘classical’ synthetic method for the production of amino acid derived *N*-Carboxyanhydrides typically involves a one-pot reaction of triphosgene and the relevant amino acid stirring for ≈ 3 hours in THF, at 50 – 60°C¹⁸. However, unique amongst amino acids, proline features a cyclic secondary amine at its α -carbon, and as such the traditional synthetic route often fails to produce the required Pro-NCA as the intermediate does not spontaneously cyclise. The use of a non-nucleophilic (typically tertiary amine) base is therefore required¹⁹.

Initially, inspiration was taken from work previously conducted by Giralt *et. al.*¹⁹, who pioneered the use of a polymer-bound tertiary amine (*N,N*-diethanolaminomethyl polystyrene), overcoming the traditional problems of triethyl ammonium salt and proline-proline diketopiperazine (Pro-Pro DKP) by-product contamination in the product.

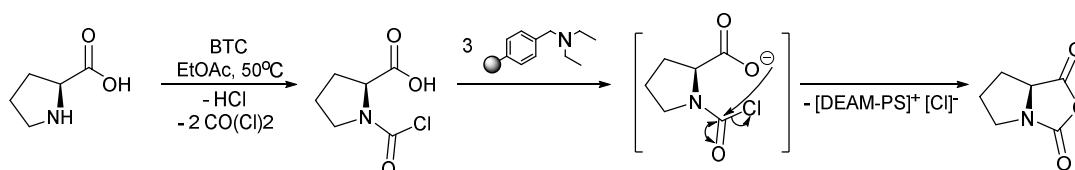


Figure 4 – BG02-01

As depicted in **Figure 4**, preliminary attempts to prepare Pro-NCA through an adaptation of the Giralt method were not fruitful, with ¹³C and ¹H NMR reporting nothing of significance. Ethyl acetate was used as the solvent in place of THF due to its properties as a non-hygroscopic dry medium, but likely failed to swell the DEAM-PS resin to any significant extent. However

primarily, it is likely that the lack of a dry nitrogen atmosphere (seemingly absent in the literature) also contributed to the reaction failure.

As such, further attempts to prepare the Pro-NCA were undertaken with 'dry' THF under nitrogen. Additionally, N-Boc-protected L-proline was substituted for the free amino acid, in order to completely prevent side reactions such as Pro-Pro DKP formation, as per work undertaken by Gkikas *et. al.*²⁰.

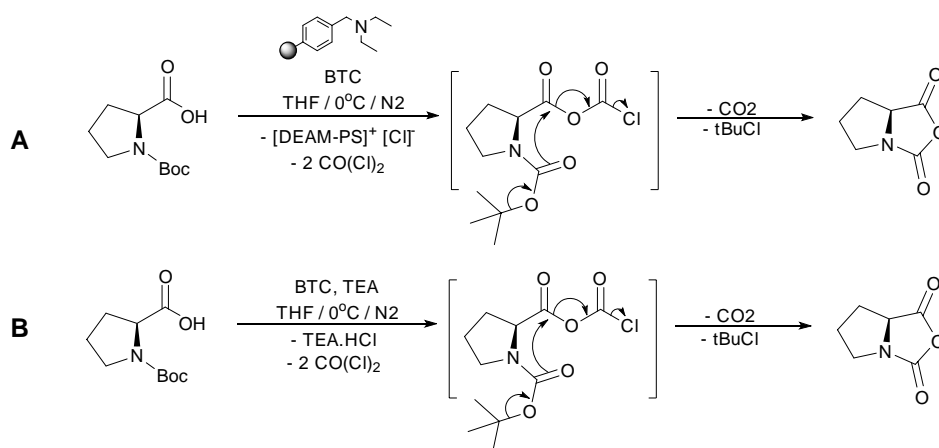


Figure 5 – BG03-01 (A) and BG06-01 (B)

Pro-NCA was successfully obtained in very low yield *via* route **A**, **Figure 5**, and confirmed as present by GC/MS (**Figure 6**), with peaks observed at 70 and 114 m/z – characteristic of Pro-NCA fragmentation products. Likewise, IR signals in the C=O region followed the general morphology of the anticipated NCA peaks. Additionally, before and after IR analysis of the DEAM-PS base demonstrated activity of the resin by the evolution of an N-H Stretch, implying quarterisation as a result of carboxylic acid deprotonation/HCl capture.

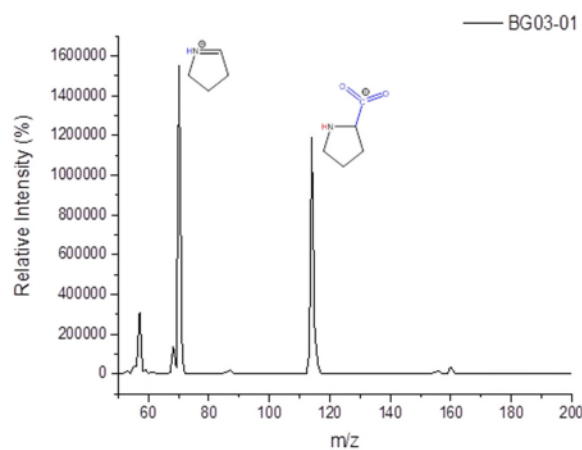


Figure 6 – GC/MS of Pro-NCA

Meanwhile, adaptation and application of the classical synthesis method to Boc-Proline, route **B**, **Figure 5**, showed little to no sign of synthetic success, with uninformative ^{13}C and ^1H NMR spectra. It was therefore clear that optimisation of route **A** would provide the most pragmatic route to Pro-NCA. However, attempts to polymerise the Pro-NCA residue obtained from route **A** in THF with Benzylamine precipitated no polymer product when centrifuged in an excess of diethyl ether, possibly indicating degradation had already occurred.

Unfortunately, despite a number of repeats, it was not possible to isolate the NCA or the polymer – with COOH ^1H NMR signals often observed in the organic crude – suggesting hydrolysis of the anhydride ring. It became evident that continued synthesis attempts on Proline NCA were impractical. In addition to the synthetic difficulty encountered in the process of converting a secondary hindered amine into an NCA, it is likely that the failures encountered with these reactions can generally be attributed to the poor stability of Proline NCA towards heat and principally, water. Being highly hygroscopic, the moisture content of anhydrous THF is likely significant regardless and the dryness of the house nitrogen cannot be guaranteed, ultimately suggesting that such a synthesis may be better suited to a glove box or similar inert environment. Additionally, due to the relatively limited number of examples in the literature, of which many are complex, would also imply that the species is not readily or easily accessible.

As such, attempts were instead made to synthesise ‘polymer like’ polydisperse oligomeric peptides which may enable us to probe polyproline’s IRI activity through a simple condensation polymerisation reaction.

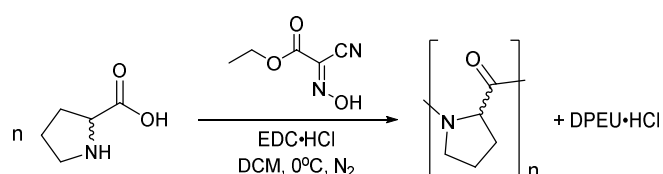


Figure 7 – BG15-01

An array of D, L, and DL-proline oligopeptides were synthesised simply through solution phase peptide synthesis utilising EDCI and OxymaPure™ in DCM under nitrogen. DMF, was avoided beyond the first attempt due to the difficulty in removing DMF at lower temperatures *in vacuo*. Primarily, the risk of inadvertently denaturing the peptide prior to activity testing was a concern. Furthermore, despite aiding the solubility of the reactive species, DMF has also been

noted to slow the rate of DCC/EDCI activation in peptide synthesis, and so maybe considered undesirable.

The crude peptide containing reaction mixtures were subsequently condensed *in vacuo* and dialysed directly for 48 hours using 1000 kDa Mw Cut-off Dialysis Tubing, before being freeze dried and the residual peptides isolated.

Oligopeptide	Mn, ^{SEC} g/mol ⁻¹	PDI
L	1737	2.12
D	2480	1.06
DL	2806	1.25

Table 1 – BG15-01. Mn/PDI Determined by RI on SEC in DMF with PMMA standards.

Of the several batches prepared, reasonable reproducibility was recorded with respect to the molecular weights of the oligomers, with peptides in the 2 – 4k Da range generally obtained. Having now collated a library of peptides, IRI activity “SPLAT” testing could be undertaken. A sample of polymer was dissolved in PBS buffer (pH 7.4) at concentrations of 5, 10, and 20 mg/mL⁻¹ and dropped 1.40 m onto a dry ice cooled glass cover slip. Upon hitting the chilled glass coverslip a thin frozen wafer was formed instantaneously, and was transferred to a cryostage held at –8°C under N₂ for 30 min. Images were taken of the initial wafer and after 30 minutes. Image processing was conducted with the five largest ice crystals in the field of view measured and the single largest length in any axis recorded. This was repeated for three independent wafers (or three wafer areas in event of insufficient material) and the average value calculated from the 15 measurements to give the mean largest grain size (MLGS).

Both the L and D-proline peptides were remarkably IRI active (L proline was particularly potent with a 74% reduction *vs.* PBS at 20 mg/mL⁻¹), whilst the DL was less so – As depicted by **Figure 8**. This observation is not unexpected – as the synthesis of a peptide with a random L/D-Proline composition will distort the secondary structure and disrupt the (amphiphilic) polyproline II helix, which is also observed in the natural AFGP 8 protein and is believed to be related to its IRI activity. Circular dichroism (CD) analysis of the synthesised peptides confirmed the secondary structure of the oligomers. It can be seen from **Figure 9** that the DL peptide is racemic, but with a slightly greater degree of D-character across the sample which is possibly due to a slight excess during preparation. Crucially however, the L-peptide is shown to adopt a very similar structure to the literature sourced²¹ CD spectrum of a polyproline (II)

helix, which in turn is largely identical to the CD of AFGP 8²². The lack of this structure in DL may explain its poorer activity. The synthesised L-proline peptide further exhibits markedly improved activity versus the commercially obtained Poly-L-Proline (1 – 10k Mw), Proline10 (1k Mw), and Proline20 (2k Mw) chains; clearly demonstrating a link with molecular weight, with longer polymer chains displaying greater IRI activity.

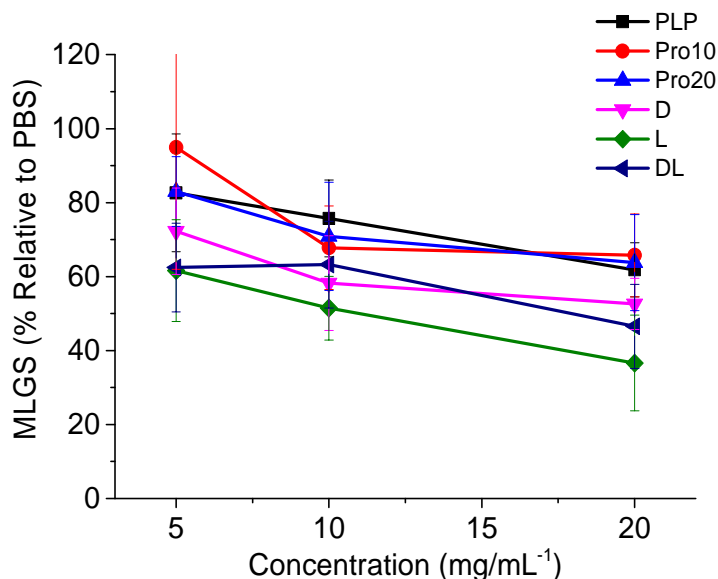


Figure 8 – Comparison of the IRI activities of Commercial Poly-L-Proline (PLP), Bespoke commercial L-Proline peptides of 10 and 20 chain lengths, and the synthesised D, L, and DL-Proline peptides.

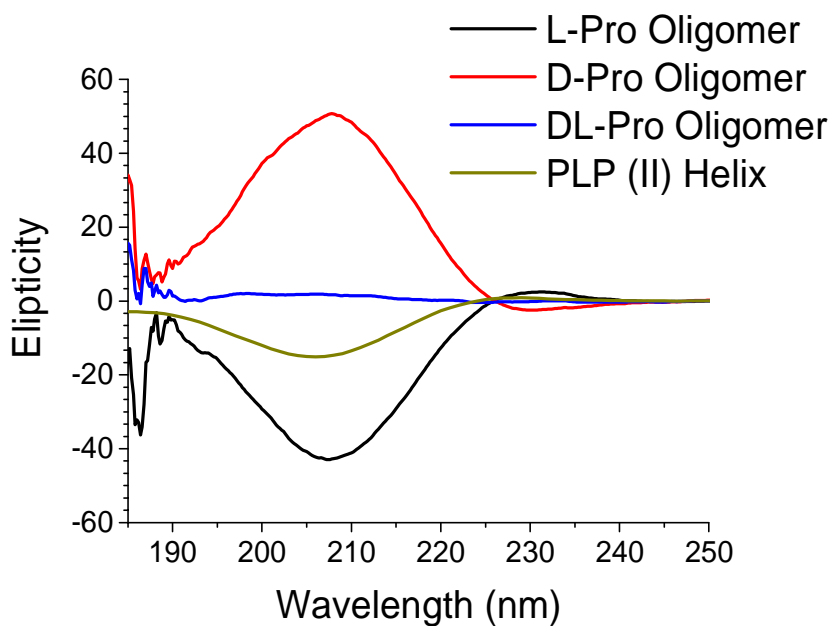


Figure 9 – CD Spectra of BG15-01 D, L, and DL derivatives.

The synthesised L-proline peptides (2 – 4k Da) displayed greater IRI activity at lower concentrations (5, 10, and 20 mg/mL⁻¹) compared to poly(hydroxy)proline (8k Da) at higher concentrations (25 and 40 mg/mL⁻¹) which has been reported previously in the literature ¹⁰ (Figure 10).

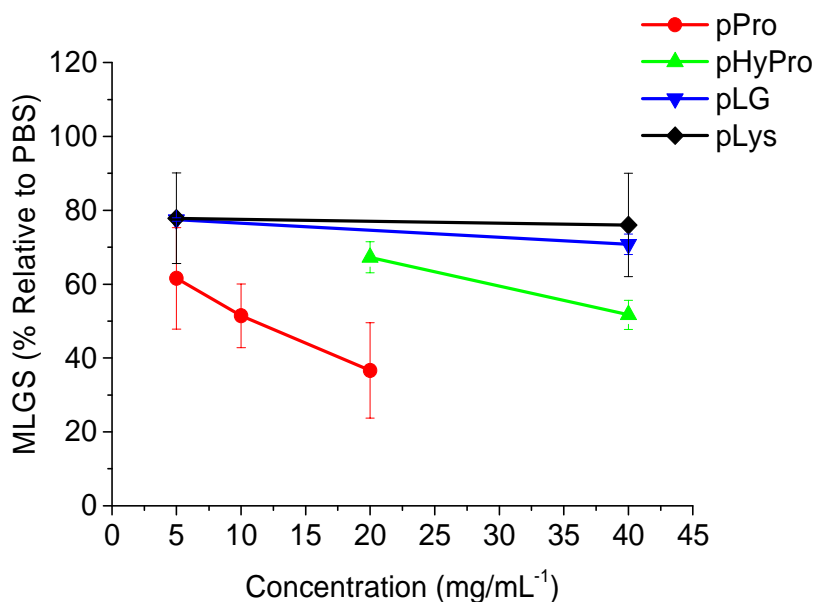


Figure 10 – Comparison of the IRI activities Poly-L-Proline with Poly-L-Hydroxyproline and two negative controls: Poly-L-Lysine and Poly-L-Glutamic acid

This is significant due to the excitement surrounding poly(hydroxy)proline, having long been recognised as a ‘potent’ IR inhibitor. Ultimately, this may suggest that hydroxyproline is not as active as the general consensus asserts. As commercial poly(hydroxy)proline is prepared biosynthetically through a post-translational modification, the abundance of hydroxyl groups is ambiguous, indicating that it may be the residual polyproline abundance that is actually responsible for poly(hydroxy)prolines apparent IRI activity.

II. Amphiphilic ROMP Monomer Syntheses and Polymerisations

Whilst the mechanism of AFGP activity remains fundamentally uncertain, AFPs and AFGPs alike have both been noted for their degrees of facial amphiphilicity;²³ the environmental presentation of distinct hydrophilic and hydrophobic regions as a result of their unique chemical structures. Similarly, the necessity for the presence of amphiphilic domains with regards to activity has also been observed with respect to antimicrobial polymers.

This correlation between the structural properties of the macromolecules and their activity, being seemingly common to both applications, provided opportunity to take inspiration from pre-existing antimicrobial polymers found in the literature¹⁷ (**Figure 11**) and to derive novel derivatives of related structure with potential IRI activity.

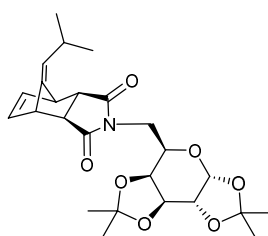


Figure 11 – Antimicrobial M1⁵

The initial synthetic route (as depicted in **Figure 12**) involved the conversion of three free sugars (glucose, galactose, and N-acetylgalactosamine) to their pentaacetates, followed by the selective anomeric deacetylation at C1 with Benzylamine. The free hydroxyl group, was then coupled *via* a Mitsunobu protocol with the nitrogen centre of the polymerisable norbornene imide, previously prepared by a simple Diels-Alder reaction. Deacetylation of the acetate protected hydroxyl groups follows, with subsequent polymerisation.

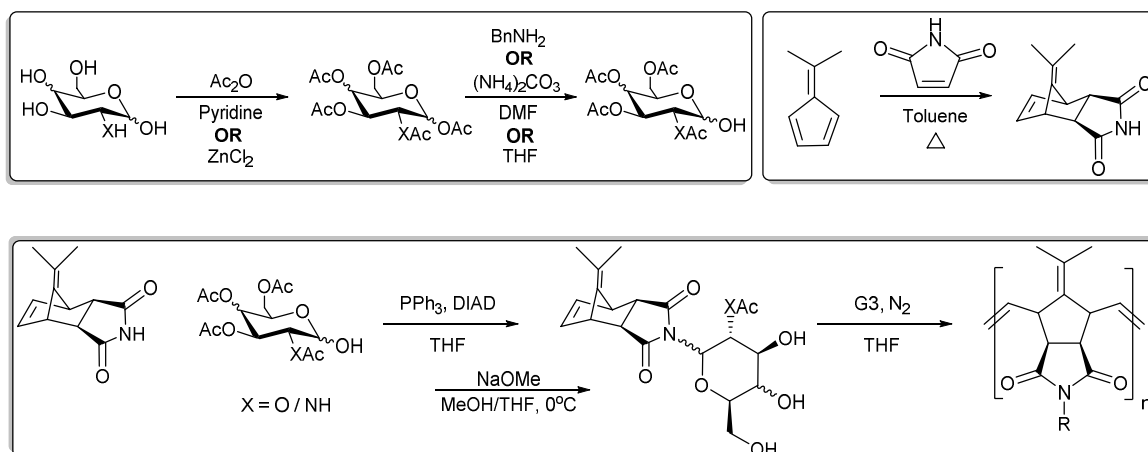


Figure 12 – Top: Sugar Prep & Diels-Alder, Bottom: Coupling of Sugar and Norborneneimide Fragments

The rate of the subsequent homopolymerisation of the product with Grubbs' third generation catalyst was judged to be rapid and non-reversible, with a ^1H NMR kinetics study of the norborneneimide starting material showing an almost instantaneous polymerisation with no ring closing metathesis observed after one hour. SPLAT tests of the polymer product showed good to very good activity at 20 mg/mL (60% reduction in ice crystal size relative to PBS). This ultimately highlights the need for further study, as it may provide the first example of a rationally designed IRI active polymer. However, whilst the coupling was synthetically successful (as confirmed by ESI-LC MS), the practicality of utilising this reaction was problematic due to the 1:1 production of Triphenylphosphine oxide (TPPO) as a by-product.

Multiple attempts at purification including column chromatography were attempted with each of the three sugar derivatives. However, the similar solubilities of TPPO and the highly polar monomer products meant that separation was not forthcoming. Whilst repeated attempts at column chromatography (possibly with exotic solvent systems such as methanol/ammonia/water) may have furnished an improved separation, this was impractical due to the small scale on which the reactions were being carried out, and ultimately would have led to significant loss of product and an impaired yield. Complexation of TPPO with hot ethanolic ZnCl_2 also failed to separate the contaminant. Equally, recrystallisation was not a viable alternative due to the two species possessing similar solubilities in polar organic solvents, and precipitation of TPPO with cold diethyl ether / cold toluene also failed to remove the contaminant. As such, it was determined that an alternative synthetic pathway was required. In addition to this, it became apparent that the anticipated β -monomer product would not be diastereoisomerically pure, resulting in a random and irregular polymer with potentially distorted IRI activity. Principally, it is unlikely that the preceding selective anomeric deacetylation of the sugar residue would exclusively yield the α -anomer, even if in great excess of the β -equivalent²⁴. A subsequent separation of the two diastereoisomers would therefore be required and would likely prove difficult due to their similarity.

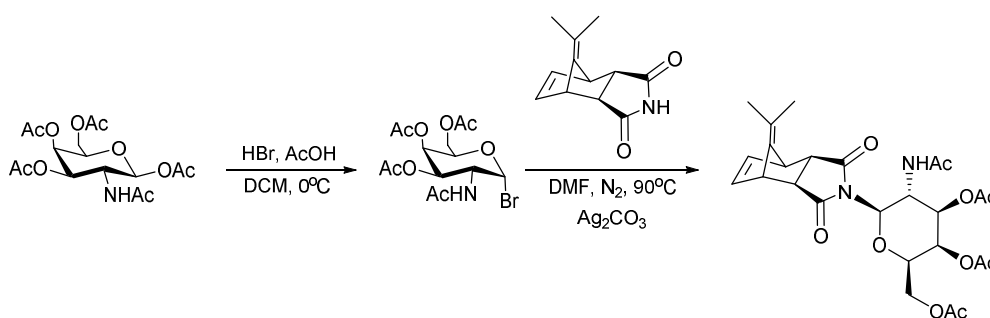


Figure 13 – BromoGalNAc Coupling with *exo*-Fulvonorborneneimide leading to a β -Monomer Product

In order to obtain the desired stereochemistry in addition to the need for an improved yield, bromosugars were utilised, providing for a column free synthetic route – **Figure 13**. Success was confirmed by ^1H NMR, with an off-white crystalline solid obtained. Silver carbonate was identified in a preliminary run as the most suitable catalyst, and was also capable of activating the imide at $\frac{1}{2}$ an equivalent without any signs of orthoester formation. β -stereochemistry of the product was ensured by the presence of neighbouring group participation at C2, preventing the formation of an α -linkage. Iodine (acting as a Lewis acid activator) and potassium carbonate (for imide activation) were also effective at carrying out the coupling reaction, but setup was initially complex and lengthy, requiring separate preparatory techniques. Likewise, the use of no carbohydrate activator was ineffective at instigating a simple $\text{S}_{\text{N}}2$ displacement, with potassium carbonate alone in DMF at 50 and at 90°C both producing no observable product. This indicated that the reaction would only proceed through an intermediary oxonium ion.

In addition to the anticipated shift in the C2 – C6 sugar signals of the ^1H NMR spectra, the disappearance of the anomeric doublet of the bromosugar at 6.61 ppm and a shift back down towards 5.98 ppm suggested a transformation in stereochemistry from alpha to beta. The proximity of the new anomeric doublet is proximal to that observed for β -GalNAc pentaacetate. Additionally, despite obtaining a low yield $\sim 10\%$, likely due to a poor workup, the NMR of the crude product was reassuring due to the complete absence of starting material implying a full conversion of the sugar.

With the synthesis method now optimised, the route can now be applied to the synthesis of the Glucose and Galactose derivatives, and other variations. An oxonorbornene derivative, **Figure 14**, has also been prepared after being identified as an appropriate model which can be used to disrupt the hydrophobicity for further study as to the necessity for amphiphilicity in IRI active systems.

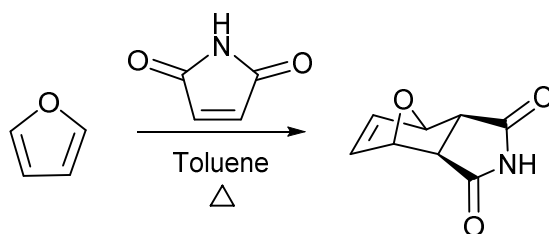


Figure 14 – Synthesis of a hydrophobically disrupted *exo*-Oxonorborneneimide

In summary, good progress has been made in the development of novel IRI active synthetic polymers, providing a suitable grounding for work in the forthcoming years. In addition to the

successful identification of proline as an IRI active species, a feasible synthetic route has been devised for the synthesis of novel amphiphilic monomers, with the potential to produce numerous modified derivatives and analogues.

Future Work and Milestones

- **First Publication:** To prepare the data acquired from the proline experiments for publication in an appropriate journal, selection of which will be dependent on the outcome of the denaturation experiment.
- **Complete the ROMP Monomer/Polymer Set:** To polymerise *via* ROMP the GalNAc-*exo*-fulvonorborneneimide monomer at 1, 25, and 50 kDa targets to observe the weight dependent effect on IRI activity, and also the GalNAc-*exo*-oxonorborneneimide monomer at the same weight fractions to ascertain whether disruption of hydrophobicity has a negative effect on IRI as hypothesised (thereby contributing to the theory that amphiphilicity is essential).
- To prepare the Gal and Glu derivatives of the same two monomers above and polymerise, in order to determine whether the greater degree of carbohydrate hydration will result in less effective IRI inhibitors, as presently thought.
- **Substitution of Carbohydrates for Hydrophilic Analogues:** Investigation of the necessity of the sugar group by the preparation of a library of non-carbohydrate containing ROMP monomer derivatives of the above, with functionalised hydrophilic pedants (e.g. alkyl-SH) substituted for the sugar residues.
- **Substitution of Standard Sugars for Peptides/Exotic Carbohydrates:** To prepare alternative derivatives of the ROMP monomers, with the potential to couple hydrophilic peptides or exotic sugars (possibly based on values for isentropic partial molar compressibilities of carbohydrates) to assess effect on IRI activity.
- **Peptide Screening and Functionalisation:** Peptide functionalisation of polymers, and the screening/synthesis of IRI active peptides.
- **To attend a major international conference:** APME 2017
- **German language module:** To undertake a language model as part of Transferable Skills

Experimental, Materials & Methods

Analytical Methods (NMR, MS, IR, GPC)

^1H (Standard and Kinetic) and ^{13}C NMR Spectra (300 MHz and 100 MHz, respectively) were recorded using a Bruker DPX-300 Spectrometer under standard NMR conditions. Chemical shifts were recorded in ppm and referenced to solvent residual peaks, using ACD Labs NMR Spectroscopy software.

ESI MS experiments were performed on an Agilent 6130B Single QUAD ESI-LC MS spectrometer in either positive or negative mode with an $\text{H}_2\text{O}/\text{MeOH}$ (80:20) eluent feed, with samples dissolved in water, methanol or ethanol, unless otherwise stated. GC MS experiments were performed on a Varian 3800 – 4000 spectrometer in an organic solvent (chloroform unless otherwise stated).

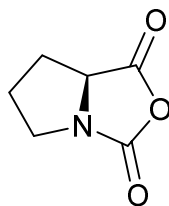
IR experiments were carried out on a Bruker Vector 22 (ATR) FTIR Spectrometer in either the solid or thin film (volatile organic solvent) phase.

SEC/GPC data was acquired on either a THF, DMF, or Aqueous (as applicable) Varian 390-LC MDS system at 30°C containing a PL-AS RT/MT2 autosampler, Shimadzu SPD-M20A microarray detector, a PL-gel 3 μm (50 x 7.5 mm) guard column and 2 x PL-gel 5 μm mixed-D columns (300 x 7.5 mm). Sample filtered and suspended in the relevant HPLC grade solvent (THF containing 2% TEA), with a flow rate of 1 $\text{mL}/\text{min}^{-1}$. Refractive index recorded. SEC data was analysed using Agilent GPC software and calibrated relative to poly(methyl methacrylate) standards (690 – 271400 Da).

SPLAT experimental technique as detailed in Results & Discussion.

Experimental

I. Attempted Syntheses of (S)-tetrahydro-1H,3H-pyrrolo[1,2-c]oxazole-1,3-dione (L-Proline NCA)



BG02-01: L-proline (0.1 g, 100 mg, 0.87 mmol) was suspended in dry EtOAc (50 mL) with stirring, and heated to 50°C. Triphosgene (0.5 eqv, 0.13 g, 0.43 mmol) was added in whole, and the reaction mixture stirred for one hour (until dissolved). The intermediate was subsequently condensed *in vacuo* and the yellow oily residue redissolved in dry EtOAc (60 mL), added to pre-swollen DEAM-PS (3 eqv, 0.82g, 2.61 mmol) in dry EtOAc (20 mL), and stirred at RT for 2 hours. The filtrate was recovered and condensed *in vacuo*, the solid having been washed with further dry EtOAc (20 mL) at the pump. The off-white crude residue obtained was redissolved in dry EtOAc (5 mL) and crystallised from pentane (20 mL), with cooling to -20°C overnight, yielding yellow crystals. Recrystallisation from EtOAc (5 mL) and cold pentane (30 mL) at -20°C yielded an off-white solid. 0.0249g (41%). ¹H NMR (300 MHz, CDCl₃) δ = 4.12 (q, EtOAc), 2.04 (s, EtOAc), 1.61 (s, H₂O), 1.26 (t, EtOAc), 0.88 (m, Unknown). ¹³C NMR (100 MHz, CDCl₃): Uninformative.

BG03-01: L-Boc-Pro-OH (0.1 g, 100 mg, 0.46 mmol) was suspended in dry THF (15 mL) with stirring under nitrogen. DEAM-PS (3 eqv, 0.44g, 1.39 mmol) was added and allowed to equilibrate for 1hr 30 minutes, followed by the slow addition of Triphosgene (0.37 eqv, 51.00 mg, 0.17 mmol) in THF (5 mL), drop wise, at -7°C. The reaction mixture was stirred for a further 30 minutes on ice, and then quickly warmed to 40°C for 5 minutes, with stirring at RT overnight. The mixture was then filtered, washed with dry THF (100 mL), and condensed *in vacuo*, yielding the crude mixture which was subsequently dried under reduced pressure (4 hrs). The crude was then dissolved in EtOAc/Pentane (15 mL, 1:2), chilled at -5°C and then extracted once with ice cold water (50 mL). The organic phase was subsequently dried over MgSO₄, filtered and condensed *in vacuo*, yielding a clear oil featuring an off-white solid. 0.032g (53%). ¹³C NMR (100 MHz, CDCl₃): δ = 27.33 (CH_x). IR (ATR): 2961 cm⁻¹ (-OH); 1744, 1720, 1700, 1650 cm⁻¹ (C=O). GC-MS (360°C): 12.67 min, m/z = 57.1 (309924, Unknown) 70.1 (1551485, -H⁺N=CHCH₂CH₂CH₂-), 114.0 (1188295, -HN-CH(CO₂⁺)-CH₂CH₂CH₂-).

BG04-01: L-proline (0.1 g, 100 mg, 0.87 mmol) was suspended in dry THF (20 mL) under nitrogen with stirring, and heated to 50°C. Triphosgene (0.5 eqv, 0.13 g, 0.43 mmol) was added in whole, resulting in a colour change from white opaque to transparent. The reaction mixture was stirred for one further hour, with the intermediate subsequently condensed under reduced pressure, and the flask evacuated and purged with nitrogen (x5) before the addition of DEAM-PS (3 eqv, 0.82g, 2.61 mmol) in dry THF (20 mL), also under nitrogen. After stirring overnight, the mixture was then filtered, washed with dry THF (10 mL), and condensed *in vacuo*.

Crystallisation from dry EtOAc (5 mL) and pentane (20 mL) followed, with cooling to -20°C for 30 minutes, yielding off-white crystals. 0.039g (65%). ^1H NMR (300 MHz, CDCl_3) δ = 4.79 - 4.43 (m, Unknown), 4.12 (q, EtOAc), 3.86 - 3.45 (m, Unknown), 2.36 - 1.68 (m, Unknown), 2.05 (s, EtOAc), 1.43 (s, H_2O), 1.30 (d, Pentane), 1.25 (m, Pentane/EtOAc), 0.89 (t, Pentane). ^{13}C NMR (100 MHz, CDCl_3): Uninformative. IR (ATR): 2940 (CH), 1746, 1640 ($\text{C}=\text{O}$), 1446 (CH), 1190 cm^{-1} (CO). GC-MS (360°C): 16.82 min, m/z = 149.2 (1821986, Unknown). m/z (ESI): Uninformative.

BG06-01: Boc-L-proline (0.5 g, 2.32 mmol) was dissolved in dry THF (20 mL) under nitrogen. Triphosgene (0.37 equiv, 0.25 g, 0.65 mmol) was added under vigorous stirring and after 10 minutes, triethylamine (1.1 equiv, 0.35 mL, 2.55 mmol) added dropwise over 15 minutes at -15°C , and the reaction mixture stirred for 1.5 hrs at RT under nitrogen. The reaction mixture was then cooled to 0°C to allow for complete precipitation of the triethylammonium salt, and was removed by filtration. The filtrate was subsequently condensed *in vacuo*, and dried under reduced pressure. The crude was dissolved in dry ethyl acetate (10 mL), chilled, and then extracted once with ice-cold water (30 mL). The organic phase was dried over MgSO_4 , filtered, and condensed *in vacuo*. The oily residue was subsequently chilled to -20°C overnight under nitrogen, yielding white crystals. 0.301g (Quant%). ^1H NMR (300 MHz, CDCl_3) δ = 6.98 (s, Unknown), 4.36 - 4.25 (m, Unknown), 4.12 (q, EtOAc), 3.56 - 3.33 (m, Unknown), 2.39 - 1.85 (m, Unknown), 2.04 (s, EtOAc), 1.48 (d, Unknown), 1.25 (t, EtOAc). ^{13}C NMR (CDCl_3): δ = 28.53 ($\underline{\text{C}}\text{H}_x$). IR (ATR): 2967, 2985, 2716 (CH), 1828, 1779, 1736, 1701, 1632 ($\text{C}=\text{O}$), 1478, 1423 (CH), $1257, 1211\text{ cm}^{-1}$ (CO). GC-MS (360°C): Uninformative. m/z (ESI): Uninformative.

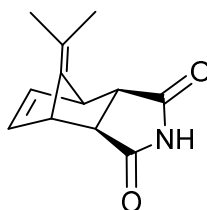
II. Attempted Syntheses of Poly-L-Proline

BG07-01: The crude material from BG06-01 (0.30 g) was dissolved in dry THF (5 mL), under nitrogen with benzylamine (0.1 mL), and stirred vigorously for 24 hours. A large excess of diethyl ether (50 mL) was added and the mixture centrifuged (10k RPM 10 minutes). No polymeric material precipitated.

BG12-01: L-proline (0.1008 g, 0.87 mmol) was dissolved in dry DMAc (50 mL) with Iodine (1 eqv, 0.2201 g, 0.87 mmol), and the reaction stirred at 70°C for 24 hours in the presence of 4\AA Molecular sieves. The dark purple solution was subsequently cooled to RT, filtered, and washed with saturated sodium thiosulphate solution (100 mL), and extracted with dichloromethane (100 mL). The aqueous phase was washed twice with dichloromethane (2 x

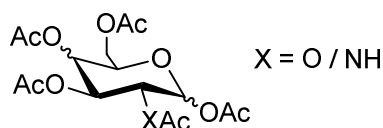
100 mL), and the combined organic and aqueous phases condensed *in vacuo*. A large excess of diethyl ether (50 mL) was added to both residues and the mixtures centrifuged (10k RPM 10 minutes). No polymeric material precipitated.

III. General Procedures for the Synthesis of (3aR,4S,7aS)-8-(propan-2-ylidene)-3a,4,7,7a-tetrahydro-1H-4,7-methanoisindole-1,3(2H)-dione (*exo*-fulvonorborneneimide)



BG08-01: 6,6-dimethylfulvene (1.2 mL, 1.06 g, 10 mmol) and maleimide (0.97 g, 10 mmol, 1 eqv) were dissolved in toluene (50 mL), and the reaction mixture stirred and refluxed at 135°C for at least 24 hours, transitioning from a translucent orange solution to opaque after 60 minutes. Reaction mixture then cooled to RT and condensed *in vacuo* to remove excess toluene and fulvene, and the solids recrystallised from hot diethyl ether to yield the pure *exo* product as an orange solid. 0.2560 g (13%), 0.6368 (31%), 2.8154 (69% x 2 Scale). ¹H NMR (300 MHz, CDCl₃) δ = 7.61 (1H, s, NH), 6.42 (2H, t, $J_1 = J_2 = 1.96$ Hz, HC=CH), 3.74 (2H, t, $J_2 = J_3 = 1.88$ Hz, Bridge 2 x CH), 2.78 (2H, s, Ring 2 x CH), 1.57 (7H, s, 2 x Me). ¹³C NMR (100 MHz, CDCl₃) δ = 206.96 (2 x HNRC=O), 137.67 (HC=CH or R₂-C=C-(Me)₂), 49.20 (C-C=O), 45.61 (C-HC=CH-C), 30.93 (2 x Me). IR (ATR): 3229 (NH), 3000 (C=C / C-H), 1759, 1705 (2 x C=O), 1369, 1345, 1182 (C-H), 689 cm⁻¹ (C=C). m/z (ESI) 202.1 (95%, R₂N⁻), 226.1 (80%, M+Na⁺). GC-MS (360°C): Uninformative.

IV. General Procedures for the Syntheses of Glucose, Galactose, and N-Acetylgalactosamine Peraacetates



BG10-01, S1 (α-D-Glucose): Zinc chloride (0.960g, 7.04 mmol) was added quickly to acetic anhydride (20 mL) and the mixture heated to and stirred at 85°C for five minutes, resulting in a dark brown solution. D-Glucose (4.000g, 22.2 mmol) was added slowly with stirring and the mixture was heated for 1 further hour. The resulting thick black oil which was poured into

chilled water (200 mL) and stirred vigorously to give a dark green solution and an off-white precipitate. The solid was recovered at the pump, washed with ice cold water (40 mL), and recrystallised from hot ethanol (20 mL). White crystals were isolated, washed with cold ethanol (10 mL) and dried *in vacuo*. 4.06 g (46.8%). ¹H NMR (300 MHz, CDCl₃) δ = 6.32 (1H, d, *J*_{1,2} = 3.7 Hz, Anomeric), 5.47 (1H, t, *J*_{3,2} = *J*_{3,4} = 9.9 Hz, H-3), 5.19 – 5.05 (2H, m, H-2,4), 4.26 (1H, dd, *J*_{6,6'} = 12.5, *J*_{6,5} = 4.1 Hz, H-6), 4.10 (2H, m, H-5,6'), 3.70 (q, EtOH), 3.48 (s, EtOH) 2.18 (3H, s, OAc), 2.09 (3H, s, OAc), 2.05 – 1.99 (9H, s, 3 x OAc), 1.24 (t, EtOH). *m/z* (ESI) 413.0 (95%, M+Na⁺).

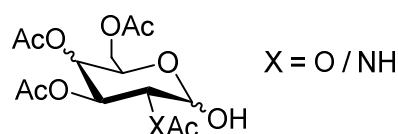
BG10-01, S1 (α-D-Galactose): Zinc chloride (0.998g, 7.32 mmol) was added quickly to acetic anhydride (20 mL) and the mixture stirred and heated to 85°C for five minutes, resulting in a dark brown solution. D-Galactose (1.005g, 5.55 mmol) was added slowly with stirring and the mixture heated for a further 2 hours, resulting in a dark brown solution, and was poured into chilled water (200 mL). No precipitation was observed with vigorous perturbation. Mixture was condensed *in vacuo* and chilled to 5°C overnight, no crystallisation was observed, and so the crude residue was extracted with dichloromethane (100 mL) and chilled water (100 mL). The aqueous phase was washed twice with dichloromethane (2 x 50 mL), and the combined organic phases washed once with chilled water (50 mL), dried over MgSO₄, filtered, and condensed *in vacuo*, yielding an orange/brown oil. *m/z* (ESI) 413.12 (100%, M+Na⁺).

BG10-01, S1 (α-N-Acetyl-D-galactosamine): Zinc chloride (0.2514g, 1.84 mmol) was added quickly to acetic anhydride (10 mL) and the mixture stirred and heated to 85°C for five minutes, resulting in a dark brown solution. N-Acetyl-D-Galactosamine (0.9996g, 4.52 mmol) was added slowly with stirring and the mixture heated for a further 2 hours. The resulting dark brown solution was poured into a mixture of crushed ice and dichloromethane (50 mL), vigorously shaken, and the organic phase extracted with saturated sodium hydrogen carbonate solution (50 mL), and the aqueous phase washed twice with dichloromethane (2 x 50 mL). The combined organic phases were then washed once with saturated sodium hydrogen carbonate solution (50 mL), dried over Na₂SO₄, filtered, yielding a translucent orange solution. The sugar was then precipitated from the crude mix with a large excess of methanol, isolated and washed with cold methanol (10 mL), yielding colourless white crystals. 1g (46%), 0.58g (16.5%), 0.62g (8.8%). *m/z* (ESI) 412.10 (100%, M+Na⁺).

BG10-01, S1 (α-N-Acetyl-D-galactosamine): To a solution of N-Acetyl-D-Galactosamine (2.0003g, 9.04 mmol) in pyridine (10 mL) on ice, acetic anhydride (10 mL) was added slowly with stirring, and allowed to warm to RT overnight. The resulting solution was cooled on ice

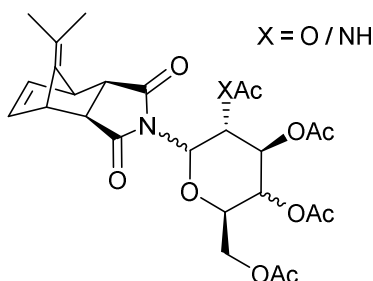
to -20°C and chilled water (50 mL) added slowly, resulting in the precipitation of a cream solid. The solution-suspension was aged on ice for a further hour before being filtered and washed with cold water (20 mL) and dried *in vacuo*, yielding a white solid. 3.21g (89%), 3.67g (Quant%), 2.42g (69%). $^1\text{H NMR}$ (300 MHz, CDCl_3) $\delta = 5.69$ (1H, d, $J_{1,2} = 8.7$ Hz, Anomeric), 5.45 – 5.25 (2H, m, NH , H-4), 5.08 (1H, d, $J_{3,2} = J_{3,4} = 11.3$ Hz, H-3), 4.45 (1H, dd, $J_{2,3} = 20.1$, $J_{2,1} = 10.4$ Hz, H-2), 4.29 – 3.93 (3H, m, H-5, $\text{H}6''$), 2.25 – 1.87 (15H, m, 1 x NHAc , 5 x Ac). m/z (ESI) 412.0 (35%, $\text{M}+\text{Na}^+$), 330.1 (100%, $\text{M}-(\text{OAc})_2+\text{CH}+\text{H}^+$).

V. General Procedure for the Syntheses of Glucose, Galactose, and N-Acetylgalactosamine Tetraacetates



BG10-01, S2: Glucose pentaacetate (1.005g) was dissolved in DMF (20 mL) with ammonium carbonate (0.5060g) or benzylamine (2 mL) and stirred at 30°C for 48 hours. Chloroform (30 mL) was added and the mixture poured into ice cold HCl (1M, 20 mL). The organic phase extracted and the aqueous washed with CHCl_3 (3 x 20 mL). The combined organic phases were washed with water (15 mL) and saturated NaHCO_3 solution (15 mL), dried over Na_2SO_4 and condensed *in vacuo* with an excess of n-Butanol, resulting in a yellow/brown gum. $^1\text{H NMR}$ (300 MHz, CDCl_3) $\delta = 5.51$ (2H, dt, $J = 7.2, 6.7$ Hz), 5.26 (1H, t, $J = 9.5$ Hz), 5.00 – 4.65 (2H, m), 4.37 – 4.06 (3H, m, H-5, $6''$), 2.13 – 1.99 (12H, m, 4 x OAc). m/z (ESI) 371.0 (100%, $\text{M}+\text{Na}^+$).

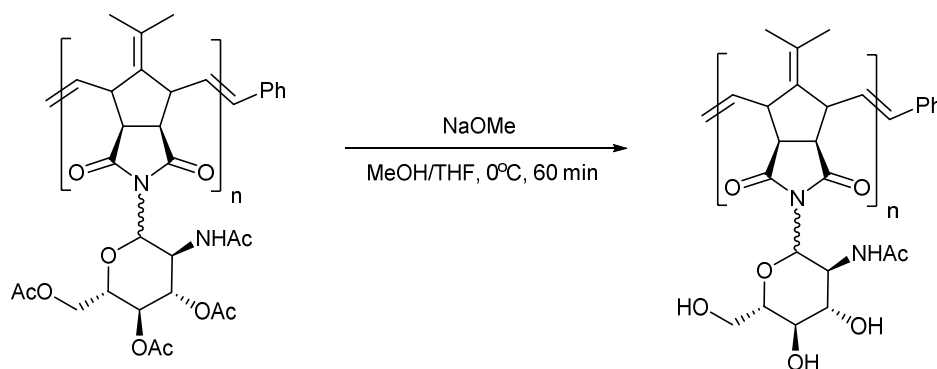
VI. General Procedures for the Attempted Syntheses of Glu/Gal/GalNAc *exo*-fulvonornborneneimide Monomers (*via* Mitsunobu Synthesis)



BG11-01: The crude mono-deacetylated Glucose/Galactose/GalNAc derivative, BG10-01 S2, (0.64g/0.44g/0.31g) and BG08-01 (0.12g, 0.63 mmol, 0.5 eqv) were dissolved in dry THF (50 mL) under N_2 with triphenyl phosphine (0.1696g, 0.63 mmol, 0.5 eqv) and cooled to zero degrees. DIAD (0.13 mL, 0.13g, 0.63 mmol, 1 eqv) was added drop wise to the reaction mixture

with stirring, and the reaction mixture kept at 0°C for five minutes, before being warmed to RT and stirred for 48 hours. The crude orange solution obtained was condensed *in vacuo* to give a dark brown syrup, and was recrystallised from hot toluene to precipitate the excess TPPO, BG08-01 and tetraacetate, and the filtrate again condensed. The residue was recrystallised from warm diethyl ether to afford an off-white crystalline product (20 mg), which was isolated and washed with cold ether (100 mL), and the remaining solution condensed down to a yellow gum. Gum subjected to column chromatography (Hexane/Ethyl acetate, 1:2), yielding a white crystalline solid. Galactose: ¹H NMR (300 MHz, CDCl₃) δ = 7.72 - 7.44 (m, TPPO), 6.41 (1H, t, *J* 1.9, HC=CH), 6.32 (s, Unknown), 5.59 – 4.83 (8H, m, Sugar), 4.47 (1H, t, *J* 6.6, Sugar), 4.27 – 3.89 (4H, m, Sugar), 3.74 (1H, t, *J* 1.9, Bridge 2 x CH), 3.48 (q, Diethyl ether), 2.77 (1H, s, Ring 2 x CH), 2.21 – 1.93 (17 H, m), 1.56 (s, H₂O), 1.43 (t, Et₂O), 1.29 - 1.20 (m, Unknown). ¹³C NMR (100 MHz, CDCl₃) δ = 132.18, 128.60, 21.96. *m/z* (ESI): 227.1 (80%, M+Na⁺), 279.1 (30%), 301.0 (100%, TPPO+Na⁺), 371.0 (55%), 579.0 (95%, M+2Na⁺-H⁺), 857.1 (90%).

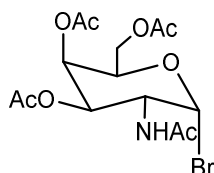
VII. General Procedure for the Polymerisation and Postpolymerisation Modification of GalNAc *exo*-fulvonorborneneimide



BG11-01 (GalNAc Derivative), Grubbs' 3 Catalyst (0.010g, 0.011 mmol) were weighed into separate flasks and purged with N₂. The monomer crude was dissolved into THF (5 mL) whilst G3 was dissolved separately THF (1 mL) and both solutions degassed, and the monomer solution transferred into the catalyst solution. The mixture was subsequently stirred for an hour at 50°C, before being terminated with the addition of Ethyl vinyl ether (1 mL, 10.4 mmol) on ice, and stirred for a further hour. Polymer precipitated with an excess of diethyl ether (45 mL) and isolated by centrifugation (10K RPM, 10 minutes), to yield the polymer product as a dark brown powder, to which a large excess of methanolic sodium methoxide (30% Soln, 2 mL) was added with stirring at 0°C in THF/MeOH (2:1, 15 mL). After 60 minutes (not exceeding

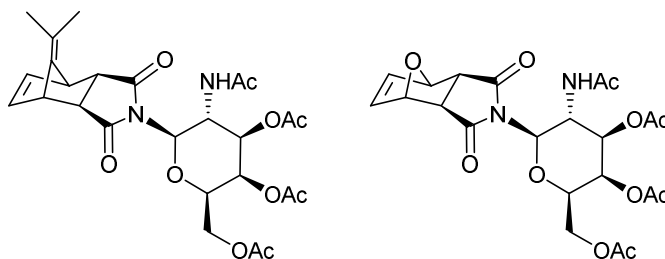
10°C), the mixture was neutralised with acidic Amberlite Ion Exchange Resin, filtered and condensed *in vacuo*. The residue was precipitated from excess diethyl ether (45 mL) and centrifuged (10K, 10 minutes) to return the deacetylated polymer, dialysed for 48 hours, to yield an off-yellow solid (28.4 mg).

VIII. BG21-01, S1: General Procedure for the Synthesis of Acetobromogalactosamine



Previously prepared peracetylated galactosamine (1.5008g, 3.85 mmol) was dissolved in anhydrous DCM (20 mL) and cooled on ice with stirring and protection from ambient light. HBr/AcOH (33%, 3.4 mL, 19.26 mmol, 5 eqv) was slowly added, dropwise, and the mixture allowed to equilibrate on ice for one hour, before being stirred at RT for 2 further hours. The translucent orange solution was washed with chilled and saturated NaHCO₃ solution (10 mL), and the organic phase washed with iced water (3 x 30 mL), and brine (30 mL), before being dried over Na₂SO₄ and condensed *in vacuo* to yield a glassy crystalline solid. 1.19 g (75.3%). ¹H NMR (300 MHz, CDCl₃) δ = 8.46 (1H, s, NHAc), 6.64 (1H, d, J = 14.50, Anomeric), 5.93 – 5.20 (4H, m, H-2,3,4), 5.30 (DCM), 4.65 – 4.04 (4H, m, H-5,6'), 2.30 (Acetone), 2.17 – 2.01 (11H, s, 4 x OAc), 1.84 (H₂O). m/z (ESI): 426.0, 429.0 (40%), 454.0, 455.1 (100%).

IX. BG21-01, S2: General Procedures for the Syntheses of GalNAc *exo*-fulvonorborneneimide and *exo*-oxonorborneneimide Monomers



Attempted promoter free synthesis of the *exo*-fulvonorborneneimide:

Two flasks containing BG08-01 (0.599g, 2.947 mmol; 0.300g, 1.476 mmol), BG21-01, S1 (0.650g, 1.5845 mmol, 1.86 eqv; 0.325g, 0.7923 mmol, 1.86 eqv), and K₂CO₃ (0.8154g, 6.16 mmol, 2.1 eqv; 0.4080g, 2.95 mmol, 2 eqv) were dissolved into dry, deoxygenated DMF (20 mL) under nitrogen, and stirred at 50°C and 90°C for 24 hours, respectively. The crude

mixtures were co-evaporated with an excess of toluene (x 3), and the solid residues extracted with DCM/Brine (1:1, 100 mL), which produced emulsions in both instances. Addition of ⁿButanol failed to resolve phases, and mixtures were recondensed – characterisation of the solid residue indicated no product, and the fulvene starting material was wholly recovered in each case. m/z (ESI): 202.0 (100%, SM-H⁺).

Iodine Catalysed *exo*-fulvonorborneneimide:

BG21-01, S1 (91.3 mg), 4Å molecular sieves, and Iodine (71 mg) were dissolved into dry, deoxygenated DMF (10 mL) under nitrogen, and stirred at 60°C for 1 hour. A separate flask was prepared concurrently of BG08-01 (50.4 mg) in dry, deoxygenated DMF (10 mL) with K₂CO₃ (17.1 mg) and was also stirred at RT for 1 hour. Upon elapse, the two mixtures were combined and stirred at 60°C overnight whilst protected from light. The reaction crude was then neutralised with Na₂S₂O₄, condensed *in vacuo* with a large excess of toluene to azeotrope DMF, and redissolved in CHCl₃. The mixture was subsequently washed with saturated NaCl solution (2 x 30 mL) and the organic phase dried over Na₂SO₄ and condensed *in vacuo* to give an orange gum 65.5 mg (55%). Characterisation as fulvene derivative, below.

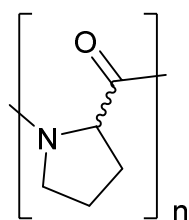
Silver Catalysed *exo*-fulvonorborneneimide and *exo*-oxonorborneneimide:

BG21-01, S1 (0.1506 g / 0.1424 g), 4Å molecular sieves, BG08-01 (83.9 mg) or BG19-01 (63 mg), and Ag₂CO₃ (57 mg / 53.4 mg) were dissolved in dry, deoxygenated DMF (10 mL) and stirred under nitrogen at RT overnight, with protection from light. The dark green reaction crude was then condensed *in vacuo* with a large excess of toluene to azeotrope the DMF, redissolved in ethanol and filtered to remove the black silver bromide by-product. The filtrate was subsequently recondensed, dissolved in CHCl₃ (30 mL) and extracted with saturated NaHCO₃ solution (2 x 30 mL), and the organic phase washed again with saturated NaCl solution (30 mL), dried over Na₂SO₄ and condensed *in vacuo* to give an off-white solid. Fulvene: 20.5 mg (10%), Oxo: 17.6 mg (10%).

Fulvene: ¹H NMR (300 MHz, CDCl₃) δ = 7.97 (s, Residual NH), 6.41 (2H, s, HC=CH), 5.99 (1H, d, *J* 6.5, Anomeric), 5.82 (1H, d, *J* 9.4, NHAc), 5.52 – 5.17 (3H, m, Sugar), 5.01 (1H, d, *J* 12.1, Sugar), 4.91 (1H, d, *J* 7.5, Sugar), 4.56 (1H, dd, *J* 20.3, 8.7, Sugar), 4.42 (1H, t, *J* 6.3, Sugar), 4.29 – 3.93 (3H, m, Sugar), 3.73 (2H, s, Bridge Base 2 x CH), 2.76 (2H, s, Ring 2 x CH), 2.21 – 2.10 (3H, m, Fulvene 2 x CH₃), 2.10 – 1.94 (10H, m, 4 x OAc), 1.71 (s, Unknown), 1.56 (s, H₂O), 1.25 (s, Unknown). ¹³C NMR (100 MHz, CDCl₃) δ = 171.06, 170.38, 137.68, 92.20, 71.82, 89.00, 67.55, 66.61, 62.09, 49.22, 45.60, 23.30, 20.75, 19.64. m/z (ESI): 370.1

(100%), 675.1 (100%) 699.2 (100%), 711.2 (75%). Oxo: ^1H NMR (300 MHz, CDCl_3) δ = 8.48 (s, Residual NH), 8.18 (m, Unknown), 8.00 (s, Unknown), 7.14 (s, Unknown), 6.51 (1H, s), 6.28 (1H, t, J = 21.3), 5.88 (1H, d, J = 9.8), 5.78 – 4.89 (4H, m), 4.83 – 3.72 (5H, m), 2.95 (s, Unknown), 2.87 (1H, s), 2.14 (5H, dd, J = 12.1, 4.4), 2.10 – 1.89 (11H, m), 1.80 (s, H_2O), 1.25 (s, Unknown), 0.83 (s, Unknown). ^{13}C NMR (100 MHz, CDCl_3) δ = 170.42, 136.58, 93.42, 92.16, 80.97, 72.57, 68.06, 67.55, 66.51, 64.96, 63.98, 61.53, 48.73, 78.08, 20.75. m/z (ESI): 352.1 (100%), 681.1 (100%) 699.2 (90%), 675.1 (100%).

X. General Procedures for the Syntheses of D, L, and DL-Proline Polypeptides/Oligomers

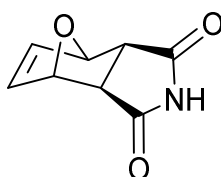


BG15-01 (L): EDCI (0.2503 g, 1.30 mmol, 1 eqv) was dissolved in dry DCM/DMF (20 mL, 1:1) and stirred at room temperature under a flow of nitrogen for 20 minutes, followed by cooling to 0°C . Within 5 minutes of cooling, L-proline (0.150 g, 1.30 mmol, 1 eqv) and OxymaPure (0.1853 g, 1.30 mmol, 1 eqv) were added together to the reaction mixture, resulting in an instantaneous colour change to yellow, followed by the addition of TEA (1.8 mL, 1.32 g, 13.0 mmol, 10 eqv) 2 minutes later. The mixture was stirred on ice under nitrogen for 1 further hour, and then warmed to RT with stirring overnight. The dark orange solution was condensed *in vacuo*, with 3 portions of n-Butanol in excess. The red oily residue was dissolved in dichloromethane (30 mL), and a large excess of toluene added to precipitate the crude material as a deep red solid. The solid was isolated, redissolved in dichloromethane (30 mL), and extracted with 1M HCl (3 x 20 mL), and the combined organic phases washed with 0.5M Na_2CO_3 (3 x 20 mL). The combined aqueous phases were saturated with NaCl and extracted with n-Butanol. The organic extracts were combined, dried over Na_2SO_4 , filtered, and condensed *in vacuo*, yielding a dark oil, which was subsequently dialysed (> 1 kDa) for 48 hours and freeze-dried to give an off-yellow solid. 4 mg (3 %).

BG15-01 (L, D, and DL): EDCI (0.5003g, 2.60 mmol) was dissolved in dry DCM (20 mL) and stirred at room temperature under a flow of nitrogen for 20 minutes, followed by cooling to 0°C . Within 5 minutes of cooling, L-proline (0.2996 g, 2.60 mmol, 1 eqv) and OxymaPure (0.3707 g, 2.60 mmol, 1 eqv) were added together to the reaction mixture, resulting in an

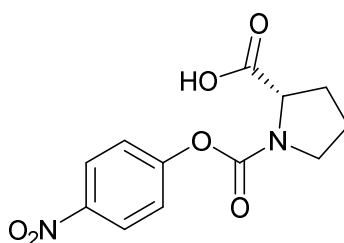
instantaneous colour change to yellow. The mixture was stirred on ice under nitrogen for 1 further hour, and then warmed to RT with stirring overnight. The dark yellow solution was condensed *in vacuo*, dissolved in water (10 mL) and dialysed (> 1 kDa) for 48 hours. The resulting solution was freeze dried, yielding an off-white solid. 31.4 mg (10.4%). Yields were generally observed for the various D, L, and DL peptides in the region of 2 – 60%.

XI. General Procedure for the syntheses of (3aR,7S,7aS)-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (*exo*-oxonorborneneimide)



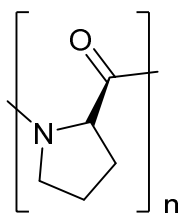
BG19-01: Furan (15 mL, 14.04 g, 206.26 mmol, 10 equiv) and maleimide (2.0007 g, 20.60 mmol) were dissolved in toluene (20 mL), and refluxed at 95°C for 48 hours, with the colourless mixture becoming white/opaque within 10 minutes. Reaction mixture cooled to RT, solids filtered, and washed with cold toluene (100 mL) to give a white solid, and dried *in vacuo*. 2.1888g (Quant%). ¹H NMR (300 MHz, CDCl₃) δ = 8.00 (1H, s, NH), 6.52 (2H, t, J₁ = J₂ = 0.91 Hz, HC=CH), 5.32 (2H, t, J₁ = J₂ = 0.92 Hz, Bridge 2 x CH), 2.89 (2H, s, Ring 2 x CH), 1.60 (H₂O). ¹³C NMR (300 MHz, CDCl₃) δ = 162.34 (2 x C=O), 136.59 (HC=CH), 80.99 (C-O-C), 48.71 (2 x C-C=O). IR (ATR): 3194 (NH); 3101, 3065, 2866, 2724 (CH); 1834, 1801; 1773, 1702 (2 x C=O); 1626, 1579 (C=C); 1345, 1301 (CH); 1148; 1065, 938, 842 cm⁻¹ (C=C). m/z (ESI): 164.1 (100%, M-H⁺).

XII. Syntheses of (4-nitrophenoxy)carbonyl-L-proline (Proline Urethane) and Poly-L-Proline



BG20-01, S1: L-proline (2.0007 g, 17.4 mmol) was suspended in ethyl acetate (40 mL) with NaHCO₃ (1.4602g), and 4-nitrophenyl chloroformate (3.53 g, 17.4 mmol) was added at RTP, and the mixture stirred at 45°C for 48 hrs. The resulting mixture was washed with distilled water (50 mL) and saturated NaCl solution (50 mL), dried over Na₂SO₄, filtered, and

concentrated *in vacuo*, yielding an off-yellow/green syrup (2.2434g, 46%). ^1H NMR (400 MHz, CDCl_3) δ = 8.37 – 8.12 (3H, m, Aromatic), 7.54 – 7.27 (2H, m, Aromatic), 6.88 (1H, d, *J* 9.1), 4.67 – 4.15 (1H, m, NCH_2), 3.91 – 3.49 (2H, m, Ring), 2.52 – 2.20 (2H, m, Ring), 2.17 – 1.92 (2H, m, Ring). ^{13}C NMR (100 MHz, CDCl_3) δ = 126.20, 125.13, 122.18, 115.62, 59.38, 59.03, 47.35, 47.30, 29.96, 24.38, 23.45. *m/z* (ESI) 281.1 (75%, $\text{M}+\text{H}^+$), 298.1 (80%, Isomer), 303.0 (65%, $\text{M}+\text{Na}^+$), 309.1 (100%, Isomer).



BG20-01, S2: The syrup obtained from BG20-01, S1 (B2) was dissolved (2.2434g) in DMAc (10 mL) and stirred at 60°C for 48 hrs under nitrogen. The mixture was poured into an excess of diethyl ether (40 mL) and centrifuged (10k RMP, 10 minutes). 4-Nitrophenol precipitated, no polymeric material observed.

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