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RESEARCH ARTICLE

A PROJECT REPORT ON DESIGN, SYNTHESIS AND NMR STUDIES OF STRUCTURES RELATED TO ANTIFREEZE GLYCOPROTEINS

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ABSTRACT

Antifreeze glycoproteins are essential for the surviving of many marine teleost fishes in polar and subpolar seawaters, where the temperature consistently are below the freezing point of physiological solutions. In this work, synthesis of structures related to antifreeze glycoproteins (AFGPs) are presented. Synthetic routes to a protected carbohydrate derivative, 2,3,4,6-tetra-*O*-benzyl- β – galactopyranosyl - (1→3) – 2 – deoxy – 2 – azido - 4, 6 – di – *O* – benzyl – β – D -thio-1-galactopyranoside, and a tBu-Ala-Thr-Ala-Fmoc tripeptide, are described. These compounds are meant to be used in the assembly of AFGPs and analogues. A Gal-GlcN disaccharide was synthesized via glycosylation between the donor, bromo-2-*O*-benzoyl-3,4,6-tri-*O*-benzyl-α-Dgalactopyranoside, and acceptor, ethyl 4,6-*O*-benzylidene-2-deoxy-2-*N*-phthalimido-β-D-1- thio-glucopyranoside, using silver triflate activation. Subsequent epimerization to a Gal-GalN disaccharide was achieved using Moffatt oxidation followed by L-selectride reduction. The tripeptide was synthesized in a short and convenient manner using solid phase peptide synthesis with immobilized Fmoc-Ala on Wang resins as starting point.

INTRODUCTION

Antifreeze proteins (AFPs) or ice structuring proteins (ISPs) refer to a class of polypeptides produced by certain vertebrates, plants, fungi and bacteria that permit their survival in subzero environments. AFPs bind to small ice crystals to inhibit growth and recrystallization of ice that would otherwise be fatal (Fletcher *et al*., 2001). There is also increasing evidence that AFPs interact with mammalian cell membranes to protect them from cold damage. This work suggests the involvement of AFPs in cold acclimatization (Jorov *et al*., 2004).

Non-colligative properties: Unlike the widely used automotive antifreeze, ethylene glycol, AFPs do not lower freezing point in proportion to concentration. Rather, they work in a noncolligative manner. This allows them to act as an antifreeze at concentrations 1/300th to 1/500th of those of other dissolved solutes. This minimizes their effect on osmotic pressure (Jorov *et al*., 2004). The unusual capabilities of AFPs are attributed to their binding ability at specific ice crystal surfaces (Walters *et al*., 2009).

Thermal hysteresis: AFPs create a difference between the melting point and freezing point known as thermal hysteresis.

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The addition of AFPs at the interface between solid ice and liquid water inhibits the thermodynamically favored growth of the ice crystal. Ice growth is kinetically inhibited by the AFPs covering the water-accessible surfaces of ice (Walters *et al*., 2009).

Antifreeze Glycoproteins

Antifreeze glycoproteins are essential for the surviving of many marine teleost fishes in polar and subpolar seawaters, where the temperature consistently are below the freezing point of physiological solutions. The AFGPs function is to inhibit the growth of ice crystals in the bloodstream of these fishes. Genetic studies have shown that AFGPs found in the two geographically distinct fish species Antarctic notothenioids and Arctic cod have evolved independently, a rare example of convergent molecular evolution. The difference between the melting- and freezing point of the ice crystals termed thermal hysteresis (TH), is used to detect and quantify the antifreeze activity. Although very little is known about the specific mechanism of the AFGPs during the depression of ice crystallization, several studies have been made to identify structure-function relationship of active AFGP derivatives. The glycoproteins consist of repeating tripeptide units (Ala-Thr-Ala)n (n ≥ 2), from which there are only minor natural variation. The hydroxyl group of the threonine residue is glycosylated to a Galβ(1→3)GalNAcα-

moiety (Figure 1). Due to the difficulties in isolating sufficient quantities of pure native AFGPs, important chemical strategies in synthesis of AFGPs have been developed.

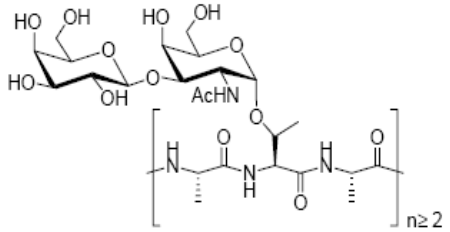


Figure 1. Stucture of a native AFGP (n ≥ 2)

AFGP derivatives have been synthesized with various structure modifications both on the tripeptide- and the disaccharide moiety to probe which residues are important for antifreeze activity. Recently it was observed that the highest TH is found when the chain length is between two- and five tripeptides long. No significant increase in activity was recorded as the chain was prolonged. When the threonine amino acid in the tripeptide was exchanged to a serenine residue, the TH was lost which indicates the importance of the methyl group on Thr for activity. Further studies from modifications on the carbohydrate moiety have revealed other interesting structure-function relationships. For example, a β-*O*-linked glycoprotein was designed to probe the importance of the terminal α-glycoside linkage. Although weak interactions between the glycopeptide and nucleated ice were found, the complete lack of TH in the Galβ(1→3)GalNAcβ AFGPs attest the essential nature of the α-linkage. Even the β(1→3)-disaccharide linkage is essential for activity. Acetylation of the hydroxyl groups on the disaccharide eliminated the TH properties, showing that at least some of the hydroxyl groups with proton donating properties are important for function. AFGP analogues have been synthesized to examine the importance of the NHAc group at C2 of the GalNAc residue, finding the NHAc-group necessary for TH activity.

MATERIALS AND METHODS

Retrosynthetic analysis of compound **1** gave key blocks **2** and **3**.

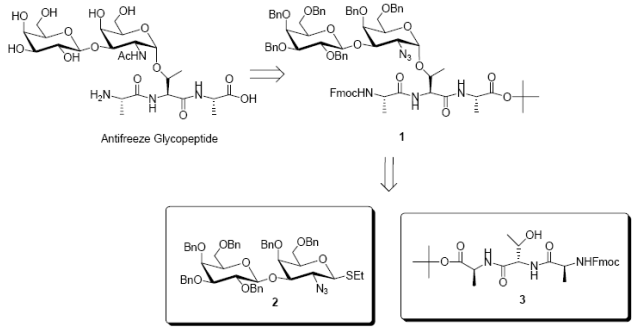


Figure 3. Retrosynthetic analysis of the monomeric antifreeze derivative

Oligosaccharide Synthesis

Synthetic strategy to compound **2** is outlined in Figure 4 and consisted of four main critical synthetic subjects:

- Formation of an efficient 2-*O*-acyl galactopyranoside donor **A**;
- Coupling of acceptor **4**11 with donor **A** to a Galβ(1→3)GlcNPhth disaccharide **B**;
- Epimerization of C4 in disaccharide **B** to a Gal-Gal compound **C**;
- Conversion of the *N*-phthalimido group to a non-participating azido group, **C** → **2**.

A more thoroughly discussion of each of these subjects will be presented below.

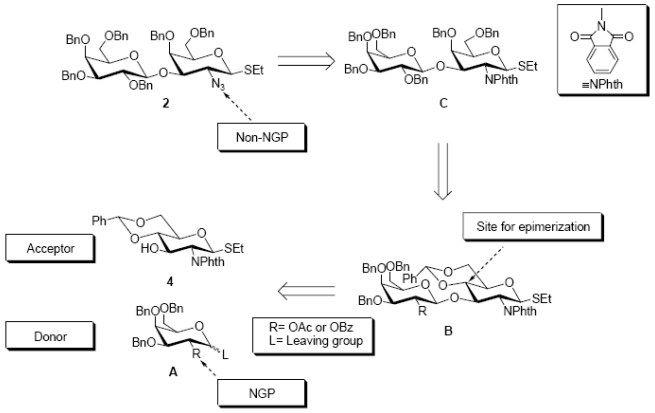


Figure 4. Synthetic strategies to compound 2. For more information see text

A convenient way to achieve a β(1→3) disaccharide link in a glycosylation is to use the neighboring group participation (NGP) effect. An 2-*O*-acyl in a donor has the ability to donate non-bonding electrons, blocking the axial approach, leading the attack to occur in an equatorial manner. Examples of substituents with the ability to participate are esters (*e.g. OAc*), amides and imides (*e.g. N-phthalimido*). The donor **A** consists of an electrophilic part which is often

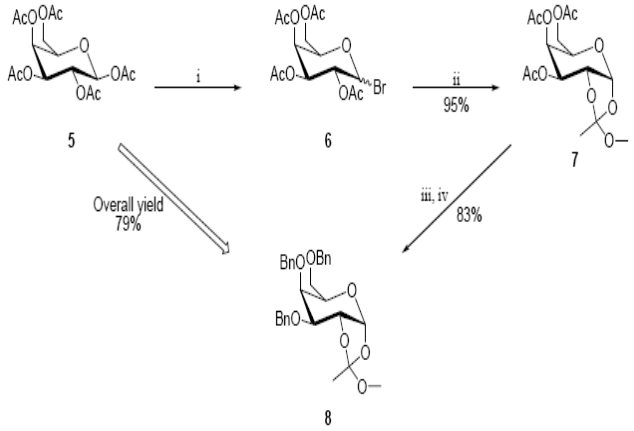
RESULTS AND DISCUSSION

Preparation of a 2-*o*-acyl galactopyranoside donor

Synthesis of compound 8

Crystalline *per*-acetylated β-galactopyranoside **5**, easily achieved via acetylation of Dgalactose, was used as starting point in the synthesis of the galactopyranoside donor to be used in the Gal-Gal disaccharide formation. In order to steer the formation to the desired β(1→3) glycoside bond in this latter reaction, a donor with a 2-*O*-acyl protecting group is required. Compound **7** with a 1,2-*O*-methoxyethylidene group has the ability to be selectively manipulated at positions 3, 4 and 6 and transformed to a donor with either a 2-*O*-acetate- or 2- *O*-benzoate group. Accomplishing a protecting group conversion from acetates to benzylethers on positions 3, 4 and 6, will increase the reactivity of the future donor and hopefully contribute to a higher yield of the disaccharide. The generation of the orthoester described by Asai and co-workers,15 was accomplished by the NGP-effect that an 2-*O*-acyl group posses (Scheme 1). Compound **5** was brominated with 33% HBr/HOAc (v/v) to give bromosugar **6** which was treated with TEA, Et4NBr and MeOH in CH2Cl2 to give compound **7** in 95% yield (step **5**→**7**). The decomposition of the acid-labile

orthoester was minimized by adding 0.1% TEA to the mobile phase when purified by FC. Compound **7** was deacetylated and benzylated to give **8** in 83% yield after crystallization from EtOAc/hexane. To summarize, a short synthetic route to a 3,4,6-tri-*O*-benzylated derivative with a potential 2-*O*acyl functionality (*i.e.* 1,2-*O*-orthoester), has been developed in very good yield.



Scheme 1. i) HBr/HOAc (33%, v/v); ii) TEA, Et4NBr, MeOH, CH2Cl2, 45ºC; iii) K2CO3, MeOH; iv) BnBr, NaH, DMF

Glycosylation between 2-*O*-acetyl galactopyranoside donor and phthalimido glucopyranoside acceptor 4

In the first attempt to synthesize the β(1→3) disaccharide, compound **8** was converted to corresponding 2-*O*-acetyl bromosugar using acetylbromide, Et4NBr and 4A MS in CH2Cl2. Coupling with compound **4** using AgOTf activation gave only traces of product (Scheme 2). Therefore this route was abandoned and we next focused on using a 2-*O*-benzoyl donor.

Summary and Conclusion

To summarize, a synthetic route to the disaccharide, Ethyl 2,3,4,6-tetra-*O*-benzyl-β- galactopyranosyl-(1→3)-2-deoxy-2-azido-4,6-di-*O*-benzyl-β-D-thio-1-galactopyranoside, has been developed. Furthermore, a tBu-Ala-Thr-Ala-Fmoc tripeptide was conveniently obtained using SPPS. These derivatives will be useful in future synthesis of glycopeptide **1**, a block opening for synthesis of AFGPs and analogues.

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