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C-Linked Galactosyl Serine AFGP Analogues as Potent Recrystallization Inhibitors

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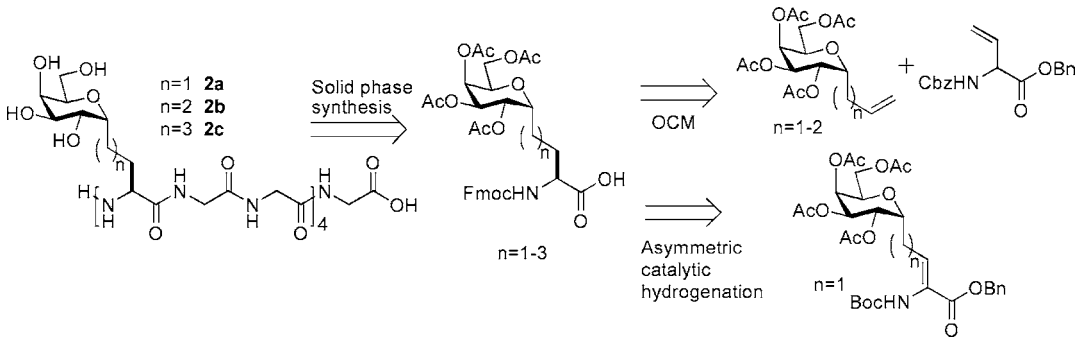
ABSTRACT



A series of *C*-linked antifreeze glycoprotein analogues have been prepared to evaluate antifreeze activity as a function of distance between the carbohydrate moiety and polypeptide backbone. The building blocks for these analogues were prepared using either an olefin cross-metathesis or catalytic asymmetric hydrogenation. Analysis of antifreeze protein-specific activity revealed that only analogue 2a ($n = 1$) was a potent recrystallization inhibitor and thus has potential medical and industrial applications.

Antifreeze glycoproteins (AFGPs) are a subclass of biological antifreezes found primarily in the plasma of deep sea polar fish.[1] These compounds have the ability to inhibit the growth of ice, thus ensuring the survival of these organisms in sub-zero environments. The typical AFGP structure is comprised of a repeating L-threonine-L-alanine-L-alanine tripeptide unit where the secondary hydroxyl group of the threonine is glycosylated with a β-D-galactosyl-(1,3)-α-*N*-acetyl-D-galactosaminyl disaccharide (Figure 1).[1g] While this structure is remarkably well-conserved among Teleost fish, lower molecular weight AFGPs may have an L-arginine residue in place of the L-threonine and/or an L-alanine residue substituted by L-proline. The AFGP mechanism of action is

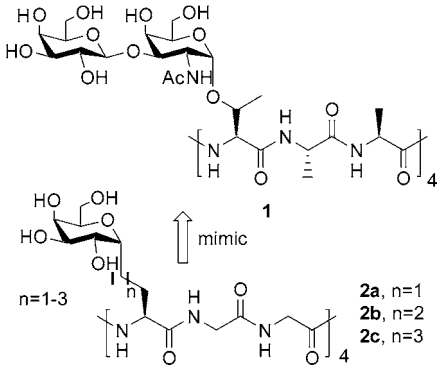


Figure 1. Structures of native AFGP 8 (**1**) and the *C*-linked AFGP analogues used in this study.

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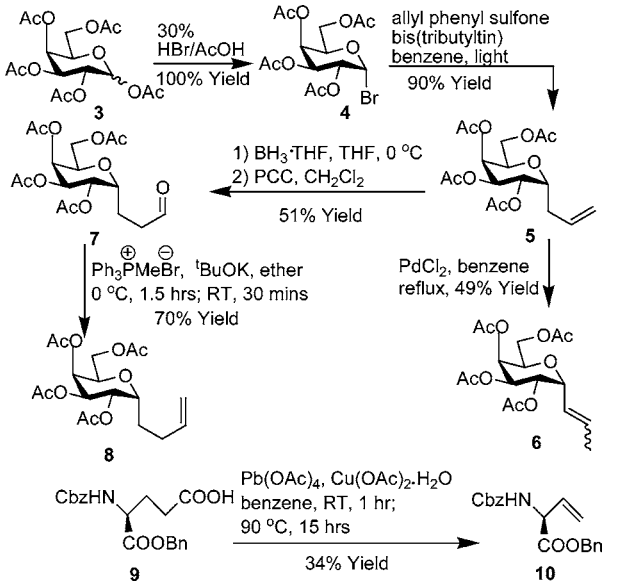
regarded as an absorption−inhibition process[1a−d,h] in which AFGPs bind tightly to the surface of an existing ice crystal. This binding ultimately inhibits the addition of other water molecules to the ice lattice resulting in a localized freezing point depression. The temperature difference between the melting and freezing point is referred to as thermal hysteresis (TH). While the protein−ice interactions of native AFGP have not been definitively identified, hydrogen-bonding interactions involving hydrophilic hydroxyl groups on the disaccharide[2] and possible hydrophobic interactions with the β-methyl group of the threonine residue are thought to be essential interations.[3] Furthermore, recent studies have identified the *N*-acetyl group, α-configuration of glycoside, and β-methyl group on the threonyl residue as important for activity.[4]

AFGPs are also potent recrystallization inhibitors. While the mechanism by which this re-organization of ice crystals is not known, this property has many potential applications in cryomedicine and the prevention of cellular damage during freezing and thawing cycles.[1i]

Unfortunately, two factors have precluded the commercialization of native AFGP for medical and industrial applications. These are the limited bioavailability and the inherent instability of the C−O glycosidic bond. Consequently, rationally designed carbon-linked or *C*-linked AFGP analogues are very attractive.[5] Toward this end, we have previously reported on the preparation of *C*-linked AFGP analogues bearing an amide bond in the side chain that demonstrated antifreeze protein-specific activity.[6] In this paper, we report the synthesis of a series of "simplified" *C*-linked AFGP analogues (general structure **2**) lacking the amide bond and correlate the distance between the carbohydrate moiety and peptide backbone to antifreeze protein-specific activity. Many of the structural features in the first-generation analogues have been incorporated into AFGP analogues **2a**−**c**. For instance, the native disaccharide has been truncated and replaced by a single galactose residue and the alanine residues replaced with glycines.

Recently, several methodologies have been developed to prepare *C*-glycosyl amino acids including olefin cross metathesis (OCM) and catalytic asymmetric hydrogenation.[7] The former approach is amenable to preparing analogues such as **2** as it requires the readily available vinyl glycine and *C*-alkenyl galactose derivatives as starting materials.[8]

Scheme 1. Synthesis of Building Blocks **5**, **6**, **8**, and **10** for OCM



Preparation of the requisite building blocks for olefin cross-metathesis are outlined in Scheme 1. *C*-Allylated galactose pentaacetate **5** was prepared via a photochemical-mediated allylation in 90% yield.[9a] *C*-Glycoside **8** was obtained by reducing **5** with borane followed by PCC oxidation and Wittig olefination with methyl triphenyl phosphonium bromide.[9b,c] *C*-(1-Propenyl) glycoside **6**, a key intermediate in the preparation of AFGP analogue **2a**, was generated via a palladium-mediated isomerization of **5**.

Vinyl glycine derivative **10** was obtained in 34% yield from the orthogonally protected glutamic acid derivative **9** by oxidative decarboxylation.[10] With the requisite building blocks in hand, olefin cross-metathesis of **10** with **5**, **6**, and **8** was conducted using the second- generation Grubbs catalyst (Scheme 2). As anticipated, building blocks **12** and

Scheme 2. Preparation of *C*-Linked Building Blocks **14** and **15**

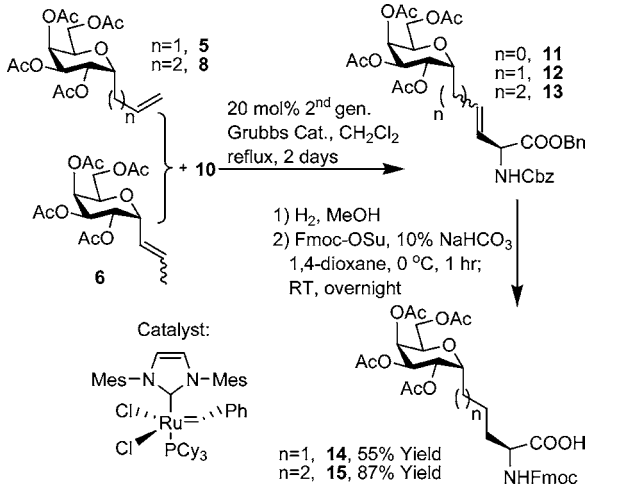


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Table 1. Olefin Cross-Metathesis To Prepare *C*-Linked Building Blocks **11**−**13**

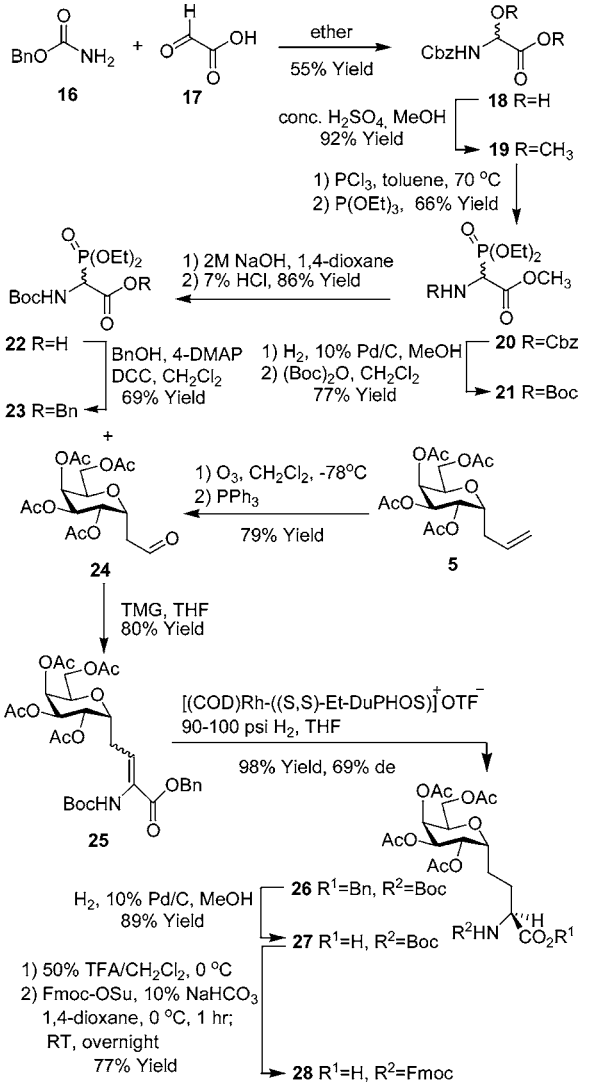
entry	*C*-alkene glycoside	product	yield, %
1	**5**	**12**	98
2	**6**	**11**	trace
3	**8**	**13**	100

13 were obtained in quantitative yield (Table 1). Unfortunately, cross-metathesis between **10** and **6** produced furnished building block **11** in only trace quantities. Presumably, this is due to the fact that the carbon−carbon double bond in **6** is too close to the pyranose ring resulting in significant steric interactions during the OCM. A similar effect has been observed by McGarvey *et al.*[8a] The carbon−carbon double bonds in enamide esters **12** and **13** were reduced by hydrogenation with palladium over carbon. Under these conditions, the Cbz and benzyl protecting groups were simultaneously removed necessitating reprotection of the amino terminus as an Fmoc carbamate to afford building blocks **14** and **15** in 55% and 85% yield, respectively.

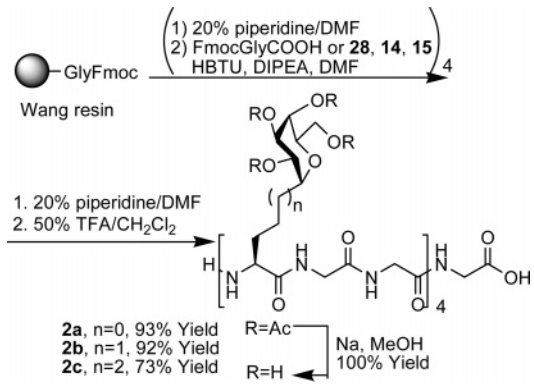
To prepare the building block for AFGP analogue **2a**, we adopted a catalytic asymmetric hydrogenation of *C*-glycosyl enamide esters first reported by Toone and co-workers.[11] The substrate for this hydrogenation (**25**) was constructed via Horner−Emmons olefination[12] with phosphonate **23**[13] and *C*-linked pyranosyl aldehyde **24**, as shown in Scheme 3. Catalytic asymmetric hydrogenation of the *C*-glycosyl enamide **25** was accomplished using cationic rhodium−DuPHOS catalyst under hydrogen atmosphere (100 psi). Hydrogenolysis of the benzyl ester followed by conversion of the Boc carbamate to a Fmoc carbamate furnished building block **28**.

Assembly of building blocks **14**, **15**, and **28** into *C*-linked AFGP analogues was accomplished by using standard Fmoc-based solid-phase synthesis protocols (Scheme 4). The protected glycopeptides were cleaved from the resin using TFA, and the acetate protecting groups on the pyranose were removed by treatment with sodium in methanol to afford

Scheme 3. Preparation of *C*-Linked Serine Building Block **28**



Scheme 4. Synthesis of *C*-Linked AFGP Analogues **2a**−**c**



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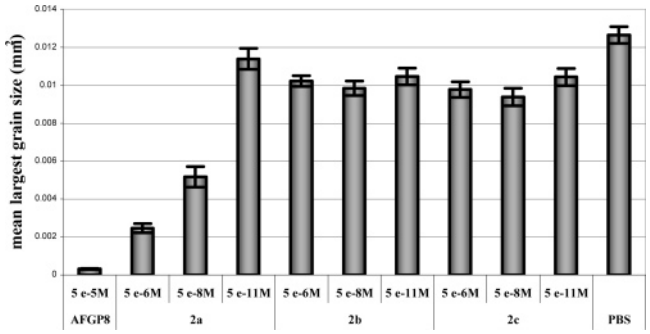

Figure 2. RI activity of *C*-linked AFGP Analogues

the *C*-linked AFGP analogues **2a**−**c** (73% to 93% isolated yield) ranging in molecular weight from 1.5 to 1.6 KDa.

AFGP analogues **2a**−**c** were assayed for antifreeze protein-specific activity using nanoliter osmometry and a recrystallization-inhibition assay.[6c] In contrast to our first-generation AFGP analogues, **2a**−**c** did not possess any thermal hysteresis or exhibit any dynamic ice-shaping ability.

However, all three analogues demonstrated recrystallization inhibition (RI) activity (Figure 2) relative to the phosphate-buffered saline (PBS) control. The *Y*-axis in Figure 2 represents the mean largest grain size (MLGS) which is an average ice crystal surface area for each sample.

Different concentrations of each analogue in PBS were assayed to rule out the nonspecific effects.[6c] AFGP analogue **2a** was the most potent with activity close to that of native AFGP8. Interesingly, when the side chain length is increased by one or two additional carbon−carbon bonds (**2b** and **2c**), these analogues showed very limited RI activity. The correlation between increased side chain length and decreased RI activity suggests that an optimal distance between the two moieties exists and plays a key role in RI activity of our *C*-linked analogues. Analogue **2a** possesses the same number of atoms between the carbohydrate and peptide moieties as native AFGP. While this analogue is not as potent as AFGP8, it appears to be a more effective recrystallization-inhibitor than type III AFP from the ocean pout (*Macrozoarces americanus*) which has an effective concentration for RI activity at 7.10×10^{-7} M compared with 5.0×10^{-8} M in **2a**.[14]

In summary, we have utilized olefin cross-metathesis and catalytic asymmetric hydrogenation to prepare a series of novel *C*-linked AFGP analogues with different distances between the carbohydrate and peptide backbone moieties. The analogue with the shortest distance between these moieties is an potent recrystallization inhibitor. This result suggests that the rational design of potent recrystallization inhibitors for medical, industrial and commercial applications is a feasible and worthwhile goal. The effectiveness of AFGP analogue **2a** in preventing cryo-injury in mammalian cell culture is currently under investigation.

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Supporting Information Available: Experimental procedures and spectroscopic data for **4**−**31**. This material is available free of charge via the Internet at http://pubs.acs.org.

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