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Aust. J. Chem. **2011**, *64*, 723–731

Synthesis of an Isotopically-labelled Antarctic Fish Antifreeze Glycoprotein Probe

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Antifreeze glycoproteins (AFGPs) are glycosylated polypeptides produced by Antarctic and Arctic fishes, which allow them to survive in seawater at sub-zero temperatures. An investigation into the postulated enteric uptake of AFGP synthesized in the exocrine pancreas of Antarctic fishes required a custom-prepared AFGP probe that incorporated seven isotopically-labelled Ala residues for detection by mass spectrometry. The AFGPs are composed of a repetitive three amino acid unit (Ala-Ala-Thr), in which the threonine residue is glycosylated with the disaccharide β-D-Gal-(1→3)-α-D-GalNAc. The synthesis of isotopically-labelled AFGP8 (**1**), as well as the optimized synthesis of the protected glycosylated amino acid building block **2**, is reported.

Manuscript received: 20 December 2010.
Manuscript accepted: 28 January 2011.

Introduction

Antifreeze proteins and glycoproteins are a class of polypeptides that inhibit ice crystal growth, allowing certain vertebrates, insects, or plants that produce them to survive at sub-zero temperatures.[1] Specifically, Antarctic notothenioid fish and northern cod produce antifreeze glycoproteins (AFGPs) that lower the freezing point of the blood and permit the fishes to live at temperatures close to the freezing point of seawater (∼−1.9°C).[1a,2] The AFGPs are present in very high quantities (up to 3.5% (w/v) or 35 mg mL^{-1}) in the plasma of the fish,[2] and are thought to operate by adsorbing onto specific faces of ice crystals, inhibiting crystal growth.[1b,3] AFGPs consist predominantly of a repetitive three amino acid unit (Ala-Ala-Thr), with the threonine hydroxyl bearing the disaccharide β-D-galactose-(1→3)-α-D-*N*-acetyl-galactosamine (identified clinically as the Thomsen-Friedenrich (TF) antigen; CD176). The different AFGPs identified to date vary in the number of repeat units present (4–50, equivalent to molar masses between 2.6 and 33.7 kDa), and the smaller variants sometimes have a proline in place of the first alanine in the trimer.[1c,2] The isoforms are

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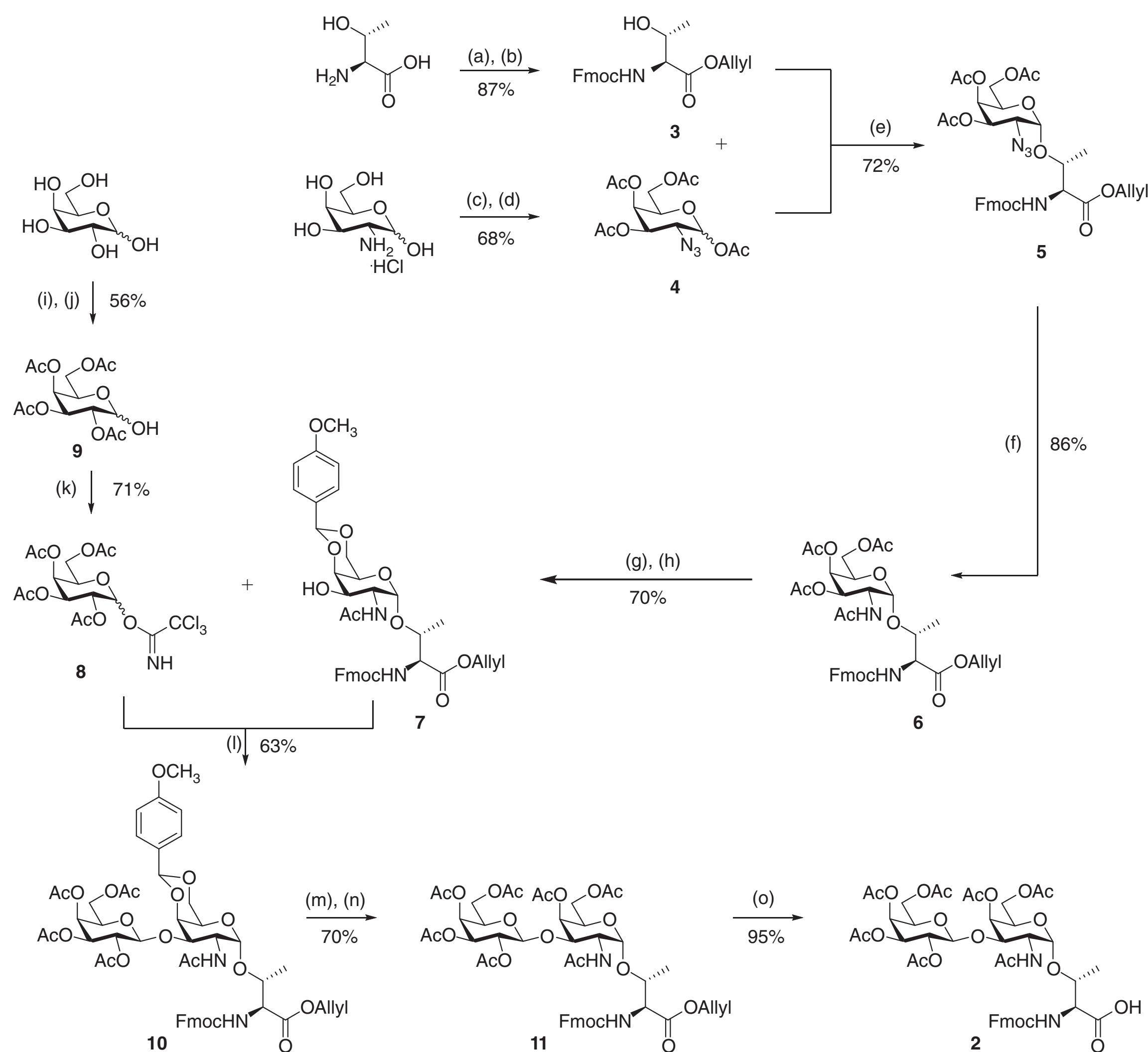


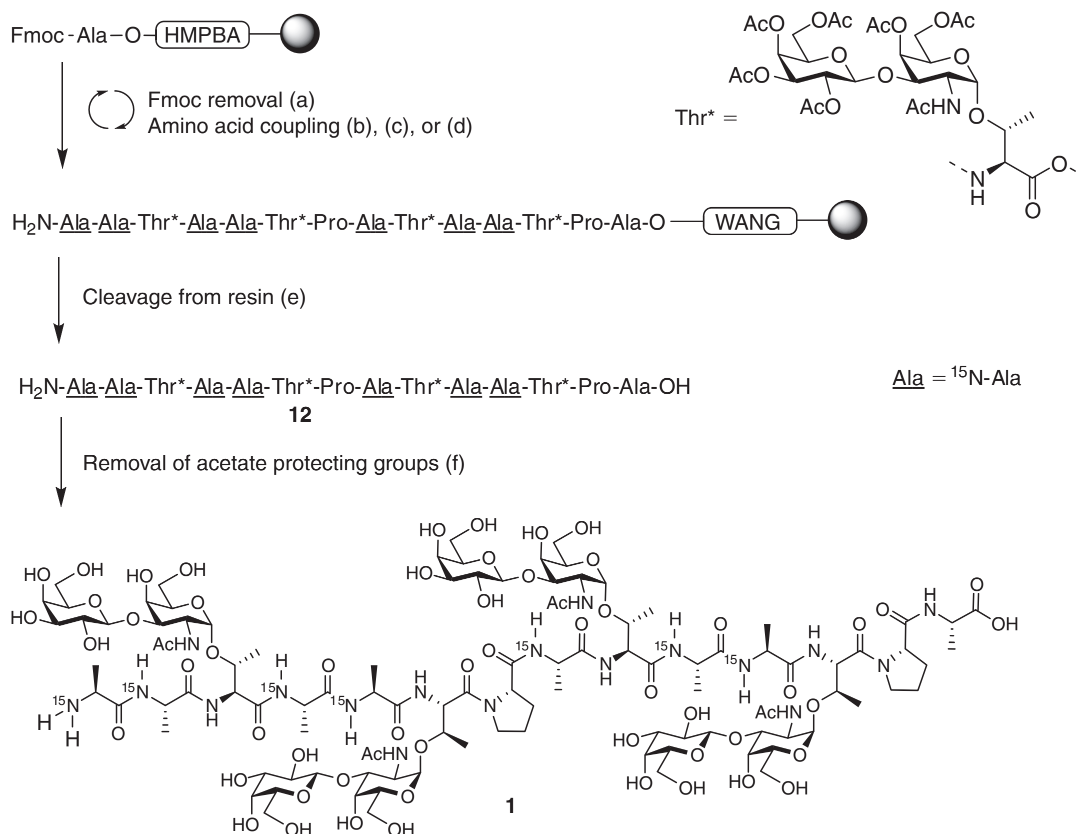
Scheme 1. Synthesis of the Gal-GalNAc-Thr building block **2**. Reagents and conditions: (a) Fmoc-OSu, Na_2CO_3, 1,4-dioxane, RT, 16 h; (b) allyl bromide, $NaHCO_3/H_2O$, $^{n}Bu_4N^+Br^-$, RT, 20 h; (c) imidazole-1-sulfonyl azide,[16] Na_2CO_3, $Cu^{II}SO_4$, $MeOH/H_2O$, RT, 2.5 h; (d) Ac_2O/Et_3N, RT, 3 h; (e) $BF_3{\cdot}Et_2O$, CH_2Cl_2, 40°C, 78 h; (f) Zn, $CuSO_4$, $THF/Ac_2O/AcOH$, RT, 30 min; (g) AcCl, MeOH, 40°C, 36 h; (h) 4-MeOPhCH$(OMe)_2$, cat. pTsOH, DMF, 55°C, 4 h, 20 mbar; (i) cat. $HClO_4$, Ac_2O, 1 h; (j) $Cu(OAc)_2$, $MeOH/H_2O$, reflux, 4 h; (k) Cl_3CCN, K_2CO_3, DCM, RT, 16 h; (l) TMSOTf, 4 Å MS, CH_2Cl_2, −10°C to RT, 2 h; (m) HOAc (80%), RT, 1.5 h; (n) Ac_2O/pyridine, RT, 16 h; (o) $Pd(PPh_3)_4$, *N*-methylaniline, THF, RT, 2 h.

grouped into eight classes (AFGP1–8) based on their size (large to small) and electrophoretic properties.

As the liver is the main source of secreted plasma proteins, it was also thought to be the site of biosynthesis of the abundant plasma AFGPs. Recently, however, Cheng et al.[4] have shown the exocrine pancreas is the major site of AFGP synthesis, with additional contributions from the oesophagus and anterior portion of the stomach. Since all three of these organs secrete into the gastrointestinal tract and not into the circulatory system, the possibility arises that AFGPs reach the blood by re-absorption intact through the intestinal wall.[5] In order to prove this hypothesis, we wish to introduce isotopically-labelled AFGPs directly into the gut (to mimic the natural secretory pathway) and monitor their subsequent uptake by mass spectrometry of blood samples. For this purpose, we require a custom-prepared AFGP that incorporates an isotopically-labelled residue (or preferably multiple labelled residues) in order for the introduced AFGPs to be distinguished from the endogenous glycoproteins by mass spectrometry.

The preparation of such large and complex molecules is a formidable challenge, hence most synthetic efforts to date have focussed on simpler AFGP analogues (see ref. [6] for a recent review). Two main approaches are usually considered for the synthesis of glycopeptides: direct glycosylation of a full-length peptide, or the incorporation of a pre-formed glycosylated amino acid building block into stepwise solid-phase synthesis of the peptide. While direct glycosylation is more convergent, it often gives poor yields due to the low reactivity of the peptide side chain hydroxyl groups.[7] The Nishimura group have used another alternative: solution-phase polymerization of a glycosylated tripeptide building block to make the glycopeptide.[8] Unfortunately, this method lacks fine control over the peptide chain length, resulting in a distribution of molecular weights rather than a single isoform, and does not permit the incorporation of proline at specific sites.

Tseng et al.[9] were the first to use a glycosylated amino acid building block in solid-phase peptide synthesis (SPPS) to generate natural AFGPs, showing that homogenous peptides can be



Scheme 2. Synthesis of labelled AFGP8 peptide (**1**). Reagents and conditions: (a) 20% piperidine/DMF, MW 75°C, 30 s then 3 min; (b) Fmoc-Pro, HBTU, $^{i}Pr_2EtN$, DMF, MW 75°C, 5 min (double coupling); (c) Fmoc-^{15}N-Ala, HBTU, $^{i}Pr_2EtN$, DMF, MW 75°C, 15 min; (d) glycoside **2**, HATU, HOAt, collidine, DMAP (cat.), DMF, MW 75°C, 20 min; (e) TFA/TIPS/H_2O (95:2.5:2.5), RT, 2 h; (f) 1 M NaOMe/MeOH, pH 9–10, RT, 3 h.

obtained by this method. In a similar fashion to Tseng et al., we have employed the glycosylated building block strategy for the synthesis of the labelled AFGP probe. We decided on an alternate route to the glycosylated amino acid, however, in order to also obtain access to the clinically relevant Tn-antigen (CD175). The Tn antigen (α-D-*N*-acetyl-galactosamine) conjugated to serine/threonine is the most prevalent of the *O*-linked glycoprotein motifs,[7] and, along with the TF antigen, they are often overexpressed in oncogenically transformed cells. Glycosylated building blocks of this nature are therefore attractive synthetic targets both for the synthesis of cancer vaccine candidates,[10] and other glycopeptides or glycoproteins.

Herein we report the synthesis of AFGP8 (**1**), the most abundant variant of the smallest natural AFGP,[11] containing seven ^{15}N-Ala residues. The glycopeptide probe was prepared through the use of the protected glycosylated amino acid building block **2** in microwave-assisted SPPS (see Schemes 1 and 2).

Results and Discussion

The synthetic route to the glycosylated amino acid building block **2** is shown in Scheme 1. First, the amino acid L-threonine was converted into a suitable glycosyl acceptor (**3**) for the initial glycosylation reaction. The amino group was protected with Fmoc using Fmoc-*N*-hydroxysuccinimide, while the carboxyl group was protected as an allyl ester (using allyl bromide with a phase transfer catalyst), as we have found this to be the most reliable protecting group.[12]

Previous work in our laboratory[13] has also established that tetra-*O*-acetyl-2-azido-deoxygalactose (**4**) was the most suitable glycosyl donor to effect glycosylation of the protected threonine. Glycosyl donor **4** was previously made[14] from galactose via the glycal, which was converted into 2-azido-2-deoxygalactosyl nitrate using cerium ammonium nitrate and sodium azide. Regrettably, this route suffers from persistent low yields obtained from the critical azidonitration step (yields of 30–40% are common).[14]

An alternative route to 2-azido-2-deoxy galactose makes use of a diazotransfer reaction,[15] using trifluoromethanesulfonyl azide as the 'diazo donor' to transform an amine (e.g. galactosamine) into the corresponding azide. TfN_3 has several problems associated with its use: it is explosive and has to be handled with care, has relatively poor shelf-life, provides inconsistent yields during preparation (which requires the expensive precursor trifluoromethanesulfonic anhydride), and also necessitates specialized workup to remove the sulfonamide by-product.[16] More recently, Goddard-Borger et al.[16] synthesized a new diazotransfer reagent, the inexpensive, shelf-stable imidazole-1-sulfonyl azide hydrochloride, which can be prepared in a simple one-pot procedure. We therefore used the imidazole-1-sulfonyl azide hydrochloride as the diazo transfer agent to effect the conversion of galactosamine to azide **4**.

The glycosyl donor **4** thus obtained was then coupled to the amino acid glycosyl acceptor **3** using boron trifluoride-diethyl etherate with gentle heating for 78 h. The progress of the glycosylation reaction was often difficult to interpret by TLC due to the similar R_f values of the reagents. The reaction was

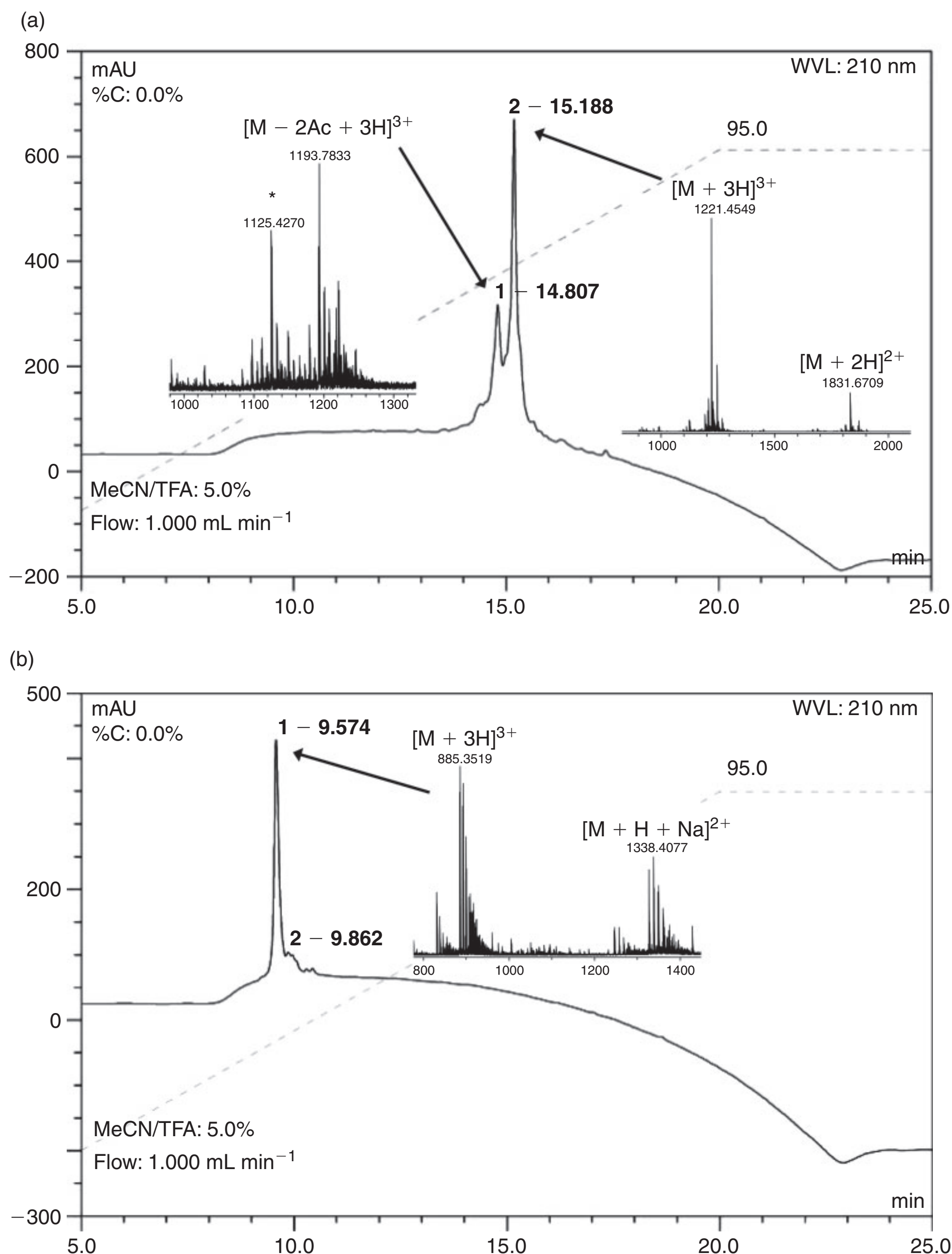


Fig. 1. Analytical data for (a) the crude, protected AFGP8 peptide **12** and (b) the final AFGP8 peptide **1**. MS data given in the insets. *Unidentified by-product.

therefore monitored by analytical RP-HPLC (isocratic flow, 60% acetonitrile in water, with 0.1% TFA). Pleasingly, the reaction proceeded with high α-selectivity, to give the azido-glycoside **5** in 72% yield after purification.

The subsequent reductive acetylation of the azide group of the glycosylated threonine intermediate **5** has previously[13] been performed using thioacetic acid and pyridine,[17] a reaction that although high yielding, involves an unpleasant workup. In the present work, the azide was reductively acetylated using zinc powder in THF/acetic anhydride/acetic acid (with addition of $CuSO_4$ to activate the zinc) in 86% yield to give Fmoc-[D-GalNAc(OAc_3)(α1-*O*)]Thr-*O*-Allyl (**6**).[18]

Our second generation synthetic protocol has resulted in a significant improvement in the yield of intermediate **6** – from 4.9% over seven steps[13] to an improved yield of 42% over only four steps. At this point, if the Tn antigen building block is desired, the building block can be deallylated to give a free acid ready for coupling in SPPS.[13] Alternatively, further manipulations can be performed to form more complex di- and oligosaccharides.

For the purpose of the AFGP8 probe, the disaccharide Gal-β-(1→3)-GalNAc was required, hence a second glycosylation reaction was performed. Compound **6** was transformed into a glycosyl acceptor suitable for glycosylation at the 3-OH position by removal of the acetates, followed by selective protection of the 4 and 6 hydroxyl groups. Deprotection of acetates was first attempted through the use of NaOMe/MeOH solution as previously reported.[8a,10b,19] Unfortunately, some loss of the Fmoc protecting group was observed, as well as significant transesterification to the threonine methyl ester, resulting in poor yields

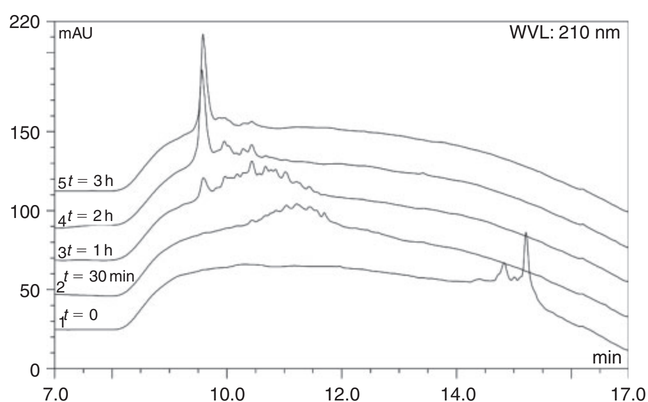


Fig. 2. LC data with detection at 210 nm for the deacetylation reaction.

for this step. Consequently, an acidic deprotection method was employed using 50 mol-% AcCl.[20] Although the reaction was much slower, no Fmoc cleavage was observed, and transesterification to the methyl ester was only observed after 3 days when the reaction was essentially complete. The resultant triol was then selectively protected using *p*-anisaldehyde dimethyl acetal under *p*-toluenesulfonic acid (pTsOH) catalysis[10b] to afford **7** in 70% yield over two steps.

With the *p*-methoxybenzyl (Pmb) protected glycosyl acceptor **7** in hand, the Helferich glycosylation procedure (galactosyl bromide with $Hg(CN)_2$) was initially attempted.[10b] Unfortunately, despite considerable effort, the reaction failed to produce the desired disaccharide, with only starting material being isolated. Consequently, attention turned to the use of the Schmidt glycosylation,[21] with galactosyl trichloroacetimidate **8** as the galactosyl donor and trimethylsilyl trifluoromethanesulfonate (TMSOTf) as the Lewis acid promoter.

The synthesis of imidate **8** required peracetylation of galactose, followed by the selective deprotection of the anomeric hydroxyl to give 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (**9**), which can be further functionalized to provide **8**. Although many routes to 1-hydroxy sugars are available,[22] all suffer from low selectivity and yield, and often require anhydrous conditions, toxic reagents, or harsh bases. Recently, Bhaumik et al.[23] reported a simple and mild method for anomeric deprotection using copper(II) acetate and methanol/water (9:1). Although the selectivity is still somewhat poor, the simplicity and mildness of the method makes this an attractive alternative. Using this procedure, the 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (**9**) was prepared from D-galactose in 56% yield over two steps. Subsequent reaction with trichloroacetonitrile in the presence of K_2CO_3[24] afforded the imidate (**8**) in 71% yield.

Acceptor **7** was coupled with the imidate glycosyl donor **8** using TMSOTf as activator, and the reaction was complete within 2 h, providing the β-(1→3)-linked disaccharide **10** exclusively (63% yield). The use of excess Lewis acid was avoided, as this leads to partial cleavage of the Pmb protecting group and subsequent undesirable glycosylations at the 4 and 6 positions.

Finally, several protecting group manipulations were required to convert **10** into a suitable building block for SPPS. The Pmb group was removed with acetic acid, and the emergent hydroxyl groups were acetylated with a mixture of acetic anhydride in pyridine to give **11** in 70% yield over two steps. Lastly, deallylation using tetrakis(triphenylphosphine)palladium(0) with *N*-methylaniline as scavenger afforded the desired Fmoc-protected building block **2** in 95% yield for this step, and in 12.3% overall yield from D-galactosamine.

The disaccharide building block **2** was then utilized in Fmoc solid-phase glycopeptide synthesis (Scheme 2). The synthesis was performed on aminomethyl resin (prepared[25] in house), to which an Fmoc-Ala-HMPBA linker was coupled using DIC. Chain elongation proceeded using a CEM Liberty microwave peptide synthesizer. For the non-glycosylated amino acids (Pro/^{15}N-Ala), the coupling was carried out using HBTU in the presence of $^{i}Pr_2EtN$; for incorporation of the glycosylated building block **2** (1.25 equiv.), HATU/HOAt with 4-*N*,*N*-(dimethylamino)pyridine and collidine was used, as developed by Kowalczyk et al.[26] A Kaiser test after coupling of the building block revealed that despite the low excess of reagent, complete reaction was observed after 20 min reaction time in the microwave synthesizer. In all cases, the Fmoc group was removed using 20% piperidine in DMF. When chain elongation was complete, the peptide was cleaved from the resin with 95% TFA, with TIPS and H_2O (2.5% each) as scavengers, giving the desired crude peptide in high purity (Fig. 1a). The major by-product was determined to be the desired peptide but with a loss of two acetate groups.

Finally, the acetate protecting groups of the sugar hydroxyl groups were removed using NaOMe in MeOH (pH ~9). The reaction was monitored by analytical RP-HPLC (Fig. 2) until completion (approximately 3 h). The solution was then carefully neutralized by addition of 1 M HCl, and lyophilized from 1:1 acetonitrile/water to give AFGP8 as a white powder. As can be seen in Fig. 1b, the final product was of sufficient purity for biological testing and hence was not purified further.

Conclusion

In summary, the isotopically-labelled AFGP8 probe was synthesized in good yield and purity, and is currently being tested in vivo in Antarctic fish; biological results will be published at a later date. Importantly, the synthetic protocol developed during this study provides facile access to both the Tn and TF antigen building blocks for the SPPS of glycopeptides of clinical relevance.

Experimental

General

All reagents were purchased as reagent grade and used without further purification. Solvents were dried and purified before use according to standard methods.[27] Solvents for RP HPLC were purchased as HPLC grade and used without further purification. ^{15}N-labelled Fmoc-Ala was purchased from Cambridge Isotope Laboratories, Inc. Analytical TLC was performed using 0.2 mm plates of Kieselgel F_{254} (Merck). Compounds were visualized by UV fluorescence and/or by staining with 5% H_2SO_4/EtOH and heating. Flash chromatography was carried out using Kieselgel F_{254} S 63–100 μm silica gel with the solvents indicated. Analytical RP HPLC was performed on a Dionex Ultimate 3000 System or a Dionex P680 System using an analytical column (Phenomenex Gemini C18, 110Å, 150 mm × 2.0 mm, 5 μm) at a flow rate of 1 mL min^{-1}. Analytical HPLC analysis of peptides was performed using a linear gradient (5–95%) of buffer B in buffer A over 15 min (buffer A = 0.1% trifluoroacetic acid (TFA) in water; buffer B = 0.1% TFA in acetonitrile). Optical rotations were determined at the sodium D line (589 nm) at 20°C with a Perkin–Elmer 341 polarimeter. IR spectra were

obtained using a Perkin–Elmer Spectrum 100 FT-IR spectrometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker AVANCE 400 spectrometer. All samples were run in $CDCl_3$ and the chemical shifts are reported in ppm relative to the tetramethylsilane signal recorded at δ_H 0.00. The ^{13}C values were referenced to the residual chloroform signal at δ_C 77.00. Mass spectra were recorded on a Bruker micrOTOFQII mass spectrometer coupled with a Dionex Ultimate 3000 HPLC with autosampler. Carrier phase consisted of 50:50 acetonitrile/water containing 0.1% formic acid at a flow rate of 0.1 mL min^{-1}. For HRMS, external mass calibration was performed with Agilent Tunemix. Data were acquired for 3 min and averaged over the region of maximum sample-ion intensity.

N-(9-Fluorenylmethylcarbonyl)-L-threonine Allyl Ester 3

L-Threonine (24.0 g, 201.4 mmol) was suspended in a mixture of 10% aqueous Na_2CO_3 (330 mL) and 1,4-dioxane (150 mL) with stirring in an ice-bath. Fmoc-OSu (71.44 g, 211.5 mmol) was dissolved in 1,4-dioxane (150 mL) with gentle heating and the solution was added dropwise to the L-threonine solution. The reaction mixture was allowed to warm to room temperature overnight and the solvent was subsequently removed under reduced pressure. The suspension was diluted with H_2O and extracted three times with EtOAc. The aqueous phase was acidified with citric acid (approximately 45 g) to pH 3.5 (pH paper). Filtration and lyophilization yielded 61.1 g (89%) of the Fmoc-threonine as a white solid that was used in the next step without further purification.

A solution of *N*-(9-fluorenylmethoxycarbonyl)-L-threonine (2.34 g, 6.84 mmol) and $NaHCO_3$ (0.58 g, 6.93 mmol) in H_2O (22 mL) was added to a solution of tetrabutylammonium bromide (2.16 g, 6.71 mmol) and allyl bromide (3.12 mL, 3.90 g, 36.05 mmol) in DCM (40 mL). The suspension was stirred vigorously at room temperature for 20 h under nitrogen. H_2O (50 mL) was added to the reaction mixture and the suspension was extracted with DCM (3 × 30 mL). The combined organic layers were dried over Na_2SO_4, filtered, concentrated under reduced pressure and the crude product (5.43 g) was purified by flash chromatography (2:3 → 3:2 → 1:1 EtOAc/hexane) to yield 2.57 g (98%) of compound **3** as a white solid (lyophilized from ^{t}BuOH); R_f 0.51 (1:1 EtOAc/hexane). ^{1}H NMR data was in agreement with that reported in the literature.[28]

1,3,4,6-Tetra-O-acetyl-2-azido-2-deoxy-D-galactopyranose 4

Imidazole-1-sulfonyl azide hydrochloride[16] (1.2 equiv., 3.27 g, 15.6 mmol) was added to D-galactosamine hydrochloride (2.72 g, 12.6 mmol), K_2CO_3 (4.80 g, 34.7 mmol), and $CuSO_4 \cdot H_2O$ (31.5 mg, 126 μmol) in MeOH/water (5:3, 80 mL) and the mixture stirred at room temperature for 2.5 h. The mixture was co-evaporated with toluene and filtered through a pad of Celite® (R_f of 2-azido-2-deoxy-D-galactose intermediate: 0.43 (0.5:2.5:7 AcOH/MeOH/DCM)). Acetic anhydride (50 mL) was added to the residue in dry NEt_3 (25 mL) and the mixture stirred for 3 h. The mixture was concentrated under reduced pressure and co-evaporated with toluene. Purification by flash chromatography (1:2 EtOAc/hexane), afforded 3.04 g of compound **4** as a yellow oil in 68% yield (ratio of α:β 1:3); R_f 0.45 (1:1 EtOAc/hexane). ^{1}H NMR data was in agreement with that reported in the literature.[15a]

N-(9-Fluorenylmethoxycarbonyl)-3-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine Allyl Ester 5

The glycosyl donor 1,3,4,6-tetra-*O*-acetyl-2-azido-2-deoxy-D-galacto-pyranose (**4**) (1.86 g, 5 mmol) and the glycosyl acceptor Fmoc-threonine allyl ester (**3**) (2.0 g, 5.2 mmol) were dried on the high vacuum for several hours. Anhydrous DCM (15 mL) was added, and the reaction flask was cooled in ice. $BF_3 \cdot Et_2O$ (3 mL, 5 equiv.) was added slowly, then the reaction was heated with stirring at 40°C under an atmosphere of N_2. Periodically, 5-μL samples were removed from the reaction, and a mini workup was performed: the sample was diluted with 500 μL of DCM, and washed with 500 μL $NaHCO_3$. The organic layer was separated and evaporated, then re-dissolved in 200 μL of 50:50 ACN/H_2O for analysis by HPLC. After 78 h, the reaction was judged complete and cooled to RT. A saturated solution of $NaHCO_3$ was added to quench the reaction, and the organic layer was diluted with DCM. The organic layer was washed with more $NaHCO_3$ solution, then brine, then H_2O, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The dark residue was purified by silica flash chromatography (2% MeOH/DCM) to yield 2.48 g (72%) of compound **5** as a pale yellow solid; R_f 0.45 (1:2 EtOAc/hexane). ^{1}H NMR data was in agreement with that reported in the literature.[29]

N-(9-Fluorenylmethoxycarbonyl)-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine Allyl Ester 6

To a solution of compound **5** (1 g, 1.5 mmol) in 50 mL of THF/Ac_2O/AcOH solution (3:2:1) was added zinc dust (1.64 g, 25 mmol) and 2.5 mL of sat. aq. $CuSO_4$, which served to activate the zinc. After 30 min of stirring, the solution was filtered and concentrated under reduced pressure. Purification by flash chromatography (2:1 EtOAc/hexane) yielded compound **6** (860.5 mg, 84%) as a pale yellow oil; R_f 0.55 (4:1 EtOAc/hexane). ^{1}H NMR data was in agreement with that reported in the literature.[29]

N-(9-Fluorenylmethoxycarbonyl)-O-(2-acetamido-2-deoxy-4,6-O-p-methoxybenzylidine-α-D-galactopyranosyl)-L-threonine Allyl Ester 7

Compound **6** (1.0 g, 1.4 mmol) was dissolved in 10 mL of MeOH, and 50 μL (0.7 mmol) of AcCl was added, and the reaction was stirred at 40°C for 36 h. The reaction was quenched by the addition of $NaHCO_3$ (sat.) solution, and diluted with DCM. The aqueous layer was extracted with DCM (2×), and the organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure, to give 794.6 mg of the crude deprotected compound. This material was then dissolved in 10 mL of DMF, and 464 μL (2.7 mmol) of *p*-anisaldehyde dimethyl acetal was added. A catalytic amount (approximately 20 mg) of *p*TsOH was added, and the reaction was stirred on the rotary evaporator at 55°C and ~20 mbar. After 2 h, a further 464 μL (2.7 mmol) of *p*-anisaldehyde dimethyl acetal was added. After 4 h, the reaction was neutralized by the addition of 5 μL of $^{i}Pr_2EtN$, and the yellow solution was partitioned between EtOAc/H_2O. The aqueous layer was washed with EtOAc, and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was dissolved in MeOH/DCM, silica gel was added, and the whole dried to a powder. This powder was then loaded onto a

flash column, and eluted with 3:1 EtOAc/hexane to yield 690 mg (70% over two steps) of the Pmb protected glycosylated amino acid **7** as a white solid; R_f 0.23 (3:1 EtOAc/hexane). $[\alpha]_D$ +40.6 (*c* 0.14 in $CHCl_3$). ν_{max}(film)/cm^{-1} 3323br (O-H), 3068w (Ar C-H), 2937m (C-H), 1721s (C=O), 1653s (vinyl C=C), 1520s (Ar C=C), 1449m (C-O-H), 1249s (C-O-C), 1045s (C-O), 742m (Ar C-H). δ_H (400 MHz) 7.77 (d, *J* 7.7, 2H, Fmoc ArcH), 7.63 (d, *J* 6.9, 2H, Fmoc ArCH), 7.44 (d, *J* 8.7, 2H, Pmb *ortho*), 7.40 (d, *J* 7.5, 2H, Fmoc ArCH), 7.31 (t, *J* 7.0, 2H, Fmoc ArCH), 6.87 (d, *J* 8.7, 2H, Pmb *meta*), 5.86 (ddt, *J* 16.9, 10.5, 6.0, 1H, allyl CH), 5.50 (s, 1H, Pmb acetal), 5.33 (dd, *J* 17.5, 1.1, 1H, allyl =CH_2), 5.27 (dd, *J* 9.9, 1.1, 1H allyl =CH_2), 4.94 (d, *J* 3.5, 1H, H1), 4.65 (dd, *J* 12.9, 5.8, 1H, allyl CH_2), 4.58 (dd, *J* 12.9, 5.9, 1H, allyl CH_2), 4.44 (m, 1H, Thr Hα), 4.42 (m, 2H, Fmoc CH_2), 4.41 (m, 1H, H2), 4.34 (qd, *J* 6.3, 1.5, 1H, Thr Hβ), 4.25 (m, 1H, Fmoc CH), 4.21 (m, 1H, H6a), 4.15 (m, 1H, H4), 4.03 (d, *J* 12.5, 1H, H6b), 3.87 (m, 1H, H3), 3.71 (br s, 1H, H5), 3.79 (s, 3H, Pmb OMe), 2.06 (s, 3H, NAc), 1.29 (d, *J* 6.3, 3H, Thr Hγ). δ_C (100 MHz) 171.7 (NAc), 170.8 (Thr C=O), 160.1 (Pmb *para*), 156.9 (Fmoc urethane), 143.7, 141.3 (Fmoc C), 131.0 (allyl CH), 130.1 (Pmb *ipso*), 127.74 (Pmb *ortho*), 127.69, 127.0, 125.1, 120.0 (Fmoc ArCH), 119.7 (allyl =CH_2), 113.5 (Pmb *meta*), 101.1 (Pmb acetal), 100.5 (C1), 76.3 (Thr Cβ), 75.6 (C4), 69.2 (C6), 68.3 (C3), 67.1 (Fmoc CH_2), 66.3 (allyl CH_2), 63.4 (C5), 58.8 (Thr Cα), 55.3 (Pmb OMe), 50.2 (C2), 47.2 (Fmoc CH), 23.1 (NAc), 18.9 (Thr Cγ). *m/z* (HR-ESI) 703.2851 $[M+H]^+$. $C_{38}H_{43}N_2O_{11}$ requires 703.2861, Δ1.0 ppm.

2,3,4,6-Tetra-O-acetyl-D-galactopyranose ***9***

To a stirred suspension of D-galactose (500 mg, 2.7 mmol) in Ac_2O (20 mL) was added dropwise 60% perchloric acid (120 µL). Additional D-galactose (4.5 g, 25.0 mmol) was added in small portions. During the additions, the temperature was kept below 40°C by occasional cooling in an ice-water bath. After the addition was complete, the reaction was stirred for 2 h at room temperature. The resulting mixture was then diluted with DCM and water, and washed twice with $NaHCO_3$ (sat) solution. The organic layer was dried over $MgSO_4$ and concentrated. The crude material (10.92 g) was then dissolved in 150 mL MeOH/H_2O (9:1). Copper (II) acetate dihydrate (5.6 g, 12.8 mmol) was added, and the reaction was heated until reflux for 6 h (monitored by TLC). The reaction was cooled, filtered, and the filtrate was concentrated under reduced pressure. The dark residue was diluted with water and extracted twice with EtOAc. Flash chromatography (1:1 EtOAc/hexane) yielded 4.67 g (56%) of **9** as a white foam (ratio of α:β 5:1); R_f 0.20 (1:1 EtOAc/hexane). ^{1}H NMR data was in agreement with that reported in the literature.[30]

2,3,4,6-Tetra-O-acetyl-D-galactopyranosyl Trichloroacetimidate ***8***

Compound **9** (3.27 g, 9.4 mmol) was dried on the high vacuum, and then dissolved in 40 mL of anhydrous DCM. To this solution was added K_2CO_3 (3.90 g, 28.2 mmol) and trichloroacetonitrile (3.77 mL, 37.6 mmol) and the reaction was stirred at room temperature under an inert atmosphere overnight. The reaction mixture was then filtered and concentrated under reduced pressure. Subsequent purification by flash chromatography (2:3 EtOAc/hexane with 0.5% Et_3N) afforded the imidate **8** (3.27 g, 71%) as a white foam (α/β 1:1); R_f 0.65 (α) and 0.34 (β) (1:1 EtOAc/hexane). ^{1}H NMR data was in agreement with that reported in the literature.[19,31]

N-(9-Fluorenylmethoxycarbonyl)-O-(2-acetamido-2-deoxy-4,6-O-p-methoxybenzylidine-3-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-α-D-galactopyranosyl)-L-threonine Allyl Ester ***10***

The glycosyl acceptor **7** (997.4 mg, 2 mmol) and glycosyl donor **8** (690.2 mg, 0.98 mmol) were combined and dried on the high vacuum for 3 h. They were dissolved in anhydrous DCM, and 4 Å molecular sieves (spatula tip) were added. The reaction flask was cooled in an ice/salt bath to approximately –10°C. TMSOTf (45 µL, 0.25 mmol) was then added to the flask. The reaction was allowed to warm to RT. After 2 h, the reaction was quenched by the addition of 100 µL of Et_3N, was filtered, and concentrated to dryness under reduced pressure. Purification by silica flash chromatography (3:1 EtOAc/hexane) yielded 637 mg (63%) of disaccharide **10** as a white amorphous solid; R_f 0.31 (3:1 EtOAc/hexane). $[\alpha]_D$ +63.0 (*c* 0.092 in $CHCl_3$). ν_{max}(film)/cm^{-1} 3352m, 3290m, 3186m (N-H), 2927m, 2852m (C-H), 1722s (C=O), 1601m (C=C), 1371m, 1226s (C-O), 1046s (C-O-C), 827s (Ar C-H). δ_H (400 MHz) 7.77 (d, *J* 7.3, 2H, Fmoc ArCH), 7.44 (d, *J* 8.8, 2H, Pmb *ortho*), 7.40 (m, 2H, Fmoc ArCH), 7.61 (br d, *J* 6.2, 2H, Fmoc ArCH), 7.31 (td, *J* 7.6, 2.4, 2H, Fmoc ArCH), 6.88 (d, J 8.7, 2H, Pmb *meta*), 5.85 (m, 1H, allyl CH), 5.73 (d, *J* 8.6, 1H, GalNAc NH), 5.67 (d, *J* 9.6, 1H, Thr NH), 5.49 (s, 1H, Pmb acetal), 5.37 (dd, *J* 3.4, 1.0, 1H, Gal H4), 5.33 (br d, *J* 17.1, 1H, allyl =CH_2), 5.28 (dq, *J* 10.4, 1.1, 1H, allyl =CH_2), 5.17 (dd, *J* 10.5, 8.0, 1H, Gal H2), 4.96 (m, 1H, Gal H3), 4.95 (d, *J* 2.9, GalNAc H1), 4.73 (d, *J* 7.8, 1H, Gal H1), 4.64 (m, 1H, allyl CH_2), 4.60 (m, 1H, GalNAc H2), 4.55 (m, 1H, allyl CH_2), 4.51 (m, 2H, Fmoc CH_2), 4.40 (d, *J* 10.5, 1H, Thr Hα), 4.27 (m, 1H, Thr Hβ), 4.26 (m, 1H, GalNAc H4), 4.24 (m, 1H, Fmoc CH), 4.21 (m, 1H, Gal H6a), 4.19 (m, 1H, GalNAc H6a), 4.11 (m, 1H, Gal H6b), 4.02 (br d, *J* 12.0, 1H, GalNAc H6b), 3.89 (m, 1H, Gal H5), 3.87 (m, 1H, GalNAc H3), 3.79 (s, 3H, Pmb OMe), 3.64 (br s, 1H, GalNAc H5), 2.13, 2.03, 2.00, 1.99, 1.96 (s, 15H, 5× Ac), 1.26 (3H, Thr Hγ). δ_C (100 MHz) 170.5, 170.4, 170.2, 170.1, 169.8, 169.4 (5× Ac, 1× C=O), 159.9 (Pmb *para*), 156.5 (Fmoc urethane), 143.5, 141.3 (Fmoc C), 130.8 (allyl CH), 130.1 (Pmb *ipso*), 127.8 (Fmoc ArCH), 127.5 (Pmb *ortho*), 127.0, 124.8 (Fmoc ArCH), 120.0 (allyl =CH_2), 119.9 (Fmoc ArCH), 113.5 (Pmb *meta*), 100.9 (Gal C1), 100.6 (Pmb acetal), 100.4 (GalNAc C1), 76.4 (Thr Cβ), 75.4 (GalNAc C4), 73.8 (GalNAc C3), 70.9 (Gal C5), 70.8 (Gal C3), 69.0 (Gal C2), 68.8 (GalNAc C6), 67.0 (Gal C4), 67.0 (Fmoc CH_2), 66.3 (allyl CH_2), 63.5 (GalNAc C5), 61.3 (Gal C6), 58.6 (Thr Cα), 55.2 (Pmb OMe), 48.0 (GalNAc C2), 47.2 (Fmoc CH), 23.3, 21.0, 20.6 (×2), 20.5 (5× Ac), 18.6 (Thr Cγ). *m/z* (HR-ESI) 1033.3839 $[M+H]^+$. $C_{52}H_{61}N_2O_{20}$ requires 1033.3812, Δ2.7 ppm.

N-(9-Fluorenylmethoxycarbonyl)-O-(2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-α-D-galactopyranosyl)-L-threonine Allyl Ester ***11***

Compound **10** (614.6 mg, 0.595 mmol) was dissolved in 10 mL 80% HOAc and the solution was stirred at room temperature for 1.5 h. The solution was neutralized with a solution of $NaHCO_3$ (sat) and diluted with EtOAc. The aqueous layer was further extracted with EtOAc (2×) and the combined organic layers were washed with $NaHCO_3$ (sat) solution. The organic phase was dried over $MgSO_4$ and concentrated. The resulting solid was dissolved in 10 mL of 1:1 pyridine/Ac_2O, and the reaction was left to stir at room temperature overnight. The reaction was quenched by the addition of ice, and was then diluted with DCM.

The aqueous layer was extracted twice more with DCM, and the combined organic phases were washed twice with $NaHCO_3$ (sat.), then with brine, dried over $MgSO_4$ and concentrated. Flash chromatography (3:1 EtOAc/hexane) afforded 556.0 mg (0.557 mmol, 94% over two steps) of **11** as a clear glass; R_f 0.30 (3:1 EtOAc/hexane). $[\alpha]_D$ +62.6 (*c* 0.099 in $CHCl_3$). ν_{max}(film)/cm^{-1} 3335m (N-H), 2981w, 2941w (C-H), 1747s (C=O), 1663m (vinyl C=C), 1536w (Ar C=C), 1372m, 1227s (C-O), 743m (Ar C-H). δ_H (400 MHz) 7.79 (d, *J* 7.5, 2H, Fmoc ArCH), 7.61 (d, *J* 7.7, 2H, Fmoc ArCH), 7.42 (t, *J* 7.4, 2H, Fmoc ArCH), 7.34 (t, *J* 6.9, 2H, Fmoc ArCH), 5.87 (m, 1H, allyl CH), 5.78 (d, *J* 9.2, 1H, GalNAc NH), 5.54 (d, *J* 9.6, 1H, Thr NH), 5.37 (m, 1H, Gal H4), 5.36 (m, 1H, GalNAc H4), 5.32 (m, 2H, allyl =CH_2), 5.10 (dd, *J* 10.5, 7.9, 1H, Gal H2), 4.95 (dd, *J* 10.3, 3.0, 1H, Gal H3), 4.86 (d, *J* 3.1, 1H, GalNAc H1), 4.67 (dd, *J* 12.4, 6.0, 1H, allyl CH_2), 4.59 (m, 1H, allyl CH_2), 4.58 (m, 1H, Gal H1), 4.53 (m, 2H, Fmoc CH_2), 4.48 (m, 1H, GalNAc H2), 4.41 (d, *J* 9.8, 1H, Thr Hα), 4.26 (m, 1H, Fmoc CH), 4.25 (m, 1H, Thr Hβ), 4.15 (m, 1H, Gal H6b), 4.16 (m, 2H, GalNAc H6), 4.12 (m, 1H, Gal H5), 3.98 (dd, *J* 11.0, 7.1, 1H, Gal H6a), 3.88 (t, *J* 6.5, 1H, GalNAc H5), 3.81 (dd, *J* 10.5, 2.7, 1H, GalNAc H3), 2.16, 2.13, 2.06, 2.02, 2.02, 2.01, 1.97 (s, 21H, 7× Ac), 1.31 (d, *J* 6.2, 3H, Thr Hγ). δ_C (100 MHz) 171.1, 170.4 (×2), 170.3, 170.14, 170.10, 169.9, 169.6 (7× Ac, 1× C=O), 156.4 (Fmoc urethane), 143.5, 141.3 (Fmoc C), 130.8 (allyl CH), 127.8, 127.1, 124.8, 120.1 (Fmoc ArCH), 120.0 (allyl =CH_2), 100.7 (Gal C1), 100.1 (GalNAc C1), 77.2 (Thr Cβ), 73.0 (GalNAc C3), 70.73 (GalNAc C5), 70.70 (Gal C3), 68.8 (Gal C2), 68.7 (Gal C4), 67.9 (Gal C5), 67.0 (Fmoc CH_2), 66.7 (GalNAc C4), 66.4 (allyl CH_2), 62.9 (Gal C6), 61.0 (GalNAc C6), 58.6 (Thr Cα), 48.6 (GalNAc C2), 47.2 (Fmoc CH), 23.3, 20.8 (×2), 20.7 (×3), 20.5 (7× Ac), 18.3 (Thr Cγ). *m/z* 999.3633 (HR-ESI) $[M+H]^+$. $C_{48}H_{59}N_2O_{21}$ requires 999.36055, Δ1.8 ppm.

N-(9-Fluorenylmethoxycarbonyl)-O-(2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-α-D-galactopyranosyl)-L-threonine **2**

Compound **11** (556.0 mg, 0.56 mmol) was dried on the high vacuum for 2 h, then dissolved in 10 mL anhydrous THF and degassed with argon for 30 min. *N*-Methylaniline (60 μL, 0.56 mmol) then $Pd(PPh_3)_4$ (6.5 mg, 0.056 mmol) were added, and then the reaction was stirred at RT under argon. After completion of the reaction (2 h), the sample was concentrated, and purified by flash chromatography (1:1 MeOH/EtOAc) to yield 506 mg (95%) of **2** as a film; R_f 0.1 (0.1% HOAc/EtOAc), 0.6 (1:1 MeOH/EtOAc). $[\alpha]_D$ +127.4 (*c* 0.11 in $CHCl_3$). ν_{max}(film)/cm^{-1} 3355br (O-H), 3072w (Ar C-H), 2930w (C-H), 1749s (C=O), 1658m (C=C), 1372m, 1228s (C-O), 1060s (C-O-C), 742m (Ar C-H). δ_H (400 MHz) 7.41 (t, *J* 7.3, 2H, Fmoc ArCH), 7.32 (t, *J* 7.3, 2H, Fmoc ArCH), 7.63 (d, *J* 6.7, 2H, Fmoc ArCH), 7.79 (d, *J* 7.5, 2H, Fmoc ArCH), 4.58 (d, *J* 7.5, 1H, Gal H1), 5.00 (d, *J* 3.0, 1H, GalNAc H1), 4.40 (m, 1H, Thr Hβ), 3.86 (m, 1H, GalNAc H3), 4.95 (dd, *J* 10.5, 3.4, 1H, Gal H3), 3.86 (m, 1H, Gal H5), 5.08 (dd, *J* 10.2, 7.9, 1H, Gal H2), 5.37 (br d, *J* 2.2, 1H, Gal H4), 4.15 (m, 1H, GalNAc H5), 5.35 (d, *J* 3.0, 1H, GalNAc H4), 4.51 (m, 2H, Fmoc CH_2), 4.00 (m, 2H, GalNAc H6), 4.17 (m, 1H, Gal H6a), 4.12 (m, 1H, Gal H6b), 4.40 (m, 1H, Thr Hα), 4.39 (m, 1H, GalNAc H2), 4.25 (m, 1H, Fmoc CH), 2.15, 2.12, 2.11, 2.05, 2.00, 1.99, 1.97 (s, 21H, 7× Ac), 1.29 (d, *J* 6.1, 3H, Thr Hγ), 6.26 (d, *J* 8.9, 1H, Thr NH), 5.72 (d, *J* 8.9, 1H, GalNAc NH). δ_C (100 MHz) 170.5, 170.4, 170.3, 170.1 (×2), 170.0, 169.9, 169.6 (7× Ac, 1× C=O), 156.6 (Fmoc urethane), 143.7, 141.3 (Fmoc C), 127.8, 127.1, 124.8, 120.0 (Fmoc ArCH), 100.4 (Gal C1), 99.5 (GalNAc C1), 77.2 (Thr Cβ), 72.5 (GalNAc C3), 70.7 (Gal C3), 70.6 (Gal C5), 68.79, 68.75 (Gal C2, Gal C4), 67.7 (GalNAc C5), 66.8 (GalNAc C4), 66.7 (Fmoc CH_2), 62.8 (GalNAc H6), 60.9 (Gal C6), 58.5 (Thr Cα), 49.1 (GalNAc C2), 47.2 (Fmoc CH), 22.7, 20.57 (×2), 20.62 (×2), 20.5, 20.0 (7× Ac), 18.3 (Thr Hγ). *m/z* (HR-ESI) 981.3109 $[M+H]^+$. $C_{45}H_{54}N_2NaO_{21}$ requires 981.3111, Δ1.1 ppm.

Solid-phase Synthesis of Protected AFGP8 **12**

Solid-phase synthesis of AFGP8 was carried out on Fmoc-Ala-Wang polystyrene resin (1.178 mmol g^{-1} loading, 0.1 mmol scale), prepared by coupling Fmoc-Ala-O-CH_2-Ph-OCH_2CH_2COOH (2 equiv.), using *N*,*N*′-diisopropylcarbodiimide (DIC) (2 equiv.) in DCM, 2 h at RT to aminomethyl resin which had been prepared according to published protocol.[25] The peptide was assembled with the use of a CEM Liberty microwave synthesizer using the following conditions: (a) Fmoc removal: 20% piperidine/DMF, MW 75°C, 30 s then 3 min; (b) Fmoc-Pro coupling: Fmoc-Pro (5 equiv.), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (4.5 equiv.), $^iPr_2EtN$ (10 equiv.) in DMF, MW 75°C, 5 min, repeated twice (double coupling); (c) ^{15}N-Ala (1.5 equiv.), HBTU (1.45 equiv.), $^iPr_2EtN$ (29 equiv.) in DMF, MW 75°C, 15 min; (d) compound **2** (1.25 equiv.), 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (1.2 equiv.), 1-hydroxy-7-azabenzotriazole (HOAt) (1.25 equiv.), collidine (4.5 equiv.) in DMF, MW 75°C, 20 min. The peptide was cleaved from the resin using TFA/triisopropylsilane (TIPS)/H_2O (95:2.5:2.5), precipitated using Et_2O, and isolated by centrifugation. The resulting pellet was dissolved in 50:50 ACN/H_2O (with 0.1% TFA) and lyophilized, to give the protected AFGP8 peptide (**12**) as a fluffy powder (206 mg, 57% crude yield). *m/z* (ESI-MS) 1221.4 $[M+3H]^{3+}$, requires 1221.8.

Removal of Acetate Protecting Groups

The protected AFGP8 peptide **12** (50 mg, 0.014 mmol) was dissolved in 10 mL MeOH. Freshly prepared 1 M NaOMe/MeOH solution was added until pH ~9–10. After 3 h at RT, the reaction was neutralized by addition of 1 M HCl, and concentrated to dryness. The residue was dissolved in 50:50 ACN/H_2O, and lyophilized to give AFGP8 (**1**) as a white powder (42.3 mg, 117% apparent yield due to NaCl salts). *m/z* (ESI-MS) 885.4 $[M+3H]^{3+}$, requires 885.6.

Acknowledgements

This research was supported by the Human Frontier Science Program (grant RGP003/2009-C). The authors thank Dr Nicole Miller for assistance in the synthesis of tetra-*O*-acetyl-2-azido-2-deoxygalactose.

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