Facile Solid-Phase Synthesis of an Antifreeze Glycoprotein

Ping-Hui Tseng, Weir-Torn Jiaang, Meng-Yang Chang, and Shui-Tein Chen*[a]

Abstract: The antifreeze glycoproteins (AFGPs) **1** are composed of a repeating tripeptide unit (Ala-Thr-Ala) in which the threonine residue is glycosylated with the disaccharide β-D-Gal-(1 $\rightarrow$ 3)-α-D-GalNAc. A new procedure for synthesizing AFGPs using Fmoc-(Ac_4-β-D-Gal-(1 $\rightarrow$ 3)-benzylidene-α-D-GalNAc)Thr-OH (**10**) as a building block has been developed. Total synthesis of the AFGPs ($n = 4$, 8) in overall yields of 61 % and 33 %, respectively, has demonstrated the usefulness of the method. The synthetic AFGPs **1** ($n = 4$, 8) showed a similar conformation to the native AFGPs in their circular dichroism spectra.

Keywords: antifreeze · glycoproteins · peptides · solid-phase synthesis · T_F antigen

Introduction

Five distinct macromolecular antifreezes (antifreeze peptide type I, type II, type III, and type IV, and antifreeze glycoproteins) have been isolated and characterized from different marine fishes.[1] The antifreeze glycoproteins (AFGPs) **1** are found exclusively in certain Antarctic fish families and in the north temperate cod.[2] These proteins are mucin-like polymers of Ala-Ala-Thr with minor sequence variations and may be expressed as Ala-[Ala-(β-D-Gal-(1 $\rightarrow$ 3)-α-D-GalNAc)Thr-Ala]$_n$-Ala.[3] Their relative molecular masses range from about 2000 to 33 000 ($n = 4 - 55$).[4] Because of their characteristic prevention of the growth of ice crystals[5], antifreeze (glyco)proteins have potential applications in cryopreservation and the hypothermic storage of materials of biomedical interest.[6–8] However, a good characterization of AFGPs is difficult, due to the heterogeneity of AFGPs which are usually isolated as a mixture.[9] There are many reports on obtaining the antifreeze peptides through molecular cloning, but not on the carbohydrate-containing AFGPs.[10] Synthesis of these antifreezes has generated great interest in research circles, owing to the difficulties encountered in obtaining large enough amounts of AFGPs in a homogenous state, especially when synthesizing AFGPs in a single sequence. In addition to reports on the synthesis of some related analogues,[11] the synthesis of high-molecular-weight mixtures of AFGP has recently been reported by Nishimura et al.[12] Unfortunately, none of these AFGPs and analogues has been tested for antifreeze-protein-specific activity. Thus, a facile and efficient synthesis of AFGPs with a biological function is urgently required in order to give a complete explanation of the antifreezing phenomenon.[11b]

In this paper, we describe a facile route for preparing homogeneous AFGPs by a solid-phase method that uses protected T_F antigen **10**[13] as building blocks. We adopted a concise synthetic strategy to prepare the protected T_F antigen **10**. The key starting point was the use of phenyl selenoglycoside as a glycosyl donor to build a T_N construct as a "cassette", which would have the α-*O*-linked amino acid prebuilt into the GalNAc. This cassette serves as an acceptor to complete the synthesis of the *O*-linked glycopeptide building block **10** and enabled us to synthesize a single-sequence AFGP ($n = 4$, 8) by using the solid-phase method.

[a] Dr. S.-T. Chen, P.-H. Tseng, Dr. W.-T. Jiaang, Dr. M.-Y. Chang
Institute of Biological Chemistry
Academia Sinica, Nankang, Taipei 115 (Taiwan)
Fax: (+886) 2-2788-3473
E-mail: bcchen@gate.sinica.edu.tw

Results and Discussion

Building block synthesis: Because the AFGPs **1** have a repeating tripeptide sequence (Ala-Thr-Ala), the solid-phase synthesis method can be applied to the synthesis of AFGPs when suitable building blocks are available. The retrosynthesis of AFGPs **1** is shown in Scheme 1. The key step in the strategy for the synthesis of AFGPs **1** is the preparation of the T_F antigen **10**. The fully protected T_F antigen has previously been prepared by a variety of methods.[13, 14] We have developed an alternative method for preparing the antigen, which is compatible with solid-phase peptide chemistry.[15] The synthetic procedure for building block **10** is as follows. The known 2-azido-2-deoxy-1-selenoglycoside (**2**)[16] (prepared from the known tri-*O*-acetyl-D-galactal in 81 % yield) was *O*-deacetylated with NH_3(aq) in MeOH, followed by regioselective 4,6-*O*-benzylidenation with $PhCH(OMe)_2$ and

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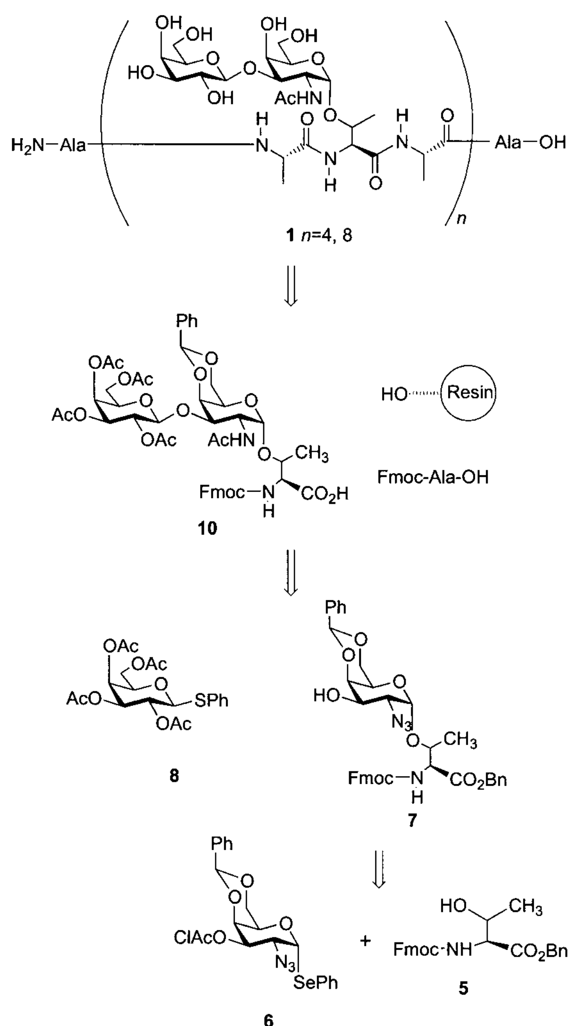
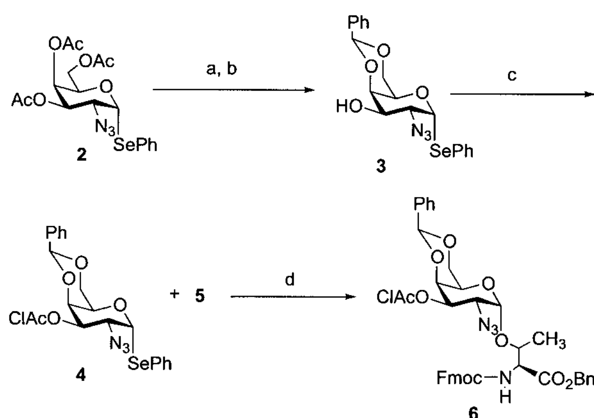


Scheme 1. Retrosynthesis of antifreeze glycopeptide **1**

para-toluenesulfonic acid (*p*-TsOH) to afford compound **3** (Scheme 2). We tried to use **3** as a glycosyl acceptor[17] in the reaction of the donor 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl imidate in CH_2Cl_2 at $-78\,^{\circ}C$ in the presence of a catalytic amount of trimethylsilyl triflate (TMSOTf), but the desired disaccharide product was not obtained. In this case,



Scheme 2. Reagents and conditions: a) MeOH, NH_3(aq). b) PTSA, $PhCH(OMe)_2$, THF, 90% from **2**. c) $(ClCH_2CO)_2O$, $NaHCO_3$, THF, quant. d) AgOTf, K_2CO_3, TMU, 87% from **4**, $\alpha/\beta = 7.2/1$.

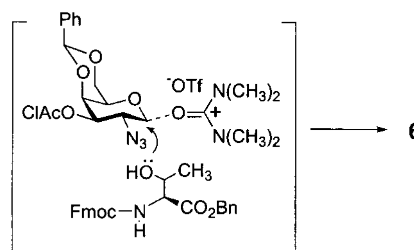
we found that the phenyl selenoglycoside **3** prefers to be a donor, as PhSe- makes a better leaving group than the imidate when TMSOTf is used as the Lewis acid.[15] On the basis of this result, we adopted an alternative strategy. Reaction of 3-*O*-unprotected selenoglycoside **3** with $(ClCH_2CO)_2O$ and $NaHCO_3$ gave **4** in quantitative yield (Scheme 2).[18] The α-phenylselenide **4** was coupled with the L-threonine derivative **5**, and the reactivity was influenced by conducting the reaction under various conditions (Table 1). We found that the better

Table 1. Glycosylation reactions of **4** with **5**[a]

	Catalyst (equiv)	Base (equiv)	T [°C][b]	α/β	yield [%]
1	TMSOTf[c]		−20		no reaction
2	AgOTf (3)	K_2CO_3 (5)	25	1/1	93
3	AgOTf (4)	K_2CO_3 (6)	−20	1.3/1[d]	94
4	AgOTf (4)	K_2CO_3 (6)/TMU (2)	25	3.8/1	89
5	AgOTf (4)	K_2CO_3 (6)/TMU (2)	−10–25[e]	7.2/1	87

[a] 1.5 equiv. [b] Reaction time 6–12 h at 25 °C or −20 °C, followed by TLC. [c] 0.05 equiv. [d] Reaction time 12 h. [e] Stirred at −10 °C, 4 °C, and 25 °C for 16 h, 12 h, and 8 h, respectively.

α/β selectivity occurred in the presence of tetramethyl urea (TMU; entries 4 and 5), and that a lower reaction temperature also led to a higher α/β ratio (entry 5). The coupling reaction of compound **4** and the L-threonine derivative **5** with AgOTf in the presence of K_2CO_3[17] and TMU[19] at lower temperatures (stirred at −10 °C, 4 °C, and 25 °C for 16 h, 12 h, and 8 h, respectively) afforded **6** as a separable 7.2:1 α/β mixture of anomers in 87 % yield (entry 5).[15] As shown in Scheme 3, the stereoselectivity could be explained by a reverse anomeric effect.[19b, 20]



Scheme 3. Possible mode of reaction of **5** with the transition state of TMU-protected **4** showing a reverse anomeric effect.

The chloroacetylated α-glycoside **6** was deblocked with thiourea to give **7** (Scheme 4).[18] Glycosylation of the threoninyl α-glycoside **7** as an acceptor with the known thioglycoside donor **8** in the presence of *N*-iodosuccinimide (NIS) and TfOH gave the desired disaccharide **9** in 93 % yield.[21] A large amount of TfOH (0.4 equiv) was used in order to avoid formation of the *ortho*-ester. In the last step, the azido moiety and the benzyl ester of **9** were hydrogenated with 5 % Pd/C under H_2 to provide the amino glycoside. The amine was converted to the acetamido derivative by Ac_2O in the presence of Et_3N to afford the building block **10** as a mixture of rotamers in 87 % yield (two steps).[14b] Thus, in the synthetic strategy to establish the protected T_F antigen **10**, it took only seven steps and the total yield from the known tri-*O*-acetyl-D-galactal was 40 %.

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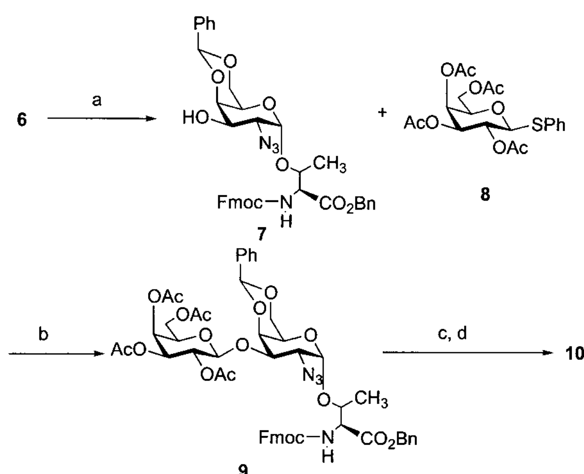
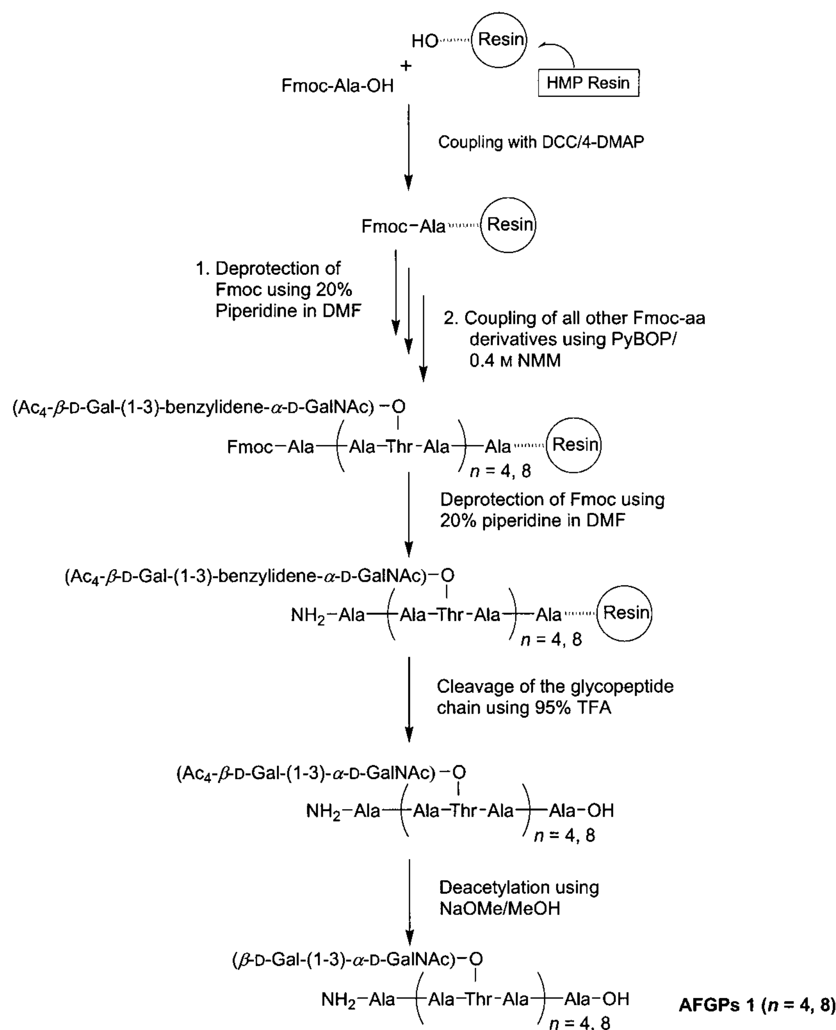


Scheme 4. Reagents and conditions: a) Thiourea, MeOH, 95% from **6**. b) NIS, TfOH, CH_3CN, CH_2Cl_2, $-45\,^{\circ}C$, 93%. c) H_2, Pd/C, MeOH. d) Ac_2O, Et_3N, 87% from **9**.

Antifreeze glycoprotein synthesis: By using **10** as a building block, the synthesis of AFGPs **1** ($n=4$, 8) was performed by the standard 9-fluorenylmethyloxycarbonyl (Fmoc) protocol[22] on an automatic peptide synthesizer. The synthetic scheme adopted for the solid-phase glycopeptide synthesis is illustrated in Scheme 5. The loading of the first amino acid to the HMP resin was achieved by the dicyclohexylcarbodiimide (DCC) method in the presence of 4-dimethylaminopyridine (4-DMAP). Apart from the first amino acid, the coupling of the amino acids was carried out by activating amino acid derivatives in situ with benzotriazole-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)[23] in 0.4 M *N*-methylmophorine (NMM). The Fmoc group was deprotected by 20% piperidine. When the synthesis of the desired glycopeptide was finished, the cleavage of the elongated glycopeptide from the resin and *O*-debenzylidenation was carried out with 95% trifluoroacetic acid (TFA). The crude glycopeptide fraction was then dissolved in dry MeOH with a catalytic amount of sodium methoxide (pH 10)[14b] to remove the acetyl groups that serve as protection for the hydroxyl groups of the disaccharide. The progress of the deacetylation was carefully monitored by analytical HPLC. The crude product of AFGPs **1** ($n=4$, 8) was purified by HPLC with a RP-C18 column. Figure 1 shows the HPLC purification profiles of synthetic AFGPs **1** ($n=4$, 8). As shown in Table 2, the total yield of AFGP **1** ($n=4$) was 61% and that of AFGP **1** ($n=8$) was 33% from Fmoc-Ala-OH attached to HMP resin until purification.

Analysis of synthetic AFGPs 1 ($n=4$, 8) by CD spectra: The CD spectra of AFGPs **1** ($n=4$, 8) are shown in Figure 2. Both spectra of these two AFGPs are similar and have positive CD bands at 218 nm and negative bands at 196 nm. Although there are several alternative models for the preferred conformations of AFGPs,[24] as well as questions as to the existence of the threefold left-handed-helix conformation, compared with the earlier reports of CD spectra of AFGPs[25] our result showed that synthetic AFGPs **1** ($n=4$, 8) have a similar secondary structure to native AFGPs.

We have shown that a homogeneous sequence of AFGP can be produced by solid-phase peptide synthesis. This differs from that of Nishimura et al.[12] who used a polymerization reaction and gel-permeation chromatography to efficiently produce a glycopolymer of a well-defined molecular weight, estimated to be 6000–7300 (10–12 repeating units). The



Scheme 5. Typical protocol used for the solid phase peptide synthesis.

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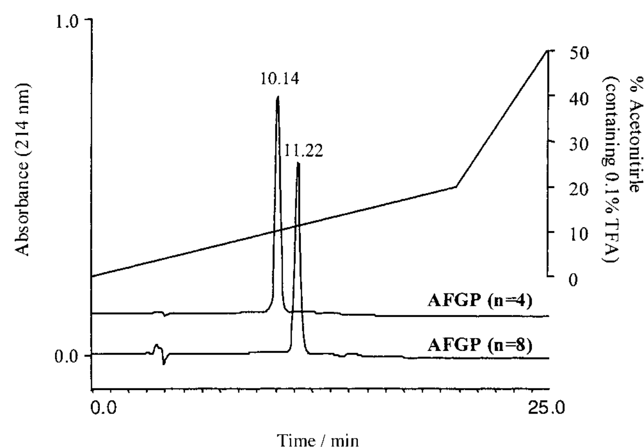

Figure 1. RP-HPLC purification profiles of synthetic AFGPs **1** ($n=4$, 8). Acetonitrile and water (both containing 0.1% TFA) were used as the solvent system at a flow rate of $1.0\,\mathrm{ml\,min^{-1}}$ on a Nucleosil C18 column (4.6×250 mm) at room temperature.

Table 2. The characteristics of the AFGPs **1** ($n=4$, 8)

	MW	Found ES-MS $[M+H]^+$	R_f value [min][a]	Yield in SPPS [mg] → [%]
AFGP ($n=4$)	2594.5	2594.0	10.14	158 → 61
AFGP ($n=8$)	5028.9	5028.0	11.22	166 → 33

[a] Each HPLC analysis was carried out by a gradient elution (see Figure 1).

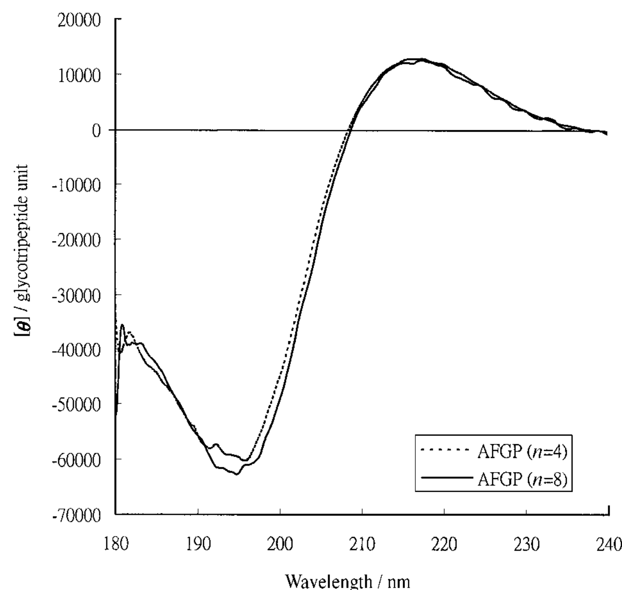

Figure 2. The CD spectra of AFGP **1** ($n=4$) and AFGP ($n=8$).

building block T_F antigen **10** was synthesized in a facile procedure with 40% yield. Both macromolecular AFGPs **1** ($n=4$, 8) synthesized in this method (61% and 33% yield, respectively) are ready for further biological study. In the future, the solid-phase approach will enable the synthesis and study of AFGP analogues in a combinatorial fashion.

Experimental Section

General procedures: The solid-phase peptide synthesis was performed on PS3 (Rainin). Column chromatography was performed on silica gel 60 (70–230 mesh, 230–400 mesh, Merck) and preparative HPLC on nucleosil C18 (4.6×250 mm). All RP-HPLC procedures were carried out with a gradient system consisting of two buffers: 0.1% TFA in MeCN/H_2O, 5:95 and 0.1% TFA in MeCN/H_2O, 95:5. Analytical TLC was carried out with silica gel 60 F254 (Merck), detected with UV light (254 nm) and with 5% $12MoO_3\cdot H_3PO_4\cdot XH_2O$ in ethanol and heated on a hotplate. Dichloromethane, acetonitrile, and TMU were dried over CaH_2 and distilled. The ^{1}H NMR spectra were recorded on a Bruker ASPECT3000 (400 MHz) spectrometer. The value of δ is expressed in ppm relative to the solvent signal as internal standard ($CHCl_3$: $\delta=7.24$, HOD: $\delta=4.7$, $HD_2C(O)CD_3$: $\delta=2.03$, $HD_2S(O)CD_3$: $\delta=2.49$). Electrospray mass spectra were measured with a Finnigan LC*Q* spectrometer. FAB mass spectra were measured with a JEOL SX-102A spectrometer. All reagents were commercially available. CD spectra were recorded at 177–250 nm on a Jasco-J715 spectrometer with quartz cells of 0.02 mm path length at 25°C.

Phenyl 3-*O*-acetyl-2-azido-4,6-benzylidene-2-deoxy-1-seleno-*α*-D-galactopyranoside (3): Compound **2** (23.5 g, 50.0 mmol) was dissolved in MeOH (120 mL), then NH_3 (aq, 25%, 50 mL) was added. The resulting solution was stirred at room temperature for 4 h and concentrated in vacuo. The residue was dissolved in dry acetonitrile (120 mL), benzaldehyde dimethyl acetal (10.3 g, 67.5 mmol) and *p*-TsOH (80 mg) were added, and the reaction mixture was stirred at room temperature for 3 h. The reaction was quenched with solid potassium carbonate (2 g). The mixture was then shaken for 30 min, filtered, and concentrated in vacuo. Flash chromatography of the residue (ethyl acetate/dichloromethane/hexane, 1:1:2) yielded **3** (19.4 g, 90%) as a light yellow liquid. ^{1}H NMR (400 MHz, [D6]acetone): $\delta=7.62-7.59$ (m, 2H, ArH), 7.52–7.49 (m, 2H), 7.35–7.29 (m, 6H), 6.09 (d, $J=5.0$ Hz, 1H, H1), 5.66 (s, 1H, PhCH), 4.39 (dd, $J=3.4$, 0.7 Hz, 1H), 4.25 (dd, $J=10.5$, 5.0 Hz), 4.17–4.14 (m, 2H), 4.00 (dd, $J=12.8$, 1.8 Hz, 1H), 3.93 (m, 1H); ^{13}C NMR (100 MHz, [D6] acetone): $\delta=139.1$, 134.5, 129.5, 129.1, 128.3, 127.9, 126.9, 101.1, 86.5, 86.4, 76.1, 70.8, 69.1, 66.1, 62.2; MS (FAB): *m*/*z* (%): 432.0 (30) $[M]^+$, 276.1 (100); HRMS (FAB) calcd for $C_{17}H_{23}ClN_3O_3Se$: 432.0571; found 432.0565.

Phenyl 2-azido-4,6-benzylidene-3-*O*-chloroacetyl-2-deoxy-1-seleno-*α*-D-galactopyranoside (4): Compound **3** (21.6 g, 50.0 mmol) and $NaHCO_3$ (16.8 g, 200 mmol) in dry THF (150 mL) were added to chloroacetic anhydride (17.2 g, 100 mmol), and the mixture was stirred at room temperature overnight. The mixture was then filtered and concentrated in vacuo. Purification of the residue by flash chromatography (ethyl acetate/dichloromethane/hexane, 1:1:3) yielded **4** (25.5 g, 100%) as a white solid. M.p. 127–129°C; ^{1}H NMR (400 MHz, [D6]acetone): $\delta=7.65-7.62$ (m, 2H, ArH), 7.50–7.48 (m, 2H, ArH), 7.35–7.32 (m, 6H, ArH), 6.25 (d, $J=5.2$ Hz, 1H, H1), 5.67 (s, 1H, PhCH), 5.13 (dd, $J=11.0$, 3.4 Hz, 1H, H3), 4.64–4.60 (m, 2H), 4.35 (s, 2H, $ClCH_2$), 4.24 (brs, 1H), 4.20 (dd, $J=12.6$, 1.8 Hz, 1H), 4.00 (dd, $J=12.6$, 1.5 Hz, 1H); ^{13}C NMR (100 MHz, [D6]acetone): $\delta=167.1$, 138.9, 134.9, 129.7, 129.2, 128.8, 128.4, 128.3, 126.8, 100.8, 85.4, 85.4, 73.7, 73.3, 73.3, 68.9, 65.5, 59.2, 41.0; MS (FAB): *m*/*z* (%): 510.1 (38) $[M]^+$, 352.0 (100), 307.1 (52), 289.0 (30); HRMS (FAB) calcd for $C_{21}H_{21}ClN_3O_5Se$: 510.0321; found 510.0317.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2-azido-4,6-benzylidene-3-*O*-chloroacetyl-2-deoxy-*α*-D-galactopyranosyl)-L-threonine benzyl ester (6)**: A mixture of AgOTf (10.3 g, 40.0 mmol), K_2CO_3 (8.30 g, 60 mmol), TMU (2.32 g, 20 mmol), threonine derivative **5** (6.47 g, 15.0 mmol) and drierite (5 g) in dry CH_2Cl_2 (100 mL) was stirred at room temperature for 30 min and then cooled to −10°C for 30 min. Compound **4** (5.10 g, 10.0 mmol) was added and the mixture was stirred at −10°C, 0°C, and 25°C for 16 h, 12 h, and 8 h, respectively. The resulting mixture was filtered through Celite. The filtrate was washed with saturated $NaHCO_3$ solution (80 mL) and water (80 mL), dried ($MgSO_4$), and concentrated in vacuo. Purification by flash column chromatography (ethyl acetate/dichloromethane/hexane, 1:1:2) yielded *α*-**6** (5.98 g, 76%) and *β*-**6** (0.83 g, 10.6%) each as a foam. *α*-**6**: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.76$ (d, $J=7.5$ Hz, 2H, ArH), 7.61 (d, $J=7.4$ Hz, 2H, ArH), 7.49–7.46 (m, 2H, ArH), 7.41–7.23 (m, 12H, ArH), 5.76 (d, $J=9.4$ Hz, 1H, NH), 5.50 (s, 1H, PhCH), 5.26–5.21 (m, 3H, H3, OCH_2Ph), 4.98 (d, $J=3.5$ Hz, 1H, H1), 4.47–4.43 (m, 4H), 4.37–4.32 (m, 1H), 4.26–4.21 (m, 2H), 4.14 (s, 2H, ClCH), 4.02–3.99 (m, 1H), 3.92 (dd,

$J = 11.0$, 3.6 Hz, 1H, H2), 3.70 (brs, 1H), 1.30 (d, $J = 6.4$ Hz, 3H, Thr-γ-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.2$, 166.9, 156.7, 143.8, 143.7, 141.3, 141.2, 137.2, 134.9, 129.2, 128.7, 128.6, 128.5, 128.2, 127.7, 127.1, 126.0, 125.2, 125.1, 119.9, 100.7, 98.7, 76.3, 72.8, 71.6, 68.9, 67.7, 67.4, 62.8, 58.6, 57.6, 47.1, 40.6, 18.6; MS (FAB): *m/z* (%): 783.3 (45) $[M]^+$, 352.0 (100), 307.0 (48); HRMS (FAB) calcd for $C_{41}H_{40}ClN_4O_{10}$: 783.2465; found 783.2470. β-**6**: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.73$ (d, $J = 7.6$ Hz, 2H, ArH), 7.60–7.58 (m, 2H, ArH), 7.46–7.45 (m, 2H, ArH), 7.36–7.22 (m, 12H, ArH), 5.78 (d, $J = 9.6$ Hz, 1H, NH), 5.46 (s, 1H, PhCH), 5.19–5.17 (m, 3H, H3, OCH_2Ph), 4.66–4.60 (m, 2H), 4.51 (dd, $J = 9.8$, 1.8 Hz, 1H), 4.40–4.30 (m, 3H), 4.23–4.20 (m, 3H), 4.14 (s, 2H, ClCH), 3.90–3.85 (m, 2H), 3.03 (s, 1H), 1.36 (d, $J = 6.4$ Hz, 3H, Thr-γ-CH_3); MS (electrospray) *m/z* (%): 783.0 (100) $[M]^+$, 531.2 (27), 432.1 (51), 415.2 (36).

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2-azido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester (7)**: A solution of compound **6** (7.83 g, 10.0 mmol), 2,6-lutidine (1.18 g, 11 mmol), and thiourea (2.28 g, 30.0 mmol) in MeOH (50 mL) was stirred at room temperature overnight. The resulting solution was concentrated in vacuo. The residue was diluted with EtOAc (100 mL), washed with saturated $NaHCO_3$ solution (80 mL) and water (80 mL), dried ($MgSO_4$), and concentrated in vacuo. Purification by flash column chromatography (ethyl acetate/dichloromethane/hexane, 1.5:1:2) yielded **7** (6.72 g, 95%) as a foam: ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.76$ (d, $J = 7.5$ Hz, 2H, ArH), 7.61 (d, $J = 7.4$ Hz, 2H, ArH), 7.49–7.46 (m, 2H, ArH), 7.42–7.25 (m, 12H, ArH), 5.79 (d, $J = 9.3$ Hz, 1H, NH), 5.55 (s, 1H, PhCH), 5.23 (s, 2H, OCH_2Ph), 4.93 (d, $J = 3.3$ Hz, 1H, H1), 4.50–4.40 (m, 4H), 4.36–4.32 (m, 1H), 4.26–4.20 (m, 3H), 4.10–4.01 (m, 2H), 3.69 (s, 1H), 3.53 (dd, $J = 10.5$, 3.4 Hz, 1H), 1.29 (d, $J = 6.4$ Hz, 3H, Thr-γ-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.1$, 156.8, 143.9, 143.6, 141.3, 137.2, 135.0, 129.4, 128.6, 128.5, 128.3, 127.7, 127.1, 127.1, 126.2, 125.2, 125.1, 119.9, 101.2, 99.2, 76.2, 75.3, 69.1, 67.7, 67.4, 67.3, 63.2, 61.1, 58.7, 47.1, 18.6; MS (FAB): *m/z* (%): 707.3 (35) $[M]^+$, 307.0 (90), 276.0 (100); HRMS (FAB) calcd for $C_{39}H_{39}N_4O_9$: 707.2732; found 707.2734.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-threonine benzyl ester (9)**: A solution of compound **7** (10.6 g, 15.0 mmol) and thioglycoside **8** (8.80 g, 20.0 mmol) in dry CH_2Cl_2 (50 mL) was cooled to $-45\,^{\circ}C$. A solution of *N*-iodosuccinimide (13.5 g, 60 mmol) in CH_3CN (60 mL) was added, followed by trifluoromethanesulfonic acid (0.90 g, 6.0 mmol) in one portion. The mixture was stirred at $-45\,^{\circ}C$ for 2 h. The resulting solution was diluted with ethyl acetate and filtered through Celite. The filtrate was washed with 10% aqueous $Na_2S_2O_3$ (120 mL), saturated $NaHCO_3$ solution (100 mL), and water (100 mL), dried ($MgSO_4$), and concentrated in vacuo. Purification by flash column chromatography (ethyl acetate/dichloromethane/hexane, 2:1:1) yielded **9** (14.5 g, 93%) as a foam. ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.76$ (d, $J = 7.5$ Hz, 2H, ArH), 7.60 (d, $J = 7.4$ Hz, 2H, ArH), 7.52–7.50 (m, 2H, ArH), 7.42–7.24 (m, 12H, ArH), 5.78 (d, $J = 9.4$ Hz, 1H, NH), 5.52 (s, 1H, PhCH), 5.40 (d, $J = 3.4$ Hz, 1H, H4′), 5.28 (m, 1H, H2′), 5.21 (s, 2H, OCH_2Ph), 5.04–5.02 (m, 1H), 4.92 (d, $J = 3.5$ Hz, 1H, H1), 4.77 (d, $J = 7.9$ Hz, 1H, H1′), 4.53–4.44 (m, 3H), 4.35–4.30 (m, 2H), 4.24–4.12 (m, 4H), 4.02–3.92 (m, 3H), 3.80–3.70 (m, 1H), 3.65 (s, 1H), 2.15, 2.04, 2.01, 1.98 (4s, 12H, $4 \times C(O)CH_3$), 1.30 (d, $J = 6.3$ Hz, 3H, Thr-γ-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.2$, 170.2, 170.1, 170.1, 169.4, 156.7, 143.8, 143.6, 141.3, 137.5, 134.8, 128.9, 128.7, 128.6, 128.5, 127.7, 127.1, 127.0, 126.1, 125.1, 125.0, 120.0, 102.3, 100.6, 99.3, 76.0, 75.8, 75.6, 71.0, 70.9, 69.0, 68.6, 67.7, 67.3, 66.9, 63.4, 61.3, 59.1, 58.7, 47.1, 20.6, 20.5, 18.8; MS (FAB): *m/z* (%): 1037.4 (23) $[M]^+$, 606.2 (52), 331.0 (100).

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-benzylidene-2-deoxy-α-D-galactopyranosyl]-L-threonine (10)**: Compound **9** (10.4 g, 10 mmol) was dissolved in MeOH (800 mL) and water (60 mL), and 5% palladium on activated carbon (4 g) was added. This mixture was vigorously stirred under H_2 (2 atm) for one day. The Pd/C was filtered off from the suspension, and the solution was evaporated in vacuo. The residue was stirred in dichloromethane (50 mL) in the presence of acetic anhydride (13 mmol) and triethylamine (30 mmol) at room temperature for 20 min. The resulting solution was diluted with dichloromethane (100 mL), washed with 10% aqueous citric acid (100 mL) and water (100 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography (ethyl acetate/ethanol/acetic acid, 90:10:1) to yield **10** (8.38 g, 87%) as a foam. Since the Fmoc amino acid derivatives exist as a mixture of rotamers, only characteristic ^1H NMR and mass data are presented. ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.89$ (d, $J = 7.5$ Hz, 2H, ArH), 7.74–7.71 (m, 2H, ArH), 7.43–7.30 (m, 9H, ArH and NH), 5.51 (s, 1H, PhCH), 5.27 (d, J = 3.4 Hz, 1H, H4′), 5.08 (dd, $J = 10.3$, 3.4 Hz, 1H, H3′), 4.90 (t, $J = 10.4$ Hz, 1H, H2′), 4.35–4.25 (m, 2H), 4.50–3.90 (m, 12H), 3.80 (dd, $J = 11.3$, 2.8 Hz, 1H), 3.71 (s, 1H), 2.01, 2.00, 1.90, 1.85 (4s, 12H, $4 \times C(O)CH_3$), 1.10 (d, $J = 6.3$ Hz, 3H, Thr-γ-CH_3); MS (FAB): *m/z* (%): 963.4 (24) $[M]^+$, 622.3 (100), 331.0 (94); HRMS (FAB) calcd for $C_{48}H_{55}N_2O_{19}$: 963.3383; found 963.3381.

Antifreeze glycopeptide (*n* = 4, 8), synthesis protocol: Amino acid couplings were allowed to run for at least 2 h. After the coupling of one amino acid or of building block **10**, the resin was washed with DMF, followed by twice deprotecting the α-amino group with piperidine (20% in DMF) for 15 min. After the resin was washed with DMF, the cycle was repeated with another amino acid or with building block **10**. During the periods of elongation, Ninhydrin reagent[26] was used to indicate the completion of the coupling or the deprotection reaction. For each AFGP, an HMP resin (1.03 mmol g^{-1}, 98 mg, 0.1 mmol) was used.

A preactivated (15 min) mixture of Fmoc-Ala-OH (125 mg, 0.4 mmol, 4 equiv) and DCC (1.0M, 0.5 mL) was added to the resin (98 mg swelled in 4 mL DMF) in the presence of 4-DMAP (0.1M, 0.36 mL). After 2 h, the resin was washed with MeOH and DMF to remove the DCU and the unreacted reagent. The coupling of other amino acids was carried out by activating 4 equiv of amino acid derivatives in situ with 4 equiv PyBOP[23] in NMM (0.4M, 5 mL) for 2 h. However, during the assembly of the glycosylated building block **10** at the desired position in the peptide sequence, only 2 equiv of **10** were used, and the coupling reaction period was prolonged from 2 h to 4 h. After the last amino acid was coupled to the resin, the Fmoc group was removed, and the resin was washed with DMF and DCM, dried by lyophilization, and then treated with a mixture of TFA/H_2O (10 mL, 95:5 v/v) for 2 h to cleave the glycopeptide from the resin and to remove the benzylidene protecting group from GalNAc. After removal of the TFA solution, the resin was washed with 10% acetic acid (200 mL) to dissolve the glycopeptide and dried by lyophilization. It was then treated for 5 h in MeOH (20 mL), to which NaOMe (0.1M) had been added until pH 10 was reached; this led to deacetylation. The crude solution of AFGP was neutralized, lyophilized, and purified by RP-HPLC. The fractions containing the correct compounds according to ES mass spectra were collected and lyophilized. The characteristics of AFGP **1** (*n* = 4, 8) are presented in Table 2.

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