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Synthesis of Fish Antifreeze Neoglycopeptides Using Microwave-Assisted "Click Chemistry"

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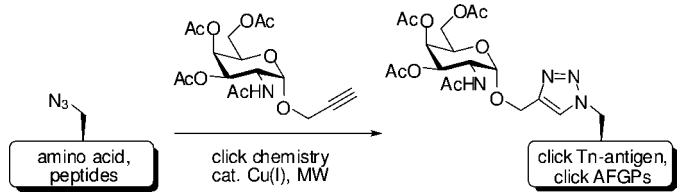
ABSTRACT



Microwave-enhanced click glycoconjugation of a propargylated α-GalNAc sugar moiety with an azido-functionalized amino acid or multiazido-functionalized peptides using a catalytic quantity of Cu(I) enabled a high-yielding and rapid synthesis of a "Tn-antigen mimic" and click analogues of antifreeze glycopeptides, thus demonstrating a valuable synthetic platform for the synthesis of biologically relevant neoglycopeptides.

Glycoproteins expressed on cell surfaces play key roles in diverse, complex biological processes such as cell signaling,[1] neuronal development,[2] immune surveillance[3] and inflammatory responses.[4] A common and well-studied motif for *O*-glycans found in higher eukaryotes is α-*N*-acetylgalactosamine (α-GalNAc) linked to the hydroxyl group of Ser/Thr, termed "Tn-antigen".[1] Thr-*O*-linked glycosides are also commonly present in antifreeze glycopeptides (AFGPs) isolated from Antarctic fish. AFGPs appear to act by binding to minute ice crystals present in the fish's body and depressing the freezing point of the fish's body fluid below the colligative freezing point. This process inhibits uncontrolled ice crystal growth and avoids tissue damage, allowing the supercooled fish to survive at subzero temperatures.[5] Although considerable research has been conducted to investigate the structure−activity relationship of AFGPs and to develop more accessible syntheses,[6] little is known about their detailed interaction with ice *in vivo*.[7]

AFGPs mainly consist of tripeptide (Ala-Ala-Thr*)$_n$ repeat units with chain lengths from $n = 4$ to 50 in which each threonine is glycosylated with a β-D-galactosyl-(1,3)-α-D-*N*-acetylgalactosamine disaccharide. In shorter AFGPs, alanine in the peptide backbone is occasionally substituted by proline (Figure 1).[5d]

Isolation of sufficient quantities of pure AFGPs from fish serum has proven difficult and natural AFGPs are difficult to synthesize in significant quantities.[7] However, it has recently been shown that complex glycopeptides are accessible using microwave-assisted solid phase peptide synthesis (SPPS), which facilitates highly efficient and fast coupling of the sterically demanding Thr-*O*-glycoside building blocks. Five trisaccharides derived from an α-*O*-linked GalNAc were incorporated into a mucin-type 20-mer[8] and AFGP analogues

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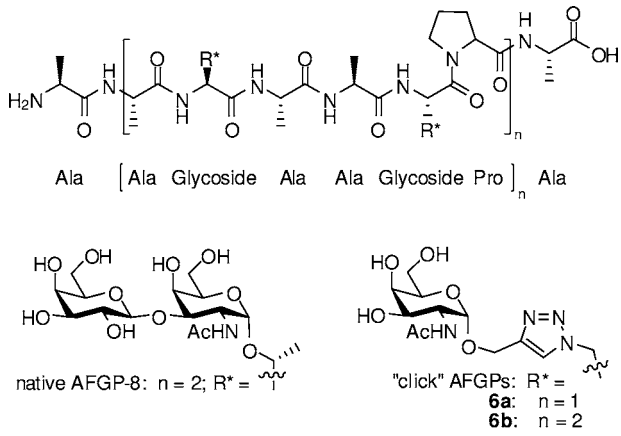


Figure 1. Structure of native AFGP-8 and neoglycopeptides (click analogues).

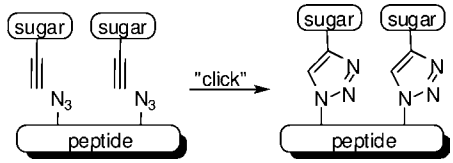
with (Ala-Ala-Thr*)$_n$-Ala-Ala sequences (n = 3, 4, 5) and proline containing AFGP analogues containing Thr-glycosylated α-GalNAc monosaccharides have also been synthesized,[9] and their activity has been quantitatively evaluated in ice recrystallization experiments.[10]

Studies on synthetic AFGP analogues by Nishimura et al.[6] showed that the presence of both an α-configured Thr-glycoside unit and an *N*-acetyl group at C-2 position are essential for activity. Furthermore, the structurally simpler α-GalNAc monoglycoside-containing AFGP exhibited comparable activity to the more complex naturally occurring AFGPs, as indicated by ice crystal morphology and measurements of thermal hysteresis.[6]

In higher organisms, different glycoforms of proteins are often generated by post-translational glycosylation, which modifies their functionality, stability and diversity. We anticipated that Cu(I)-catalyzed Huisgen azide−alkyne cycloaddition would present a powerful method for effecting glycosylation of a preformed peptide, enabling incorporation of any sugar of choice to afford the neoglycopeptide mimic of the natural glycopeptide for further study.[11,12]

We demonstrate herein that using microwave-assisted synthesis, analogues of AFGPs can be generated using SPPS *via* click reaction of an azide-functionalized peptide with an alkynylated sugar moiety (Scheme 1), thus providing a flexible platform for the synthesis of a wide range of neoglycopeptides.

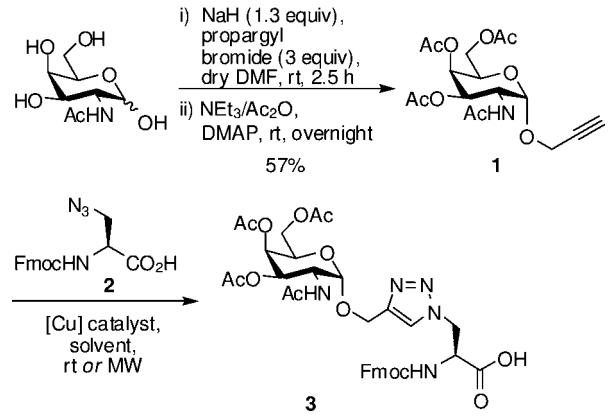
Scheme 1. Click Chemistry to Append Glycosides to an Azide-Functionalized Peptide Backbone



To demonstrate proof of principle, the sequence chosen was the shortest native AFGP (AFGP-8, Figure 1). For comparative purposes, it was proposed to synthesize the neoglycopeptide by clicking GalNAc residues onto the peptide backbone and by using SPPS incorporating the neoglycoside monomer at the appropriate place in the amino acid sequence.

Initial synthesis of the acetylene-functionalized sugar component, α-propargylated GalNAc(OAc)$_3$ **1**, was achieved in 57% yield over 2 steps on a 20 mmol scale from commercially available GalNAc (Scheme 2).

Scheme 2. Synthesis of Neoglycosyl Amino Acid Building Block



Selective deprotonation at the anomeric position using sodium hydride and subsequent alkylation with propargyl bromide in DMF afforded **1** after exhaustive acetylation. Interestingly, the proportion of α-anomer generated under these conditions was >90%[13] with the α-anomer being readily isolated by flash chromatography. This method proved superior to Fisher-type glycosylation using H_2SO_4-activated silica, which suffered from

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(13) Ratio of α:β-**1** was determined from the ^{1}H NMR spectra of the crude reaction mixture. The same conditions were applied by Beau et al. to the propargylation of GluNAc(OH)$_4$, yielding 65% β- and 9% α-*O*-propargyl-GluNAc(OAc)$_3$: Vauzeilles, B.; Dausse, B.; Palmier, S.; Beau, J. M. *Tetrahedon Lett.* **2001**, *42*, 7567–7570.

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Table 1. Synthesis of **3** *via* Click Reaction at Room Temperature (A) or under Microwave Irradiation at 80 °C (B)[a]

entry	catalyst	solvent	condition	time	conversion[b] (%)
	2 mol % $CuSO_4$·5 H_2O	CH_3CN/H_2O 2:1	A	18 h	0
1	5 mol % Na ascorbate				
	2 mol % CuI	H_2O/*t*BuOH 1:1	A	16 h	17
	5 mol % ascorbic acid				
2	7 mol % DIPEA				
	2 mol % $CuSO_4$·5 H_2O	H_2O/*t*BuOH 1:1	A	16 h	53
3	5 mol % Na ascorbate				
	2 mol % $Cu(OAc)_2$	H_2O/*t*BuOH 1:1	A	16 h	70
4	5 mol % Na ascorbate				
	2 mol % $CuSO_4$·5 H_2O	H_2O /*t*BuOH/DCM	A	18 h	100(81)[c]
5	5 mol % Na ascorbate	1:1:1			
	2 mol % $CuSO_4$·5 H_2O	H_2O/*t*BuOH/DCM	A	22 h	100(82)[c]
6[d]	5 mol % Na ascorbate	1:1:1			
	5 mol % $CuSO_4$·5 H_2O	H_2O/*t*BuOH	B	<5 min	100 (76)
7[d]	15 mol % Na ascorbate	1:1			
	5 mol % $CuSO_4$·5 H_2O	H_2O/*t*BuOH/DCM	B	<5 min	100 (80)
8	15 mol % Na ascorbate	1:1:1			
	5 mol % $Cu(OAc)_2$	H_2O/*t*BuOH	B	<5 min	100 (87)
9	15 mol % Na ascorbate	1:1			

[a] Reactions performed on 0.1 mmol scale with a ratio **1** to **2** of 1.5:1. [b] Conversion based on consumption of azidoalanine **2**. [c] Isolated yield of **3** in parentheses. [d] Scale (0.5 mmol).

low reactivity when using $GalNAc(OAc)_4$.[14] The second component, Fmoc-L-azidoalanine (**2**), was synthesized in 88% yield by diazo transfer[15,16] from commercially available *N*-α-Fmoc-L-diaminopropionic acid using a modification of the method of Cho *et al.*[17] in which DCM was substituted for MeOH/water as solvent.

With both building blocks in hand, attention turned to the synthesis of neoglycoside **3** (Scheme 2). A number of conditions were screened in which solvent and catalyst were varied at room temperature (Table 1, entries 1−6). $CuSO_4$·5 H_2O and $Cu(OAc)_2$/sodium ascorbate were found to be suitable catalysts yielding 53 and 70% of **3** after 16 h (entries 3 and 4). The yield could be further improved to 81 and 82% of **3** using DCM as cosolvent with H_2O/*t*BuOH (1:1:1) and 2 mol % $CuSO_4$·5 H_2O or $Cu(OAc)_2$ (entries 5 and 6).

Encouraged by these results, we sought to improve the reaction further using microwave irradiation, as has been previously demonstrated.[18,19] It was found that the rate of conversion accelerated dramatically using microwave irradiation: 100% conversion in less than 5 min was observed at 80 °C with 5 mol % $CuSO_4$·5 H_2O and $Cu(OAc)_2$ and 15 mol % sodium ascorbate (entries 7−9). Under microwave conditions use of $Cu(OAc)_2$ appeared to be the best catalyst system, yielding **3** in 87% yield in H_2O/*t*BuOH (entry 9). Moreover, even using just 2 mol % Cu catalyst and 5 mol % sodium ascorbate, a considerably lower catalyst loading than previously reported in the literature,[20] full conversion took place within 30 min (Table 2). $CuSO_4$·5 H_2O in H_2O/*t*BuOH gave just 42% conversion, whereas an excellent yield of 88% was obtained using DCM as cosolvent (entries 1 and 3). Other catalyst systems such as $(EtO)_3PCuI$ or CuI did not result in full conversion (entries 4−7).

Table 2. Synthesis of **3** via Click Reaction under Microwave Irradiation at 80 °C[a]

entry	catalyst	solvent	conversion[b] (%)
	2 mol % $CuSO_4$·5 H_2O	H_2O/*t*BuOH	42
1	5 mol % Na ascorbate	1:1	
	2 mol % $Cu(OAc)_2$	H_2O /*t*BuOH	100 (54)[d]
2[c]	5 mol % Na ascorbate	1:1	
	2 mol % $CuSO_4$·5 H_2O	H_2O/*t*BuOH/DCM	100 (88)[d]
3[c]	5 mol % Na ascorbate	1:1:1	
	10 mol % $(EtO)_3PCuI$	Toluene	18
4	20 mol % DIPEA		
	5 mol % CuI	H_2O/*t*BuOH 1:1	63
	15 mol % ascorbic acid		
6	20 mol % DIPEA		
	10 mol % $(EtO)_3PCuI$	H_2O/*t*BuOH 1:1	49
7	20 mol % DIPEA		

[a] Reactions performed on 0.1 mmol scale with a ratio **1** to **2** of 1.5:1 under 30 min microwave irradiation at 80 °C. [b] Conversion based on consumption of azidoalanine **2**. [c] Scale (0.5 mmol). [d] Isolated yield of **3** in parentheses.

With the glycosylated amino acid **3** in hand, the syntheses of peptides **4a** and **4b** were next examined (Scheme 3). In order to monitor the efficiency of the coupling steps *per*-acetylated AFGP **4a** containing two neoglycoside units was synthesized by manual Fmoc-SPPS using Fmoc-Ala preloaded Wang resin, with couplings performed under standard conditions (HBTU, DIPEA). Subsequently, *per*-acetylated

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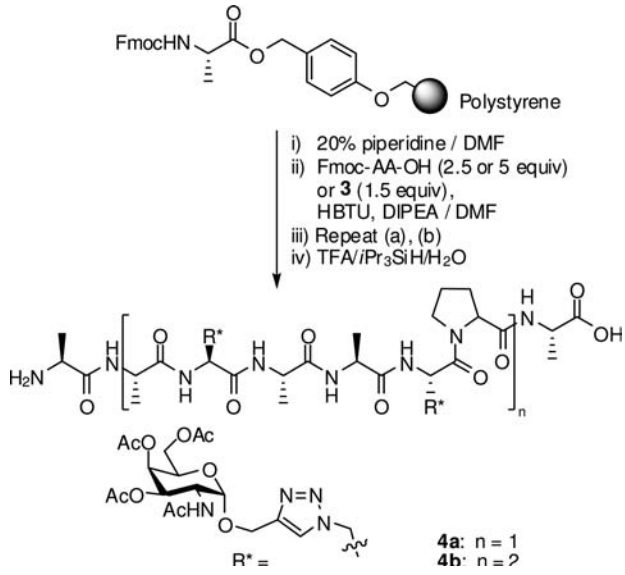
(19) Moorhouse, A. D.; Moses, J. E. *Synlett* **2008**, 2089–2092.
(20) Catalyst loadings of 1−5 mol % in presence of sodium ascorbate in H_2O/tBuOH are standard conditions for click reactions at room temperature.[14,21] However, higher catalyst loadings of 10 mol % are used for microwave reactions.[18,19]
(21) Baron, A.; Bleriot, Y.; Sollogoub, M.; Vauzeilles, B. *Org. Biomol. Chem.* **2008**, *6*, 1898–1901.

Scheme 3. Fmoc-SPPS of **4a** and **4b**

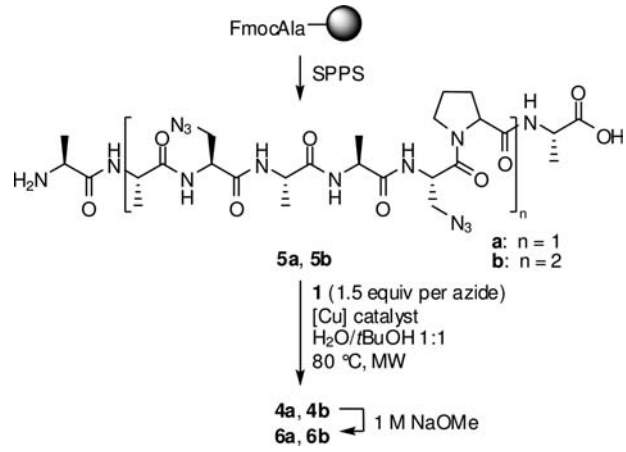


AFGP **4b** was synthesized by microwave-enhanced automated Fmoc-SPPS.

The second synthetic strategy was next investigated (Scheme 4). This procedure entailed incorporation of azidoalanine **2**[17] into the peptide backbone under standard SPPS conditions to afford peptides **5a** and **5b** followed by click reaction with **1**. Click reaction of azidoalanine containing peptides **5a** and **5b** with **1** was performed under microwave irradiation at 80 °C in H_2O/*t*BuOH (1:1) with 1.5 equiv of **1** per azide group monitoring the reaction by HPLC. Full conversion of model compound **5a** to **4a** was obtained in less than 10 min with 10 mol % $CuSO_4$·5 H_2O and 25 mol % sodium ascorbate. Similarly, **5b** was converted to **4b** with 20 mol % $CuSO_4$·5 H_2O and 50 mol % sodium ascorbate. Previously, such microwave-enhanced reactions on peptide[11,22] and peptoid[23,24] backbones were performed with large excesses of copper. Deacetylation of **4a** and **4b** with 1 M NaOMe solution in methanol at pH 11.3 afforded AFGP analogues **6a** and **6b** after purification in ca. 93% and 97% purity.

In summary, we have developed an efficient synthesis of a valuable α-GalNAc-containing Tn-antigen mimic and used this building block in microwave-enhanced automated Fmoc-SPPS of analogues of AFGPs. In a second strategy, the glycoconjugation was performed on a multiazido-functionalized peptide backbone using only a catalytic quantity of Cu(I). Use of microwave irradiation enabled high-yielding and rapid syntheses of the neoglycopeptides providing a platform for the generation of a more extensive range of neoglycopeptides of biological relevance. The antifreeze activity of the synthesized peptides will be assessed to establish whether the sugar moiety tethered remotely from the peptide backbone *via* the triazole linker affords similar biological activity.

Scheme 4. Click Chemistry on an Azide-Functionalized Peptide Backbone



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Supporting Information Available: Experimental procedures, copies of NMR spectra, and LCMS Data. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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