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SYNTHESIS OF AN OXIME-LINKED NEOGLYCOPEPTIDE WITH GLYCOSYLATION-DEPENDENT ACTIVITY SIMILAR TO ITS NATIVE COUNTERPART

Lisa A. Marcaurelle, Elena C. Rodriguez and Carolyn R. Bertozzi*

Department of Chemistry, University of California, Berkeley, CA 94720

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Abstract: Neoglycopeptides containing an oxime sugar-peptide linkage can be generated by coupling an aminooxy sugar with a peptide bearing a keto-amino acid. The coupling reaction can be executed in aqueous milieu without need for protecting groups on the peptide or saccharide, or auxiliary coupling reagents. Using this method, an oxime-linked analog of an antimicrobial peptide with glycosylation-dependent function was prepared and found to have similar bioactivity to the native glycopeptide. Thus, replacement of the sugar-peptide bond with an unnatural but synthetically facile linkage can produce functional neoglycopeptides.

An appealing strategy for the assembly of glycopeptides is the convergent coupling of an oligosaccharide with a pre-formed peptide. While this strategy has been implemented in the synthesis of *N*-linked glycopeptides,[1] *O*-linked glycopeptides have been prohibitive due to the difficulty in forming a glycosidic bond in the presence of multifunctional proteins and carbohydrates. However, if the native glycosidic linkage between the sugar and the peptide backbone were replaced with an alternate non-native linkage, a convergent synthesis of *O*-linked glycopeptides could be realized. To date, this strategy has not been widely explored, perhaps due to the suggestion that the proximal GalNAc residue of *O*-linked glycoproteins plays a role in modulating local peptide conformation.[2] However, the importance of the native sugar-peptide linkage for the *bioactivity* of *O*-linked glycopeptides, especially those with glycosylation-dependent function, has yet to be addressed. It may be that in some cases this linkage can be substituted without dramatically altering global structure or function. The complexity of glycopeptide synthesis could certainly be reduced by replacing the sugar-peptide linkage with a more facile bond.

We synthesized an *O*-linked glycopeptide with glycosylation-dependent activity (**1**) and a neoglycopeptide analog (**2**) in which the sugar and peptide are linked by an oxime (Fig. 1). Glycopeptide **1**, named drosocin, is an antimicrobial substance produced by insects in response to immune challenge, and its potency in blocking bacterial growth is enhanced approximately five-fold by the presence of a GalNAc residue at Thr11.[3] A similar glycosylation-induced enhancement in potency is observed for other drosocin glycoforms,[3,4] suggesting that the glycan exerts a conformational influence on the peptide.

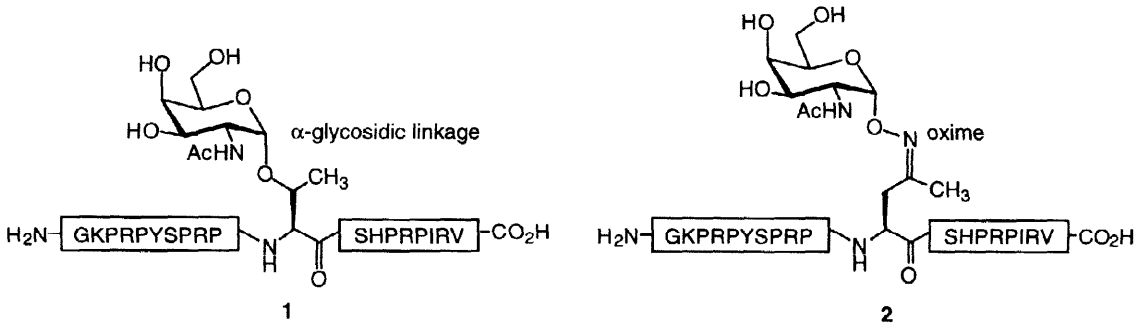

Figure 1. Native drosocin (**1**), bearing a proximal GalNAc residue attached in an α-glycosidic linkage to Thr11, and a drosocin neoglycopeptide (**2**) bearing an oxime-linked α-GalNAc residue.

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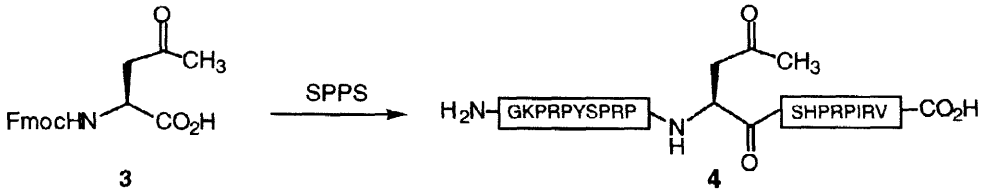
We envisioned that the oxime-linked neoglycopeptide (**2**) could be obtained from the highly selective reaction of an aminooxy sugar with a peptide bearing an unnatural ketone side chain. The ketone group is chemically orthogonal to all natural amino acid side chains and reacts with aminooxy groups in a highly specific manner, allowing site-specific conjugation without the requirement for protecting groups on the sugar or peptide. While methods for the synthesis of neoglycopeptides bearing other non-native linkages are well known,[5] the majority of these methods involve the coupling of electrophilic carbohydrate derivatives with nucleophilic amino acids. For example, bromoacetamides[5d] or isothiocyanates[5e] can be coupled with exposed cysteine or lysine residues, respectively. However, in the presence of several copies of these nucleophilic residues, such methods are not site selective.

As described in the companion paper,[6] unprotected ketone groups can be incorporated into a peptide using Fmoc-protected (2*S*)-aminolevulinic acid (**3**) by solid-phase peptide synthesis (SPPS) (Scheme 1). We generated keto-drosocin (**4**) in this fashion, in which the (2*S*)-aminolevulinic acid residue replaced Thr11, the natural site of glycosylation.

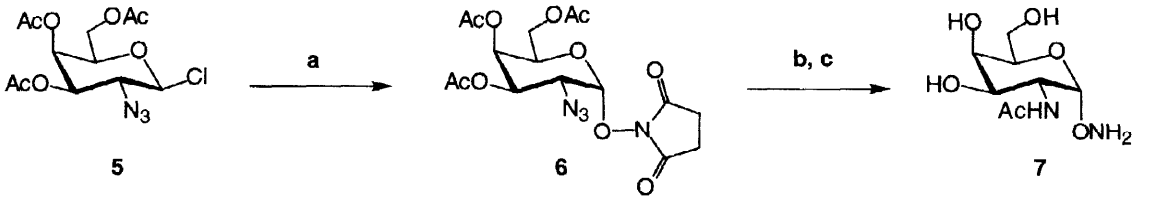
Scheme 1



3 **4**

The aminooxy sugar, α-GalNAc derivative **7** (Scheme 2), was generated from glycosyl chloride **5**[7] using a method similar to that reported by Roy and coworkers.[8] Compound **5** was reacted with *N*-hydroxysuccinimide (NHS) to afford the α-NHS glycoside **6**. Reductive acetylation of the azide, followed by deprotection of the acetyl esters and succinimido group provided the desired aminooxy sugar (**7**).[9]

Scheme 2



5 **6** **7**

Reagents and conditions: (a) *N*-hydroxysuccinimide, $Bu_4N(HSO_4)$, 1:1 CH_2Cl_2/1 M Na_2CO_3, 52%; (b) H_2, 10% Pd/C, Ac_2O, 100%; (c) $H_2N_4{\cdot}H_2O$, 71%.

The coupling reaction of keto-drosocin (**4**) with aminooxy GalNAc (**7**) was carried out in 1.0 M NaOAc buffer, pH 5.5 at 37 °C (Fig. 2A). The progress of the reaction was monitored by reversed-phase HPLC (Fig. 2B). The reaction was essentially complete after eight hours, as indicated by the presence of a single peak in the HPLC trace. No significant byproducts were observed, and the identity of the drosocin neoglycopeptide (**2**) was confirmed by electrospray mass spectrometry (ES-MS).

In order to evaluate the functional consequences of replacing the glycosidic linkage at Thr11 with an unnatural oxime linkage, we evaluated the bacteriostatic activity of neoglycopeptide **2** and compared its activity to both native (**1**) and unglycosylated drosocin.[10] As shown in Fig. 3, the oxime-linked neoglycopeptide (**2**) was found to be four-fold more potent in blocking bacterial growth ($IC_{50} = 0.16 \pm 0.04$ μM) than unglycosylated drosocin ($IC_{50} = 0.63 \pm 0.05$ μM), and similar in potency to native drosocin (**1**) ($IC_{50} = 0.10 \pm 0.02$ μM). These results indicate that the native sugar-peptide linkage in drosocin is not essential to achieve glycosylation-dependent enhancement in potency. We are interested in exploring the extent to which this observation extends to other *O*-linked glycopeptides.

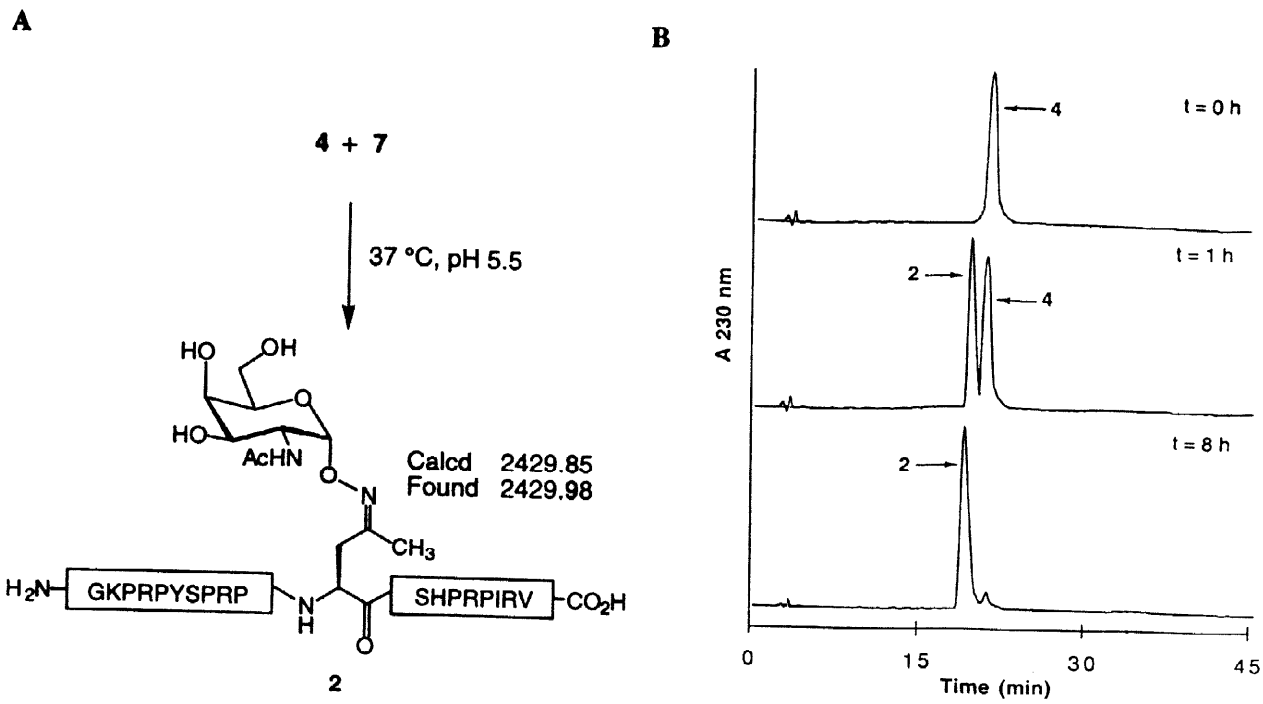


Figure 2. (A) Coupling reaction of keto-drosocin (4) with aminooxy GalNAc (7) to give oxime-linked neoglycopeptide 2. (B) Reversed-phase (C_{18}) HPLC analysis at t = 0, 1 and 8 h. Elution gradient: CH_3CN (B) in H_2O (A), both with 0.1% TFA (10 → 30% B, over 30 min).

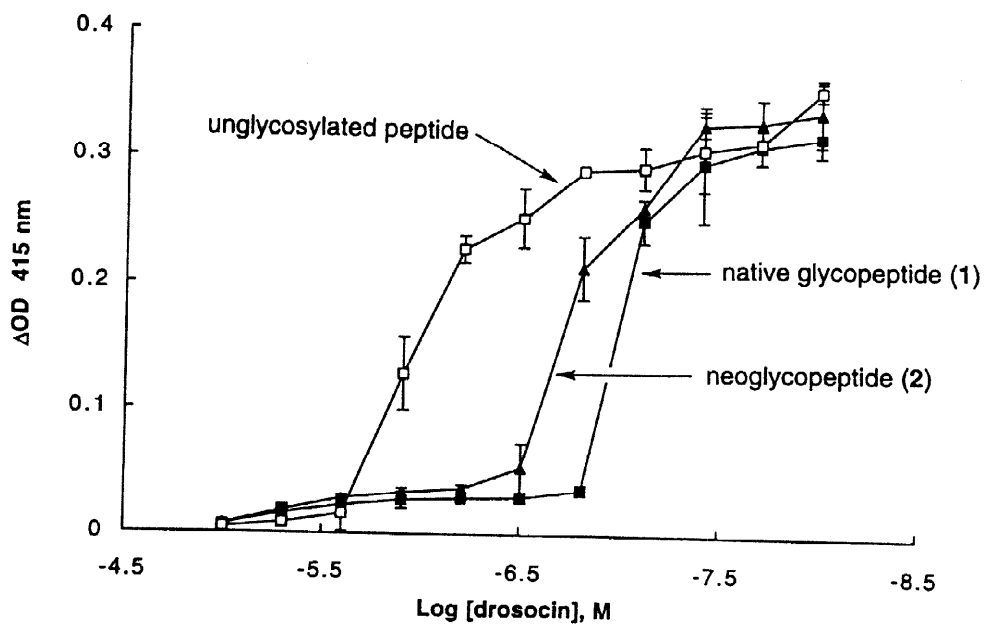


Figure 3. Inhibition of bacterial growth by drosocin neoglycopeptide 2, native drosocin (1) and unglycosylated drosocin. Bacterial growth was determined by measuring the change in optical density (ΔOD) at 415 nm over a 24-h period. Error bars represent the standard deviation of two or three measurements.

In summary, we have shown that a functional neoglycopeptide can be synthesized by the condensation of an aminooxy sugar with a keto-peptide. There are several convenient features of this approach. First, this method obviates the need for the cumbersome assembly of glycosylated amino acids typically used in the synthesis of native *O*-linked glycopeptides.[12] Second, the carbohydrate can be installed at a user-defined location within any given peptide without concern for differential protection of amino acid side chains. Third, the highly selective coupling reaction is carried out under aqueous conditions without use of auxiliary coupling reagents. Furthermore, the only byproduct of the reaction is water, minimizing the need for purification. Finally, a variety of neoglycopeptides can be obtained from a single keto-peptide using this convergent synthetic strategy.

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9. Characterization of aminooxy-2-acetamido-2-deoxy-α-D-galactopyranoside (**7**): ^{1}H NMR (300 MHz, D_2O): δ 4.97 (d, 1 H, J = 4.0), 4.23 (dd, 1 H, J = 11.3, 4.0), 4.00 (m, 2 H), 3.81 (m, 3 H), 2.06 (s, 3 H); ^{13}C NMR (100 MHz, D_2O): δ 174.55, 100.55, 70.93, 68.34, 67.49, 61.07, 49.11, 21.83; FAB-HRMS calcd for $C_8H_{17}N_2O_6$ ($M+H^+$) 237.1087, found 237.1084.
10. The growth inhibition assay was performed essentially as described by Bulet *et al.*[3] Sterile 96-well plates were used, with a final volume of 100 μL per well. This volume consisted of 90 μL of mid-logarithmic phase culture of *E. coli* D22 in LB media containing streptomycin (50 μg/mL), added to 10 μL of serially diluted peptide (native drosocin (**1**), unglycosylated drosocin or neoglycopeptide **2**) in water. Peptide concentrations were determined based on tyrosine content by measuring the absorbance at 280 nm and assuming ε = 1.490 $M^{-1}cm^{-1}$ as described by Pace *et al.*[11] Final peptide concentrations ranged from 10^{-8} to 10^{-5} M. Plates were incubated for 24 h at rt. Bacterial growth was determined by measuring the absorbance at 415 nm on a BioRad 550 microplate reader. Measurements were taken at t = 0 h and t = 24 h, and the change in absorbance (optical density) was recorded.
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