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Bioorganic & Medicinal Chemistry Letters 17 (2007) 2459–2464
Bioorganic & Medicinal Chemistry Letters

Synthesis of soluble multivalent glycoconjugates that target the Hc region of botulinum neurotoxin A

Ramesh R. Kale,[a] Colin M. Clancy,[b] Rebecca M. Vermillion,[a] Eric A. Johnson[b] and Suri S. Iyer[a,*]

[a]*301 Clifton Court, Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221, USA*
[b]*1925 Willow Drive, Department of Food Microbiology and Toxicology, Food Research Institute, University of Wisconsin–Madison, Madison, WI 53706, USA*

Received 26 December 2006; revised 6 February 2007; accepted 9 February 2007
Available online 14 February 2007

Abstract—The design, synthesis, and initial inhibitory studies of di- and tetravalent glycoconjugates that target the heavy chain of botulinum neurotoxin A are reported.

Botulinum toxin, produced by the gram-positive, spore forming *Clostridium botulinum*, is one of the most poisonous proteins found in Nature.[1] It has been estimated that a gram of aerosolized botulinum neurotoxin A (BoNT/A) could potentially cause over a million deaths in a densely populated area.[2] The clinical manifestations of botulism are based on observation alone and are subject to misdiagnosis, particularly in the early stages of intoxication. Current treatment is limited to administration of neutralizing antibodies and life support to prevent respiratory failure. Furthermore, effective small molecule inhibitors are not available as therapeutics or prophylactics. The ease of transmission and the paralyzing nature of the toxin have led the CDC and NIAID to classify botulinum as a Category A Select Agent. The high potency of the toxin coupled with recent bioterrorist events and lack of small molecule drugs has led to a renewed interest in developing therapeutics and diagnostics for these toxins.

All seven serotypes of botulinum toxin consist of two fragments, a 50 kDa catalytic domain and a 100 kDa binding and translocation domain. Several recent reports have targeted the 50 kDa light chain to develop small molecule inhibitors of the zinc protease,[3] however, suitable drug candidates specific to the heavy chain would also be valuable.[4] The 100 kDa fragment consists of H_N and H_C fragments, of which the H_C is responsible for binding to complex glycolipids, GD1a/b and GT1a/b. Indeed, polysialylated GT1b has been shown to inhibit synaptosomes and to quench BoNT/A fluorescence.[5,6] Recent crystal structures of clostridial neurotoxins provide the first direct identification and characterization of the binding sites to their natural ganglioside receptors.[7] It is obvious that multivalent binding is prevalent, as X-ray structures reveal that the binding sites for individual units (monosaccharide) of glycolipid are widely separated. The terminal sialic acid of the two branches of the gangliosides is crucial in binding and it induces cross-linking by binding to two distinct sites on the Hc fragment. The structure of the GT1b is shown in Figure 1 (left). Our interest in this area is twofold: (1) Using the structure of the ganglioside as a starting point combined with a modular synthetic approach, we were interested in developing a panel of glycoconjugates that could potentially block the toxin from cell entry. (2) The tailor-made soluble GT1a/b analogues could be used for Bot epitope mapping to define the intricate mechanism of infection. Tailor-made soluble glycoconjugates are important for structural biology studies; recent studies have shown that the structural differences are present in the different serotypes which can be exploited to differentiate between the serotypes, and develop better inhibitors.[7a,17a–c] Thus, this report describes our efforts to develop soluble glycoconjugates that could be used for structural biology

Keywords: Glycoconjugates; Multivalent; Botulinum A.
* Corresponding author. Tel.: +1 513 556 9273; fax: +1 513 556 9239; e-mail: suri.iyer@uc.edu

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doi:10.1016/j.bmcl.2007.02.028

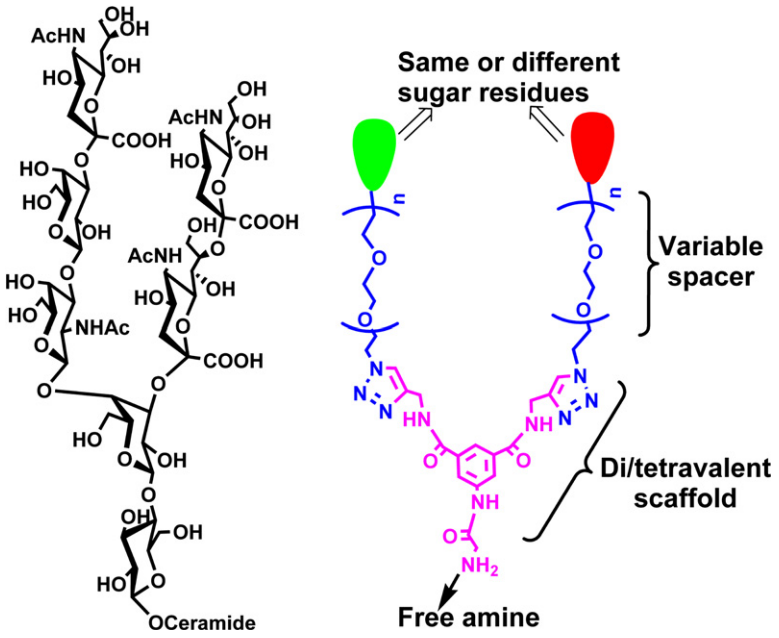


Figure 1. Structures of GT1b (left) and designer divalent ligand (right).

studies or as competitive inhibitors to prevent toxin binding or find use in diagnostics as front end capture ligands.

We reasoned that a tailored bivalent molecule with carbohydrate residues at the termini of variable spacers would mimic the two arms of the natural ganglioside. The designer molecule is shown in Figure 1 (right). The three components of the molecule are the carbohydrate residues, the oligoethylene oxide spacers, and the dimeric scaffold bearing a protected amine at the meta position. The first critical component is the variability of the oligoethylene spacers. We have chosen tetraethylene glycol spacers as our starting material; however, we can vary the length of the spacers to potentially control the binding stoichiometry. Shorter spacers should result in aggregation as the terminal sugars will be prevented from wrapping around the molecule for a perfect fit. The bifunctional oligoethylene glycols are designed to terminate in an azide, which allows for facile 1,3 dipolar addition to an alkyne bearing molecule.[8] The second component is the dimeric scaffold. The dimeric scaffold bears two alkyne functionalities and a protected amine. 5-amino-isophthalic acid was chosen as the rigid core because the two acid functionalities are in the meta position, reducing the possible formation of a lactone byproduct. The amine functionality is critical as it can be used to tether the molecule to a surface/magnetic bead/fluorophore or extended to a higher order dendron. The third and most important piece of the molecule is the recognition element. While we have concentrated on the development of high affinity ligands for botulinum neurotoxins, any azide bearing sugar/antibody/aptamer can be used. This modular design strategy was utilized to synthesize five compounds (Fig. 2).

The synthesis of the dimeric scaffold is shown in Scheme 1. First, 5-amino isophthalic acid was reacted with chloroacetyl chloride and subsequently reacted with aqueous ammonia and the amine formed was protected with a CBz group in high yields.[9] Next, the acid groups were activated using CDMT/NMM and reacted with 2.2 equivalents of propargyl amine to yield **5**. The terminal alkynes of **5** resonated at 3.15 and 66.0 ppm in the ^{1}H and ^{13}C NMR spectra in DMSO-d_6, respectively (HRMS $M+H^+ = 447.2182$). We were also interested in studying the binding affinity of multiple sialic acid residues with the toxin and therefore, we synthesized a tetrameric scaffold as shown in Scheme 2. The amine group of **1** was protected using Boc anhydride and the acid groups were activated using CDMT/NMM and reacted with 2.2 equivalents of propargyl amine to give **7**. Deprotection of the Boc group, followed by sequential treatment with bromoacetyl bromide and methanolic ammonia, furnished amine **9**. Two equivalents of **9** was reacted with the carboxylic acid residues of the amino protected isophthalic acid derivative **4** in the presence of CDMT/NMM to yield a dendron, **10**, bearing four terminal alkynes, in good yields. The terminal alkynes resonated at 3.16 and 66.0 ppm in the ^{1}H and ^{13}C NMR spectra in DMSO-d_6, respectively (HRMS $M+H^+ = 961.3275$).

Next, we synthesized two carbohydrate–oligoethylene conjugates bearing an azide functionality at the terminus of an oligoethylene oxide spacer. Briefly, the anomeric acetate of lactose octaacetate **11** was specifically cleaved in the presence of hydrazine acetate and activated to trichloroacetimidate **13** using K_2CO_3. Reaction with a tetraethylene glycol spacer in the presence of TMSOTf yielded the desired β-lactoside **14** in reasonable yields as shown in Scheme 3. The anomeric protons resonated at 4.53 ppm ($J_{1,2} = 8.0$ Hz) and 4.44 ppm ($J_{1,2} = 8.0$ Hz) in the ^{1}H NMR spectrum, respectively, which is in good agreement with expected values. While a similar methodology could be used to attach an oligoethylene oxide spacer to the sialic acid residue, we chose to synthesize the thiosialoside for two reasons. One, the

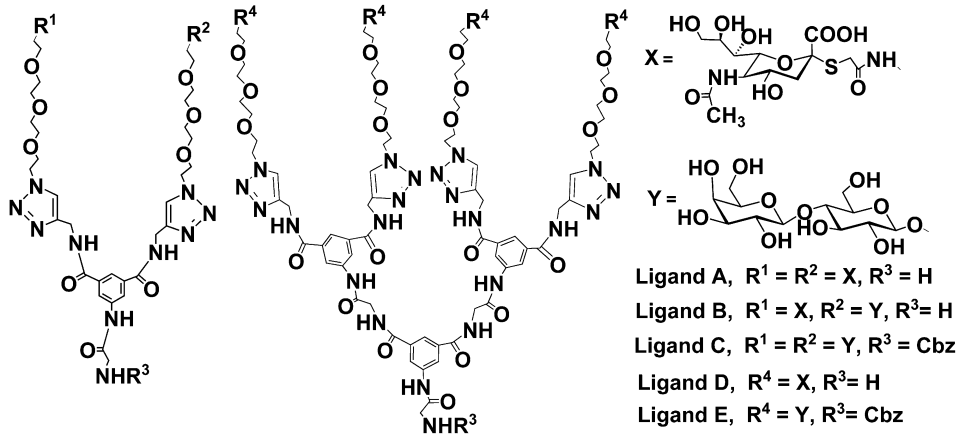
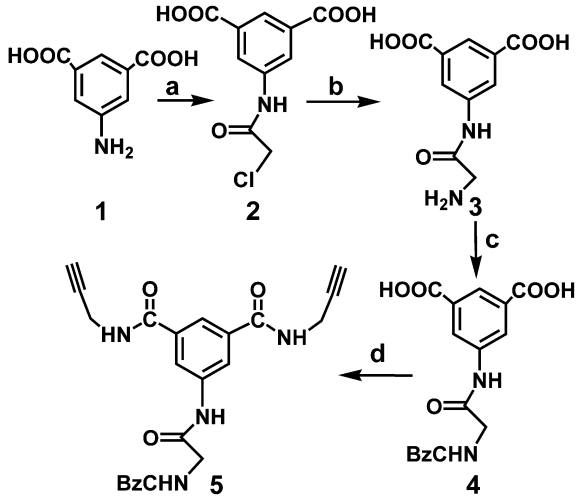


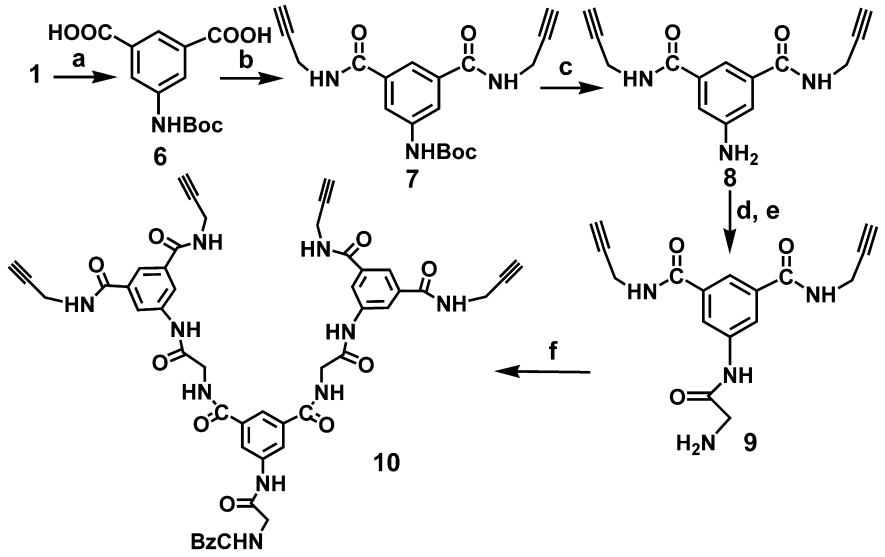
Figure 2. Structures of homodimers (A and C), mixed heterodimer (B), and tetramers (D and E).



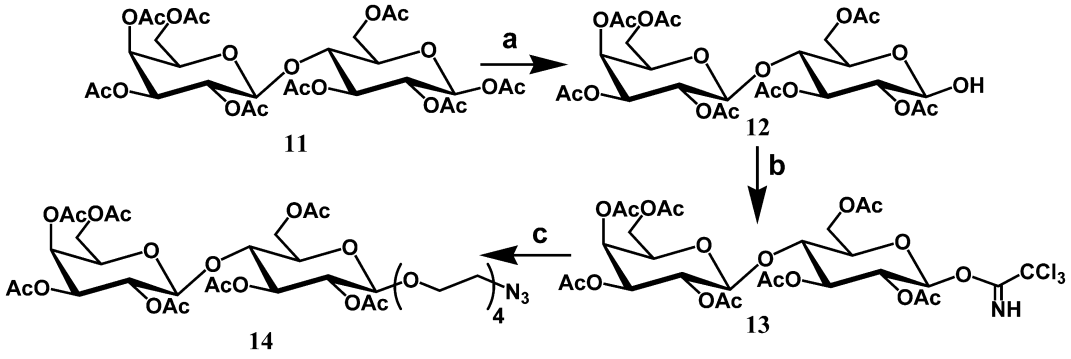
Scheme 1. Reagents and conditions: (a) chloroacetyl chloride, 4 M NaOH 82%; (b) aq NH_3, 12 h, rt, 91%; (c) CBzCl, $NaHCO_3$, rt, 12 h, 79%; (d) propargyl amine, CDMT, NMM, THF/DMF, 0 °C → rt, 32 h, 81%.

formation of the undesirable β-glycoside is limited and second, the thioglycoside is more stable to cleavage reactions.[10] Thio-*N*-acetyl neuraminic acid **15**[11] was subsequently reacted with a bifunctional oligoethylene linker **16** as shown in Scheme 4 to yield a sialic acid - azide terminated oligoethylene oxide conjugate **17**. The reaction was monitored by ^1H and ^{13}C NMR spectroscopy; loss of the peak at 2.28 ppm in the ^1H NMR and observation of a concomitant peak in the ^{13}C NMR spectrum at 32.6 ppm confirmed the formation of the product (HRMS $M+H^+$ = 766.2814). The coupling of the carbohydrate bearing azides to the dimeric or the tetrameric scaffold was carried out in a mixture of THF/H_2O in the presence of Cu(I) as catalyst.[8] Briefly, reaction of 2.2 or 4.2 equivalents of the carbohydrate–oligoethylene conjugates with the dimeric (or tetrameric) scaffold yielded the desired products. A representative example is shown in Scheme 5. The dimeric scaffold **5** was treated with 1.1 equivalent of azide **17** which furnished 57% of the monomeric compound **18**. The proton of the triazole ring resonated at 8.23 ppm and integrated very well with the acetate protons in the ^1H NMR. **18** was treated with 1.1 equivalent of azide **14** to give mixed heterodimer which was subjected to acetate deprotection and deesterification using NaOMe and aqueous NaOH, respectively. The final product was purified using a Biogel P-2 column and lyophilized to yield a white foam. The presence of characteristic signals in the ^{13}C NMR spectrum at 174.9 (–COOH) along with resonance signals at 102.0 and 102.9 ppm (C-1 and C-1′ from the lactose unit) and 85.2 ppm (C-2 from the sialic acid unit) further corroborated the formation of compound **19** (HRMS = 1505.4847 for $C_{57}H_{87}N_{12}Na_3O_{29}S$). We are currently furthering the synthetic methodology to mix and match sugars on different multivalent scaffolds. All other ligands were synthesized and purified using a similar strategy.

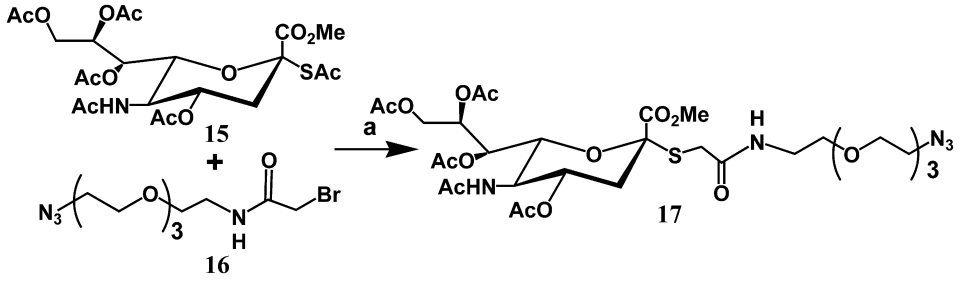
The five compounds (Fig. 2) were examined for cleavage of SNAP-25 by BoNT/A. SNAP-25 (SyNaptosomal-Associated Protein, 25 kDa) is the intracellular protein target selectively cleaved by BoNT/A[1,2] and cleavage of SNAP-25 by BoNT/A provides a convenient in vitro high throughput assay to test the efficacy of potential inhibitors.[12] Briefly, 250 μM solutions of the compounds were dissolved in PBS buffer and added to 500 pg of BoNT/A. The solutions were incubated at 37 °C for 1 h and added to single wells of a cell culture dish containing spinal cord cells harvested from rats. After incubation for 24 h at 37 °C, LDS buffer was added and subjected to Western blot analysis using polyclonal antibodies to SNAP-25. The results are shown in Figure 3. Lanes A, B, C, D, and E represent compounds **A**, **B**, **C**, **D**, and **E** of Figure 2, respectively. Lane F represents the mixture of ligands. Lane G contains no sample. Lanes H, I and J are controls; H has anti-toxin, I represents only toxin, and J has no toxin. The Western blot analysis indicates that significant inhibition of BoNT/A was achieved with ligands A and D, and when a mixture of ligands (Lane F) was used. Compounds A and D, and the mixture of compounds caused significant inhibition of BoNT/A mediated cleavage of SNAP-25.



Scheme 2. Reagents and conditions: (a) NaOH, di-*tert*-butyl-dicarbonate, DMF/H_2O, rt, 30 h, 67%; (b) propargyl amine, CDMT, NMM, THF/DMF, 0 °C → rt, 32 h, 85%; (c) TFA, triisopropylsilane, rt, 8 h, 80%; (d) bromoacetyl bromide, Na_2CO_3, CH_3CN, rt, 96%; (e) NH_3 in MeOH, rt, 81%; (f) **4**, CDMT, NMM, THF/DMF, 0 °C→ rt, 32 h, 78%.



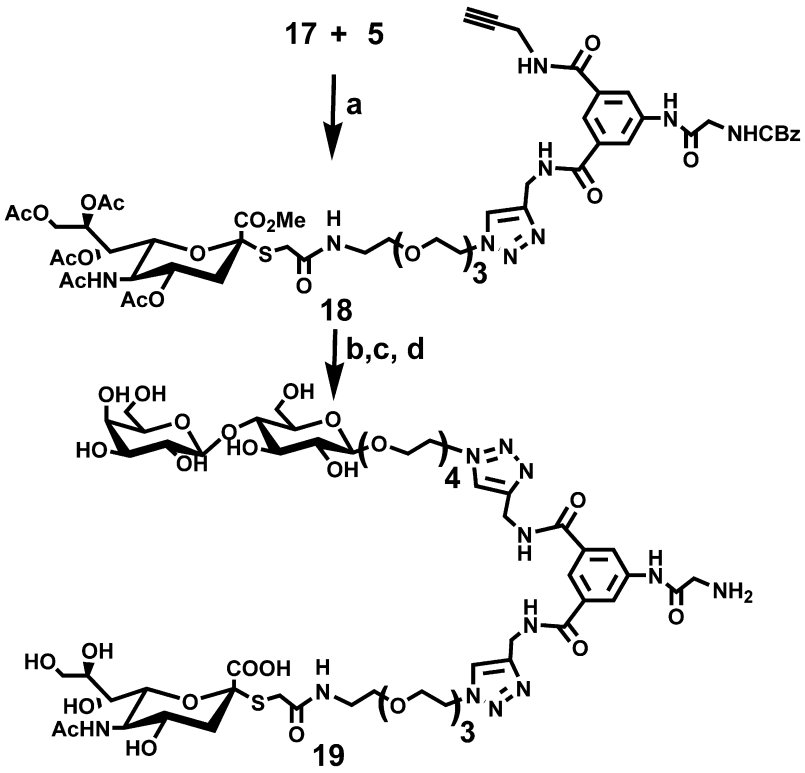
Scheme 3. Reagents and condition: (a) NH_2NH_2·AcOH, THF, 85%; (b) Cl_3CCN, K_2CO_3, DCM, 82%; (c) $H(OCH_2CH_2)_3N_3$, TMSOTf, DCM, 0 °C, 74%.



Scheme 4. Reagents: (a) Et_2NH, DMF, 92%.

This study indicates that the penultimate sugars are critical for tighter binding and the binding pockets are deeper. In general, carbohydrate–protein interactions can be characterized as weak and multivalent binding is necessary to increase the binding affinity.[13] However, in this situation, increase in the valency of the sialic acids does not lead to an increase in complete inhibition, which is in good agreement with a recent report that characterizes the binding of botulinum neurotoxin A with gangliosides as unimolecular.[14] Thus, the penultimate sugars are more critical for tighter binding as opposed to multivalency, which is in contrast to pathogens such as Influenza virus, where multivalency is critical for capturing the pathogen.[15]

In summary, we have used a rational approach to synthesize robust, carbohydrate based ligands for botulinum toxin. In this report, we have outlined our



Scheme 5. Reagents: (a) $CuSO_4$, sodium ascorbate, THF/H_2O, 57%; (b) **14**, $CuSO_4$, sodium ascorbate, THF/H_2O, 71%; (c) NaOMe, MeOH ; (d) aq NaOH, 86% (over two steps).

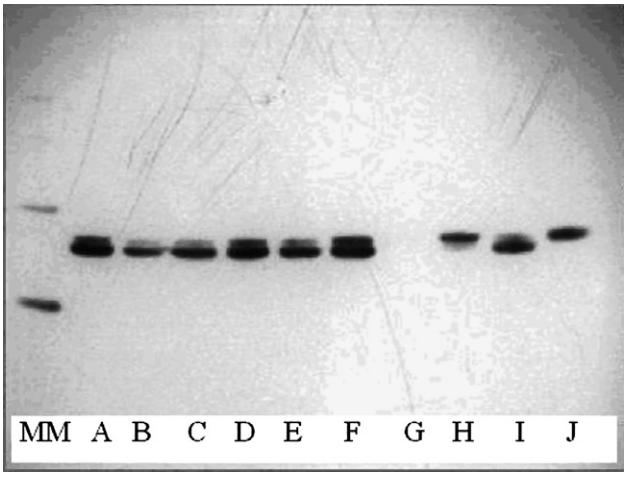


Figure 3. Western blot analysis depicting the inhibitory capacity of the ligands. Lanes A, B, C, D, and E represent compounds **A**, **B**, **C**, **D**, and **E** of Figure 2, respectively. Lane F represents the mixture of ligands. Lane G contains no sample. Lanes H, I, and J are controls; H has anti-toxin, I represents only toxin, and J has no toxin (Please see text for details) MM is the molecular marker.

modular synthetic strategy and successfully synthesized dimeric and tetrameric glycoconjugates. Select first generation compounds show significant inhibition of SNAP-25 of BoNT/A in a cell culture based assay. The compounds could potentially be used to reduce the toxicity when Bot NT/A is used to treat disorders associated with the central nervous system.[1a,16] However, for complete inhibition, a chimeric ligand incorporating a peptide and a ganglioside mimic is essential. This is based on recent crystal structures of botulinum neurotoxin B with cell surface receptors, which suggests a double receptor model, where synaptotagmin and the ganglioside synergistically bind the neurotoxin.[17] Our modular synthesis, presented here, allows us to introduce or delete sugars/peptides and develop high affinity inhibitors. We are currently exploring these options in addition to obtaining quantitative binding affinity data to develop future generation ligands.

Acknowledgments

We thank the Chemical and Biological Sensors Group at the University of Cincinnati for funding. We also thank Dr. Steven Macha for obtaining the mass spectral data and Dr. Alison Weiss for valuable suggestions.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.02.028.

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