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Heterobifunctional Multivalent Inhibitor-Adaptor Mediates Specific Aggregation between Shiga Toxin and a Pentraxin

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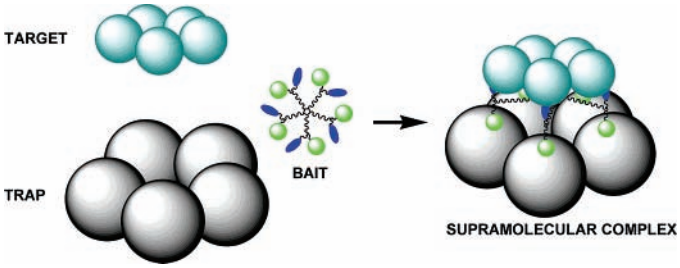
ABSTRACT



The first example of a multivalent heterofunctional inhibitor-adaptor, called "BAIT", is described. This multivalent inhibitor-adaptor is able to capture a "target" receptor (Shiga toxin) through its recognition of one ligand of a heterobivalent headgroup while the other ligand binds to an endogenous "trap" protein (serum amyloid P component, SAP). BAIT showed markedly enhanced inhibition of toxin activity. An efficient synthesis of this multivalent cluster containing heterobifunctional ligands was accomplished by chemical and chemoenzymatic approaches.

It is well-documented that inhibition via specific aggregation can increase the inhibitory power of antagonists of multivalent receptors.[1−4] Specific aggregation achieves effective blocking of the target receptor, even if some binding sites are not actually engaged in an interaction, by creating occluded binding sites incapable of interaction with its native epitopes.

To be successful, specific aggregation requires sufficient concentration of the target protein and tight control over the protein/inhibitor ratio. In contrast to conventional inhibition, specific aggregation occurs in a limited range of inhibitor concentrations, which depends on total concentration of the target protein.[2]

To solve this problem, an abundant second protein is required to serve as a "trap" that captures a scarce "target" receptor, this interaction being mediated via a heterofunctional ligand,[5] which we call "BAIT". Use of multifunctional ligands that mediate aggregation between the serum pentraxin, serum amyloid P component (SAP), and pathogenic proteins was originally suggested by Mark Pepys,[6] and the application of a bifunctional ligand comprising binding fragments for two proteins, serum amyloid P component (SAP) and cholera toxin, has recently been reported.[7]

Recognition of mammalian cells by AB_5 bacterial toxins, such as cholera toxin and Shiga toxins (Stx), is mediated by the oligosaccharide component of glycolipids (in the case of Stx, P^k-trisaccharide, 1,4′-α-D-galactopyranosyl-lactose). SAP is a highly conserved innate immune system protein

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that belongs to the pentraxin family and binds a number of self- and foreign antigens in a Ca^{2+}-dependent fashion. In humans, SAP is one of the most abundant proteins in serum at concentrations of up to 20−40 mg/L.[8] Similar to SAP, the binding subunit, B_5, of bacterial toxins is a pentameric, radially symmetric protein assembly of 5 protomers that presents all binding sites on one face of the pentamer. Despite a large disparity in size between SAP and bacterial toxins, the radial distance for the Ca^{2+}-dependent binding site in SAP matches that for binding sites of toxin ligands. This provides an opportunity for efficient design of a short linker between binding fragments when the respective proteins are positioned face-to-face.

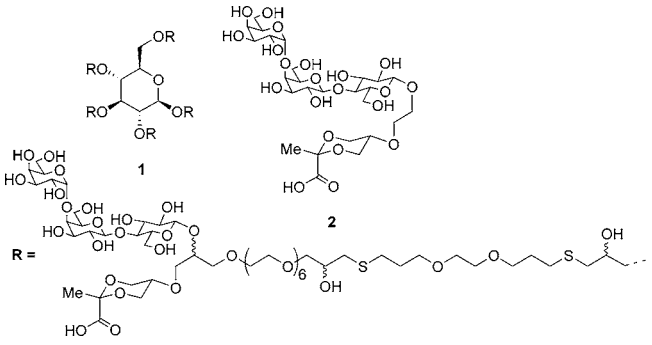
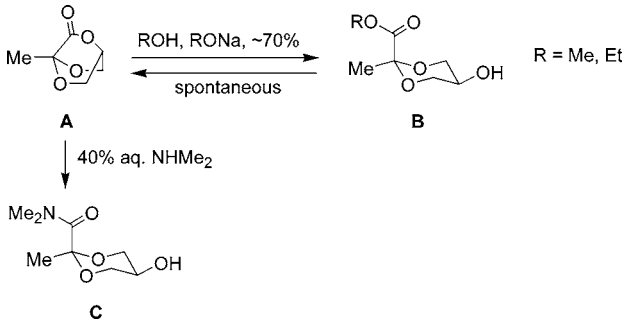


Figure 1. Structure of the heterobifunctional ligand-terminated glycocluster **1** and its univalent analogue **2**.

Prior to the report of Liu et al.[7] we had independently developed an inhibition system utilizing SAP as a trap protein in combination with a heterobifunctional ligand for neutralization of Shiga toxins. Preliminary binding data for compound **2** (Figure 1) showed results similar to those reported for cholera toxin.[7] However, although amplification could be achieved in the presence of SAP, the inhibition of the toxins with **2** is still far from being practical. In this respect, we explored the possibility of additional activity enhancement via a tailored multivalency effect.[1] Here we present the synthesis and activity evaluation of a pentavalent cluster **1** containing heterobifunctional end groups designed to induce formation of a supramolecular complex between Stx and SAP.

Previously, we have developed a ligand for the Ca^{2+}-dependent binding site of SAP that is based on a cyclic pyruvate of glycerol,[2] which represents a small binding fragment of one of the SAP native ligands, 4,6-*O*-(1-carboxyethylidene)-*β*-D-galactose. The synthetic block **B** (Scheme 1), which is readily available from lactone **A**, provides a convenient attachment point, a hydroxyl group for further conjugation. Unfortunately, in solution at non-neutral pH, the ester **B** undergoes rapid spontaneous lacton-

Scheme 1



ization that impedes alkylation of the hydroxyl group. Therefore, we chose dimethyl amide **C** for conveniently stable protection of the carboxyl group. Since deprotection of this functionality requires rather stringent conditions, a hydrolytically stable scaffold had to be designed. Accordingly, we decided to assemble a cluster that would contain only ether and thioether links. The key step of the assembly would be nucleophilic opening of epoxide with a thiol.

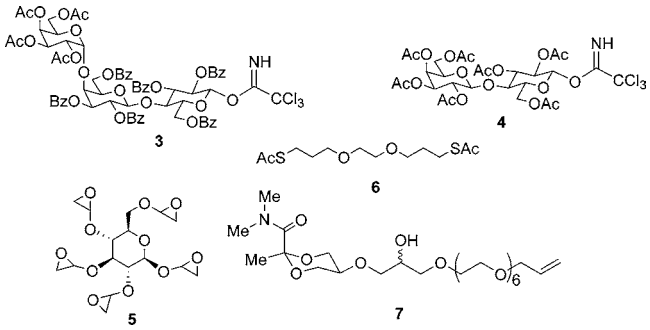


Figure 2. Structure of the main building blocks for synthesis of cluster **1**.

The chemical synthesis of **1** was accomplished in a series of efficient synthetic steps including glycosidation of the P^k-trisaccharide trichloroacetimidate **3**[9] by alcohol **7**, which contains a derivative of the cyclic pyruvate of glycerol and a double-bond-terminated spacer arm (Figure 2). The double bond was then converted to an epoxide, and conjugation of the construct **12** with the core fragment **5** was accomplished via a homobifunctional linker **6**. Alternatively, the key intermediate **13** was obtained by a chemoenzymatic approach via the lactose intermediate **15**, which functioned as a substrate for α-(1,4)-galactosyl transferase, which utilized UDP-Gal produced from UDP-Glc by a UDP-Gal/Glc epimerase. Both enzymes were expressed as a single fusion protein.[10]

Compound **5** was prepared in one step from pentaallylglycoside **8**[11] by epoxidation with *m*-CPBA in 70% yield (Scheme 2). Diallylation of monoethylene glycol **9** followed by UV-assisted radical addition of thioacetic acid to the

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(6) Pepys, M. B. International patent WO 03/013508 A1, 2003.
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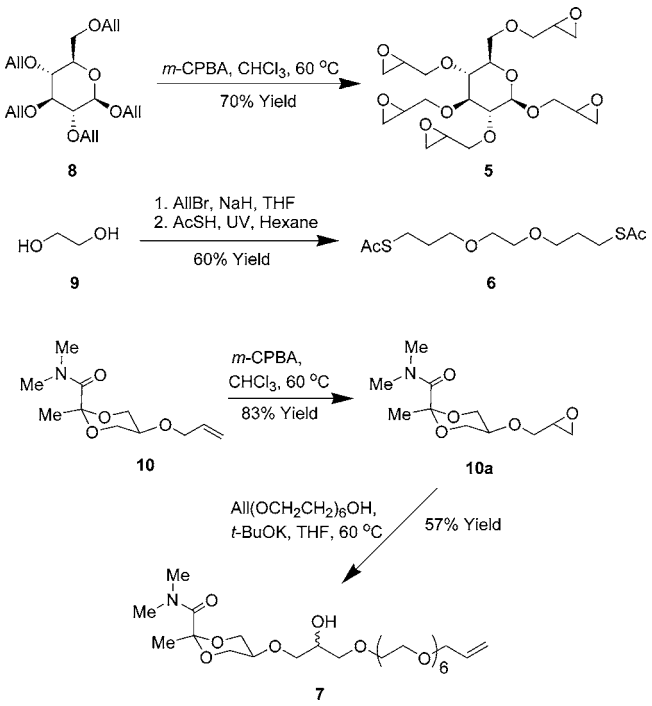
double bond afforded building block **6** in 60% yield. The amide **10**[2] was epoxidized by the same methodology as compound **8**, and the resultant epoxide **10a** was opened with readily available monoallyl (hexaethylene glycol) in basic conditions to produce the target alcohol **7** as a mixture of enantiomers in 47% yield over two steps.

Scheme 2. Synthesis of Building Blocks **5**−**7**



The final assembly of the target multivalent cluster **1** was achieved initially by a chemical pathway and then repeated using a chemoenzymatic approach (Scheme 3). For the chemical route to **1** the alcohol **7** was glycosylated using trichloroacetimidate **3** to afford compound **11** in 77% yield. Subsequent epoxidation of the double bond with *m*-CPBA yielded compound **12** (62%). The epoxide of **12** was opened under basic conditions using an excess of compound **6**, and simultaneous deprotection of hydroxyl groups led to thiol **13**, which was isolated in moderate yield following reversed-phase HPLC purification. Compound **13** was reacted with penta-epoxide **5** to produce the pentavalent cluster with the amide-protected carboxyl groups that were subsequently hydrolyzed to afford target compound **1** in 67% yield over the last two steps.

In the chemoenzymatic approach, the glycosyl acceptor **7** was glycosylated using lactosyl trichloroacetimidate **4** to afford compound **14** in 74% yield. Compound **14** was treated with *m*-CPBA, and the resulting epoxide was opened under basic conditions with **6** to produce thiol **15**, which was isolated using reverse-phase HPLC chromatography in 67% yield over two steps. Compound **15** was galactosylated enzymatically using the α-(1,4)-galactosyltransferase/UDP-4′-Gal-epimerase fusion protein to provide thiol **13** in 85% yield. The two final steps were conducted as above to give cluster **1**.

The inhibitory activity of the synthesized compounds **1** and **2** was evaluated by a solid-phase assay (ELISA), in which the inhibitor competes with immobilized P^k-trisaccharide ligand for binding to Shiga toxin Type 1 (Figure 3).

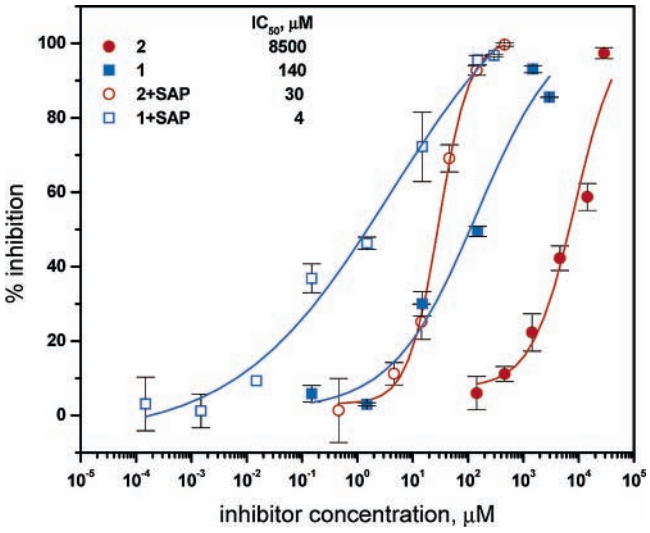


Figure 3. Inhibition of Shiga toxin Type 1 by compounds **1** and **2**. Error bars represent standard deviations for triplicates. Inhibitor concentrations are expressed with respect to the whole molecule.

In the presence of SAP and Ca^{2+}, at close to physiological concentrations, both compounds show substantial activity amplification. Although the type of multivalent scaffold chosen here for compound **1** does not apparently support a large multivalency effect seen in other pentavalent P^k-trisaccharide Stx antagonists,[12] the observations presented here confirm the notion of ligand-induced aggregation as a viable inhibition mechanism for multivalent receptors. The combination of *supramolecular* effect, as the one observed here for heterobivalent ligand **2**, with a *multivalency* effect, as previously shown for multivalent inhibitors,[1,2] does result in activity enhancements as exemplified by the multivalent BAIT-TRAP system, **1**-SAP.

In conclusion, we have developed a convenient and concise synthesis of a multivalent ligand presenting heterobifunctional headgroups on a hydrolytically stable scaffold. The synthesis was accomplished both chemically and by a chemoenzymatic approach in a series of efficient steps, the key one of which was opening of an epoxide by a thiol. The enhancement of the inhibitory power of the ligand in the

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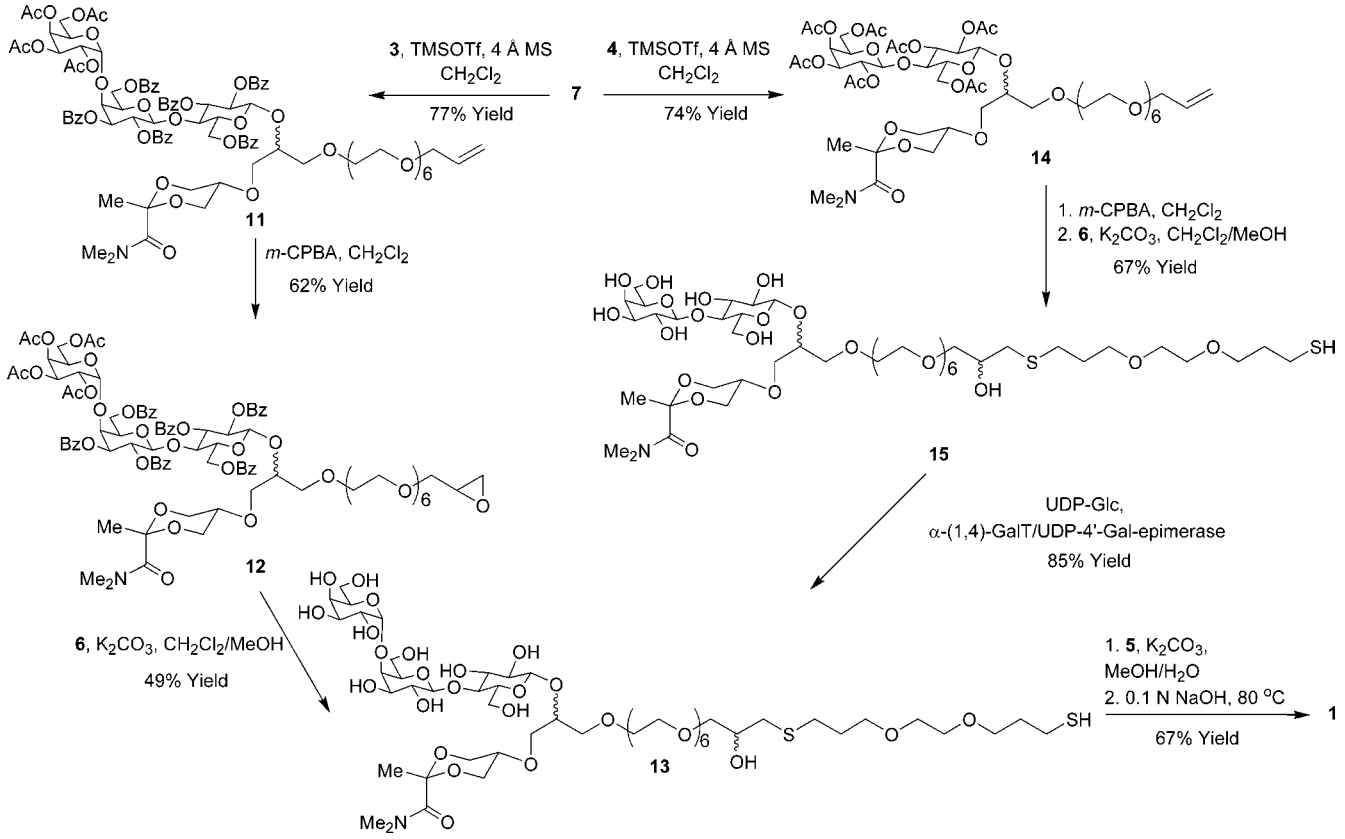
Scheme 3. Assembly of the Cluster **1**



presence of the second trapping protein was observed. We have shown that multivalency is a potentially valuable tool in pharmaceutical design for enhancing activity for toxin antagonists applied in tailored "trap-BAIT" inhibition format. Ongoing work is being performed to improve the activity of the BAIT by focusing on modifications of the multimeric scaffold.

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Supporting Information Available: Experimental procedures for the preparation of compounds **1** and **2** and a solid-phase inhibition assay protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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