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A Synthetic Divalent Cholera Toxin Glycocalix[4]arene Ligand Having Higher Affinity than Natural GM1 Oligosaccharide

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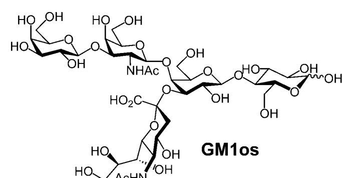
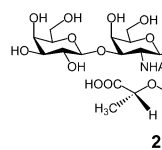
Multivalent interactions[1] are often observed in biological systems where they appear to accomplish the enhancement and fine-tuning of signal response. Many of the known examples of multivalent binding involve interactions between proteins and carbohydrates. In this case, the affinity enhancement due to multivalency has also been termed the "*glycoside cluster effect*".[2−4] Multivalent glycoligands have been synthesized and have often shown remarkable enhancement factors.[2,5−8]

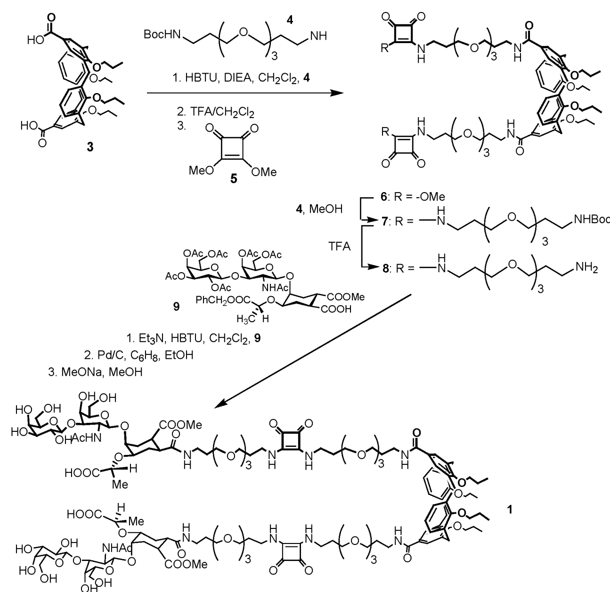
The cholera toxin (CT) is a pentavalent sugar-binding protein belonging to the class of AB_5 toxins. The doughnut-shaped B pentamer (CTB) presents five identical sugar-binding sites on a single face and is responsible for cell-surface binding. The cell-surface ligand of CT is ganglioside GM1 [Galβ1−3GalNAcβ1−4(NeuAcα2−3)Galβ1−4Glcβ1−1Cer]. The GM1 oligosaccharide (GM1os) interacts with the toxin via the terminal galactose and the sialic acid residues.[9−11] Impressive results have been recently reported in the design of multivalent ligands for AB_5 toxins.[7,8,12−16] In particular, polyvalent CT binders have been developed starting from weak ligands, such as lactose or galactose.[8,14−16] Huge affinity enhancements (10^5-fold) compared to that for the monosaccharide were reported by Fan and co-workers, who prepared and screened a series of galactose-based pentavalent ligands with linkers of various lengths.[14−16]





In recent years, we have been working on the design of mimics of the GM1 pentasaccharide.[17−19] One of the second-generation ligands developed is compound **2**, a 190 μM ligand of CT, which can be synthesized in multigram scale and can be easily conjugated to polyvalent aglycons using the carboxy groups on the dicarboxy cyclohexanediol (DCCH) moiety. Polyvalent versions of this GM1 mimic based on dendrimer scaffolds have been recently reported.[20] The scaffolds we selected for the present study are calixarene polycarboxylic acids. Calixarenes are synthetic macrocycles derived from the condensation of phenols and formaldehyde whose shape, size, and conformational properties can be fine-tuned by varying the number of phenol rings and the steric hindrance of the substituents on the OH groups (*lower rim*).[21] Calix[*n*]arenes have been recently used by us[22,23] and others[24] to synthesize neoglycoconjugates and study their interactions with proteins.

Scheme 1. Synthesis of the Divalent Ligand **1**



The first scaffold we focused on is the calix[4]arene diacid **3** fixed in the *cone* conformation. This allows the introduction, on the *upper rim*, of sugar units projected in the same portion of space, thus mimicking, to some extent, a small portion of the cell surface presenting a series of glycosylated residues on the exterior of the lipophilic region.

For the synthesis of the divalent compound **1**, the calixarene core was functionalized by reaction of the diacid **3**[25] with the monoprotected amine **4** using HBTU as the coupling reagent (Scheme 1). Deprotection from Boc and treatment with squaric acid dimethyl ester **5** afforded **6** which, in turn, was condensed with a second monoprotected diamine **4** yielding product **7** in 45% overall yield. The amine groups of calixarene **7** were deprotected and coupled (35% yield) with the pseudotrisaccharide **9**.[20] The benzyl groups were removed under transfer hydrogenation (84%), and the subsequent removal of the acetate groups with MeONa in MeOH gave the divalent calixarene ligand **1** in 66% yield. The interaction of **1** with CT was studied using fluorescence spectroscopy (Figure 1). Ligand binding to CTB is known to induce variations in the protein fluorescence intensity whose extent depends on the structure of the ligand.[26,27] The titrations were performed by irradiating the sample at 280 nm and collecting the data at the maximum of the Trp emission curve at ca. 350 nm. Interference of the calixarene

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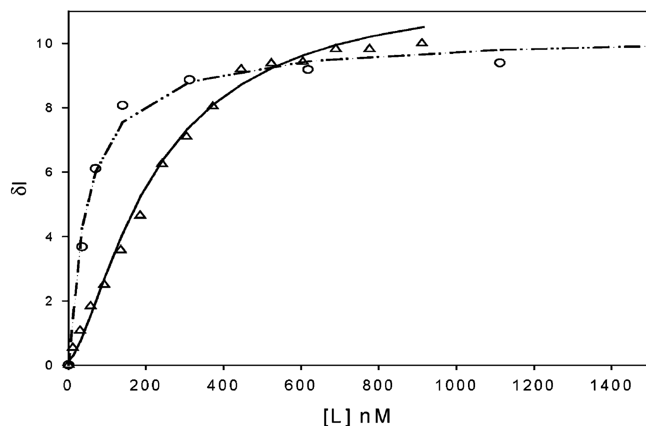

Figure 1. Fluorescence titration curve ($T = 25$ °C) of CTB 0.1 μM in H_2O (Tris buffer, pH 7.5) with the divalent ligand **1** (dashed line, circles) and GM1os (solid line, triangles) (δI vs total ligand concentration).

scaffold was ruled out by examining its emission spectra upon excitation at 280 nm (see Supporting Information).

The changes in the fluorescence emission intensity (normalized to $\delta I_{max} = 10$) of a 0.1 μM solution of CTB upon titration with **1** (dashed lines, circles) or with the natural ligand GM1os (solid line, triangles) are collected in Figure 1. Curve interpolation using SigmaPlot yielded a value of concentration of the ligand at 50% saturation[28] of 48 ± 5 nM for **1** and of 219 ± 1 nM for GM1os. Hence, **1** displays a slightly higher affinity for CT than the natural GM1 oligosaccharide, as it is clearly evident in Figure 1.[29] Further confirmation of this result came from ELISA tests performed using GM1-coated plates (see Supporting Information). The plates were coated with 0.5 μg/well of GM1 ganglioside and then treated with solutions of horseradish peroxidase (HRP)−CTB conjugate (0.025 μg/mL) preincubated with increasing concentrations of GM1os or **1**. After washing, the presence of GM1-bound toxin in the wells was colorimetrically detected by treatment with *o*-phenylendiamine (OPD). In this assay, GM1os was clearly capable of inhibition, although concentrations of over 200 μM were required for full inhibition, due to the multivalent GM1 display on the ELISA plate. At low inhibitor concentration, (below 200 μM) the efficiency of the divalent compound **1** is superior to that shown by GM1os. For instance, at 20 μM **1** gives 54% inhibition vs 20% with GM1os. However, above 200 μM the inhibition power of **1** is apparently saturated at ca. 40%. This behavior appears to result from adsorption of the calixarene **1** on the plate which occurs at high ligand concentration (see Supporting Information). Thus, a high-potency inhibitor of CT binding has been obtained by exploiting a combination of structure-based design of monovalent ligands and affinity enhancements by multivalent presentation using a calixarene scaffold. The observation of an affinity increase is consistent with our expectations about the ability of the calixarene core to project the two monovalent ligands in the appropriate direction for simultaneous interactions with two distinct binding sites on the toxin. In the X-ray structure of the GM1os:CTB complex[9] the distance between adjacent ligands, as measured between the anomeric oxygens of the terminal galactoses, is 31 Å. Molecular graphics shows that the length of the linkers should be sufficient for the two pseudosugars to span even two nonadjacent binding sites (see Supporting Information). The affinity enhancement estimated by fluorescence spectroscopy for the divalent ligand **1** relative to that for the monovalent **2**,[30] roughly 4000-fold (2000-fold per sugar mimic), is exceptionally high and much higher than the one normally measured for a divalent ligand interacting with a polyvalent receptor.[1,5] Of course, this is also due to the relatively high affinity of mimic **2** for CT, which however, cannot be enough to explain the results since, for instance, a divalent version of **2** based on a dendrimer core showed (by SPR) only a 17-fold affinity enhancement (9-fold per sugar mimic) in CT-binding experiments.[20] A more detailed thermodynamic analysis of the role of multivalency in this system, according to recently proposed models,[31,32] is not possible at the moment since it requires the accurate determination of K_d or IC_{50} for a set of analogous multivalent ligands. We are currently varying the number of branches and their length to shed light on this point.

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Supporting Information Available: Synthetic procedures and spectroscopic data of all new compounds; 1D- and 2D-NMR, MS, fluorescence and absorption spectra of compound **1**; experimental detail of spectrofluorimetric titrations and ELISA tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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