Natural and Synthetic Cholera Toxin Antagonists

I.A. Velter[a,§], M. Politi[b], C. Podlipnik[#,c] and F. Nicotra[a,*]

[a]*Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, I-20126 Milano, Italy,* [b]*Departamento de Estructura y Función de Proteínas Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid,* [c]*Dipartimento di Chimica Organica e Industriale, Università degli Studi di Milano,* Via *Venezian 21, I-20131 Milano, Italy*

Abstract: Cholera Toxin (CT) recognizes the cell membrane through specific interactions with ganglioside GM1. Recent structural elucidation of the CT/GM1 complex has allowed the rational design of artificial receptors for the toxin, which could function as anti-cholera drugs. The efforts towards the rational design of Cholera Toxin inhibitors will be presented.

Key Words: Cholera toxin, ganglioside GM1, catechins, galactose, multivalency.

INTRODUCTION

Cholera is a disease that causes watery diarrhea as a result of intestinal infection by the gram negative bacillus *Vibrio cholerae*. Detailed accounts of the history of cholera are available so only a brief summary is provided here [1]. Records of a cholera-like disease go back to the times of Hippocrates and Buddha and maybe even earlier [2], while the modern history of cholera began in 1817 with the first pandemic outbreak in south-east Asia. During the 19th century, six cholera pandemics took place, ending in 1923 and affecting mostly the continents in the southern hemisphere, as well as North America and Europe [2]. In 1961, the seventh pandemic began in Indonesia, then spread to the Indian subcontinent and the Middle East, then moved on to Africa in the 1970s and finally reached South America in the early 1990s [3]. The etiologic agent responsible for cholera was identified in 1883 when Robert Koch demonstrated that this disease is produced by a bacterium that he referred to as a 'comma(-shaped) bacteria' [4], later designated *Vibrio cholerae*. Since Koch's discovery of the infectious cause of cholera, different specific strain variants have been identified and named *V. cholerae* serogroupO1 of biotype 'classical' and biotype 'El Tor', as well as *V. cholerae* serogroup O139 that was responsible for a large epidemic in Bangladesh and India [5-7] described as the eighth pandemic [3]. As a result of continuing scientific and medical efforts directed at combating cholera, major improvements in medical treatment as well as a better understanding of the molecular processes involved in the virulence of *Vibrio cholerae* have been achieved [8]. During the 1960s intensive research aimed at identifing the basis of the cholera disease at a molecular level were performed in different laboratories [9-12], until a protein-toxin was recognized as the major factor that causes the massive fluid release in cholera infection [13,14]. In 1977 Finkelstein and co-workers described a method for the production of cholera toxin crystals suitable for X-ray diffraction analysis [15], while the efforts to exhaustively elucidate the complete structure of this protein-toxin were concluded during the 1990s [16-18].

CT- STRUCTURE AND MODE OF ACTION

Cholera toxin (CT) is a heterohexameric AB_5 complex (Mr = 85,620) belonging to a class of microbial toxins that are composed of structurally independent A (enzymatic) and B (targeting) subunits. Cholera Toxin is closely related to the heat-labile enterotoxin from enteroxigenic *Escheria coli* (LT), with which it shares 80% sequence homology and to which it has a very similar structure and mode of action. The toxin A and B peptide chains are encoded by two genes, and are synthesized in the cytosol [19]. Then both the A and B subunits of the toxin are translocated across the inner membrane into the bacterium periplasm, where one A subunit and five B subunits assemble into one hetero-hexameric holotoxin [19]. The toxin is actively secreted across the outer membrane by the extracellular protein secretion (Eps) and the general secretion pathway (Gsp) export apparatuses of *Vibrio cholerae*, which is an example of the type II bacterial protein secretion system [20-21]. Once in the lumen of the gastrointestinal tract of the human host, the B subunits, arranged to form a regular pentamer (LTB5 or CTB5) with five identical receptor binding sites, recognize the receptor ganglioside GM1 (Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc-ceramide) on the host cell surface [22] and trigger endocytosis (Fig. (**1**)). The binding capability to cell-surface receptors of B pentamer retains even in absence of the A-subunit. However, the complete AB5 holotoxin is required for actual intoxication [23].

The structure and function of the AB5 toxins have been reviewed on detail in several occasions [24]. Several high-resolution structures of AB5 toxins with or without bound ligands are reported [25], as well as binding data obtained by various biochemical and biophysical techniques [26]. The interaction of GM1 with LT and CT is, therefore, fully characterized [27]. Biochemical data and data from structural studies indicate that the two sugars at the non reducing end of GM1, galactose and sialic acid, are essential for binding.

*Address correspondence to this author at the Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, I-20126 Milano, Italy; E-mail: francesco.nicotra@unimib.it

[§]Present address: Johnson & Johnson, Pharmaceutical Research & Development Medicinal Chemistry, Turnhoutseweg 30, B-2340 Beerse, Belgium

[#]Present address: Faculty of Chemistry and Chemical Technology, Askrceva 6, 1000 Ljubljana, Slovenia

1389-5575/07 $50.00+.00

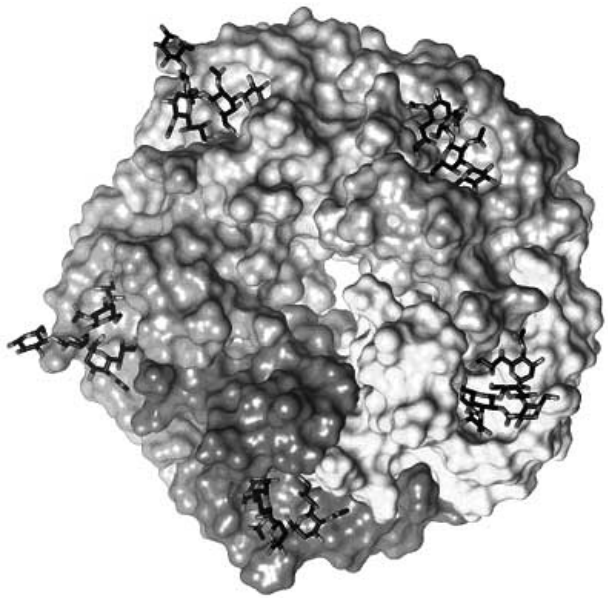


Fig. (1). Structure of CTB pentamer complexed with GM1-OS.

The 1.25 Å resolution structure deposited for the CTB-GM1 complex shows a bivalent interaction of the branched GM1 pentasaccharide. Binding of the terminal galactose group is very specific; there are numerous hydrogen bonds between galactose hydroxyls and protein residues and in addition hydrogen atoms from alpha face of galactose interact with aromatic ring of Trp 88 (CH/pi interaction) (Fig. (**2**)). Galactose binding pocket is shielded from the solvent, the rest of the toxin binding site is shallow and solvent exposed. The sialic acid moiety is placed less specifically than terminal galactose, sugar ring of sialic acid makes hydrophobic interactions with Tyr 12, whereas carboxylic acid, hydroxyl, and N-acetyl substituents form hydrogen bonds with protein backbone. The glycerol tail is only involved in water mediated hydrogen bonding. In terms of buried protein surface area, the terminal Gal and Neu5Ac residues contribute more than 80% of intermolecular contacts; the rest and minor part of protein surface is buried with GalNAc.

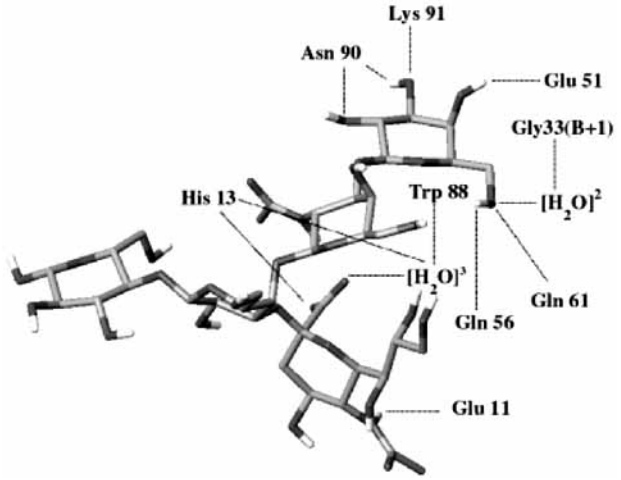
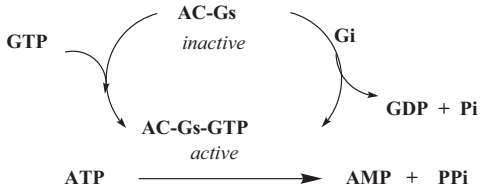


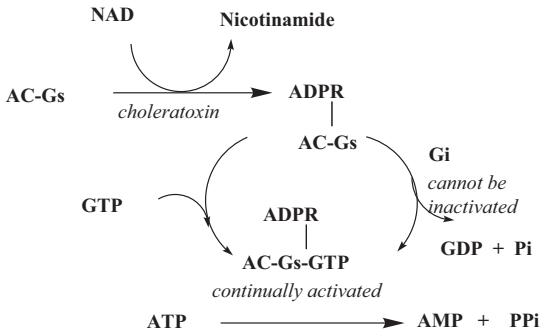
Fig. (2). Interaction map of CT:GM1 complex.

The threatening action of CT is initiated by binding of the B subunits to the GM1 ganglioside on intestinal epithelial cell membranes. This binding event is followed by nicking of the A chain, and disulfide bond reduction, which yield the two fragments A1 (23.5 K Daltons) and A2 (5 K Daltons). Trafficking of the toxin inside the host cell is a fascinatingly complicated and not yet fully understood process [28,29]. After translocation across the membrane, the enzymatic A1 fragment of the A subunit of CT enters the cytosol, where it catalyzes a transfer of an ADP from an NAD^+ to a component of the adenylate cytosol system. Adenylate cyclase (AC) is activated normally by a regulatory protein (Gs) and GTP; however activation is normally brief because another regulatory protein (Gi) hydrolyzes GTP. The normal situation is described in Scheme (**1**).



Scheme (1). Normal biochemical path of adenyl cyclase.

Cholera toxin catalyzes transfer of ADP ribose (ADPR) to adenyl cyclase cycle. Ribosylation of Gs stabilizes the GTP bound form of the protein and stays continually activated. This situation is presented in Scheme (**2**).



Scheme (2). Mechanism of action of Cholera toxin.

The resulting elevated level of AMP causes the activation of the sodium pumps in the lumen of the cell through an AMP dependent kinase pathway, forcing the Na^+ ions out. The electrochemical imbalance is then compensated by driving out Cl^- and H_2O. The process of action of cholera toxin is demonstrated by the enormous loss of fluids, which may lead to death by dehydration.

At the moment there are no effective prophylactics to prevent this toxin-caused diarrhea, no vaccines with long-lasting protective effects. Although oral rehydration can significantly reduce the fatality rate, it is very labor intensive and requires supplies of clean water. This makes the task of disease control difficult because cholera outbreaks usually occur in areas with contaminated water sources. Therefore, there is a need for pharmacological approaches to treat cholera. Once intestinal infection occurs, there are at least four

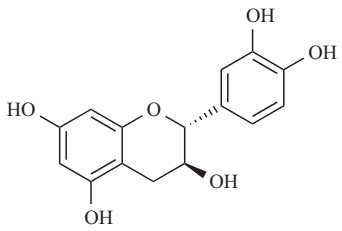
potential pharmacological strategies to reduce intestinal fluid loss in cholera, including the elimination of *Vibrio cholerae* bacteria through host immunity and/or antibiotics, inhibition of increased cAMP levels [30], inhibition of Cl^- secretion, and inhibition of CT-bindings to cells [31]. The latter is the subject of the present paper and is considered a recent and promising approach [32].

It is of note that, because the primary symptoms of cholera are caused by CT, it was originally assumed that *Vibrio cholerae* deleted for *ctx* (or just the toxic *ctxA* portion) would make a good vaccine. Nevertheless, such strains still caused significant disease symptoms (reactogenic) in humans, indicating the presence of additional virulence factors [33-35]. CT remains, however, a potent target in pharmaceutical research on cholera, and current advances on inhibitors of CT activity are reviewed in the following sections.

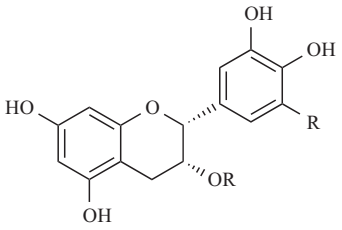
CT INHIBITORS: NATURAL PRODUCTS AS POTENTIAL SOURCES

For a long time, cholera was treated with plants traditionally used as medicament [36]. Because a crude extract from a plant, like infusions or decoctions that represent common ways to administer natural remedies, is a mixture of different natural products, the treatment of cholera infection with the use of medicinal plants can occur through different pharmacological action mechanisms; from the direct antimicrobial activity against *Vibrio cholerae*, for example, to CT inhibition. The understanding of the CT toxicity and the further set up of useful biological assays for the screening of potential CT inhibitors, was allowed in recent years the identification of different classes of bioactive natural products against CT. We report here the cases of different polyphenols extracted from apples [37], hop bract [38], and the Chinese rhubarb rhizome [1] (*Rhei rhizome*, used in China and Japan for many centuries in the so called "Kampo formulation", a herbal medication against diarrhea diseases such as cholera). Moreover, is one of our recent investigations, a polysaccharide extracted from garlic was recognized as a potential inhibitor of CT activity [39].

In 1992, Toda and co-workers [40] reported that tea polyphenols inhibit the fluid accumulation induced by CT in mice and rabbits. Monomeric galloyl-catechins derivatives like epigallocatechin gallate, epigallocatechin, and epicatechin gallate (Fig. (**3**)) were the major inhibitory substances recognized in tea polyphenols. Further pharmacological details on the molecular mechanism of action and effects of other polyphenols, however, were not elucidated until recently. In 2002, Saito and co-workers [2] confirmed the biological activity (inhibition of the fluid accumulation) of natural polyphenols extracted, in this case, from immature apples. Moreover, they described the dose-dependent inhibitory effect of apple polyphenols extract (APE) on CT-catalyzed ADP-ribosylation of agmantine, measuring by this method, the inhibition of the enzymatic activity of the A subunit of CT. The concentration of APE needed to inhibit 50% of the enzymatic activity of CT (15 µg/ml) was approximately 8.7 µg/ml. Bioassay oriented fractionation of APE indicated that the highly polymerized catechins, also named procyanidine polymers, are the major inhibitory components of this apple extract. Other constituents like the non-catechin-type polyphenols (chlorogenic acid, phloridzin, phloretin, caffeic acid, and *p*-cumaric acid) and the monomeric catechins (catechin and epicatechin) show no inhibitory activity. The inhibition starts to be observed from the dimeric and trimeric catechins (procyanidin B1, B2, and C1 respectively). Details on the kinetic effect of APE on the ADP-ribosyltransferase activity indicate that APE may a negative allosteric effect on CT. The result indicates that APE disturbs the biological activity of the CT *in vivo* also, but not only, through the inhibition of the enzymatic activity of the A subunit. An additional explanation for the *in vivo* effect of APE can be the protection, due to the polymerized catechins, on the mucosa of the intestine, where the secretory activity is reduced. Hor and coworkers [41] also reported this effect due to the proanthocyanidins (same group of catechin polymer, see Fig. (**4**)) [42] extracted from *Guazuma ulimifolia*, a medicinal plant used in Mexico to treat diarrhea.



(+)-Catechin, **1**



	R	R_1
(-)-Epicatechin (**2**)	H	H
(-)-Epigallocatechin (**3**)	OH	H
(-)-Epicatechin gallate (**4**)	H	galloyl
(-)-Epigallocatechin gallate (**5**)	OH	galloyl

Fig. (3). Catechin derivatives from tea polyphenols.

Oi and collaborators [36] described the bioactivity of the rhubarb galloyl tannin (RG-tannin), a compound isolated from *Rhei rhizome* and characterized by a polygallate structure, against different CT activities including ADP-rybosilation and fluid accumulation. This kind of heterologous polyphenol-gallate inhibits, fluid accumulation in mouse and rabbit ileal loops, as well as the catalytic activity of CTA. RG-tannin had no effect on the binding of CTB subunit to the receptor ganglioside GM1 or on endogenous ADP-ribosylation of membrane proteins. A small library of synthetic gallate (sugar moieties esterified with galloyl groups, see the example in Fig. (**5**)) was prepared and tested for the inhibitory effect of CTA catalytic activities. The authors highlight the possibility to developing non-toxic synthetic gallate derivatives as CT inhibitors to use as an adjunctive therapy for the treatment of cholera in anepidemic area as well as a preventive strategy.

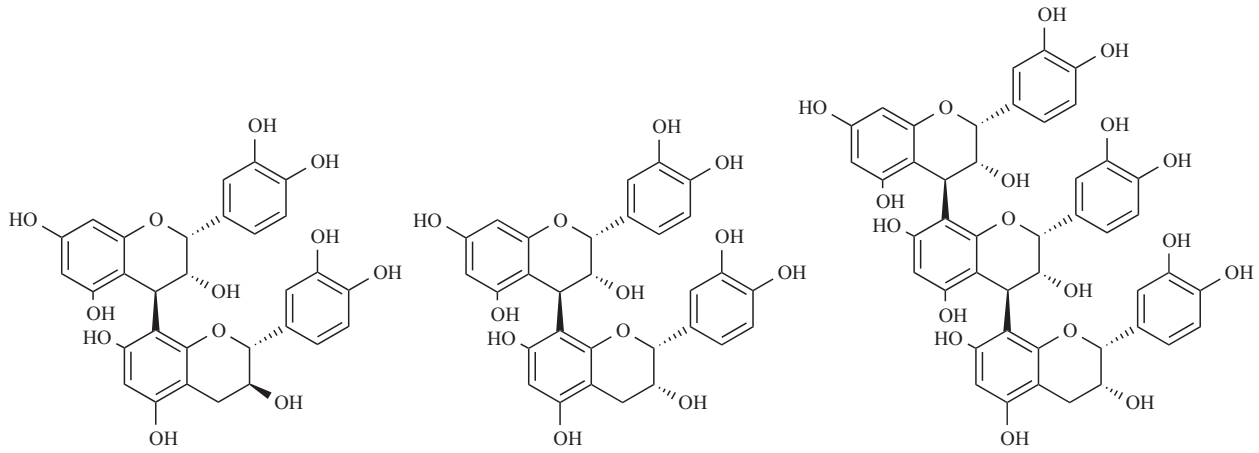


Fig. (4). Procyanidins.

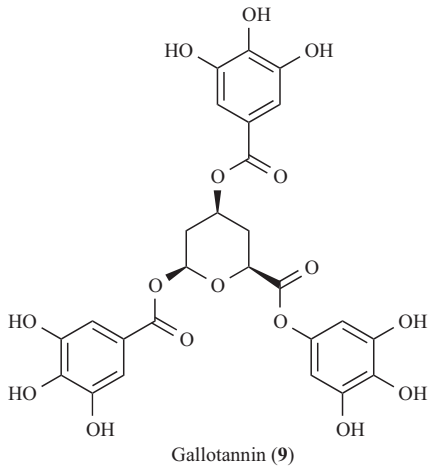


Fig. (5).

The major bioactive constituents from apple polyphenol extract are procyanidine polymers, (less than 15-mer and mostly 3-6-mer). RG-tannin polymer (mainly 8-mer) from the Chinese rhubarb rhizome, contains mostly galloyl groups. Other procyanidine polymers (about 10-30-mer with a molecular weight around 6,000) were extracted from the hop bract and tested as potential CT inhibitors in comparison with the other two polyphenols. The authors report that RG-tannin does not inhibit CT binding to cells but forms complexes with CT and thereby suppresses internalization from the cell surface. On the other hand, the procyanidine polymers extracted from apple and hop bract form large aggregates with CT that interfere with its binding to and internalization by cells, indicating that the mechanism of RG-tannin action is different. The different interactions of RG-tannin and other polyphenols with CT may reflect the presence in RG-tannin, but not in the procyanidine polymers extracted from apple and hop bract, of galloyl moieties.

In our recent study [39], binding activity between a high molecular weight galactan (polysaccharide of around 60 kDa) extracted from garlic, and the B subunit of the CT (CTB) was detected by Saturation Transfer Difference (STD) experiments, one of the NMR methods used to measure the binding activity between ligands and target receptor [43]. The interaction was confirmed by florometric binding assay. The high estimate for the stoichiometry suggests multiple binding sites in the galactan ligand.

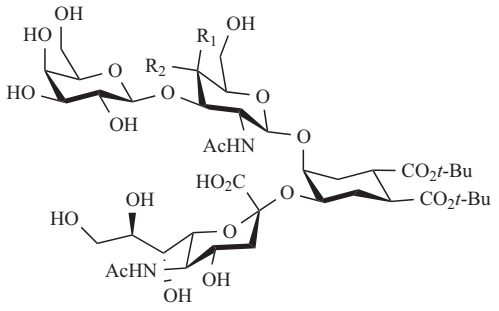
A direct antimicrobial activity of garlic extracts against *Vibrio cholerae* has been reported [44]. This work indicates a second possible pharmacological mechanism for the treatment of cholera infection with garlic products (possible inhibition of the CT binding to cells). Many plants are known to contain galactans and galacan-containing polysaccharides [45], and, following the same direction of previous reports, concern the potential use against cholera of polyphenols extracted from different natural sources. Other plants polysaccharides could be tested in the future for the binding activity with CTB.

Many others plants have been used for centuries everywhere around the world, and especially in the developing countries, as natural remedies against cholera infection. Most current pharmacological studies on some of these medicinal species have been performed by testing the antibacterial activity of the plant extracts or isolated compounds against *Vibrio cholerae*, while direct investigation of natural products as potential CT inhibitors is still very rare, and this research field remains at the moment largely untapped.

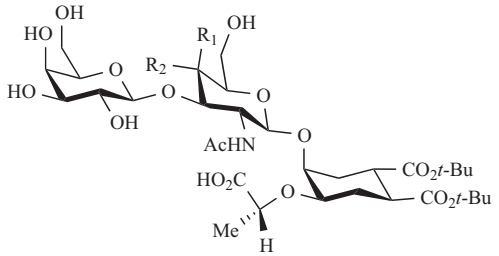
RATIONAL DESIGN OF CTB ANTAGONISTS: FROM MONOVALENT TO MULTIVALENT LIGANDS

Monovalent Ligands

While synthesis of the ganglioside GM1 itself is a long and complex process [46], one of the strategies of impeding the binding of CTB to the cell surface involves the design and synthesis of functional and structural mimics of GM1. In this regard, Bernardi and coworkers designed a first generation of GM1-mimics, **10-11** in (Fig. (**6**)) [47], in which the GalII residue of GM1 was replaced with a conformationally restricted *cis*-1,2-cyclohexanediol. The binding of these



10, R_1= OH, R_2 = H
11, R_1= H, R_2 = OH



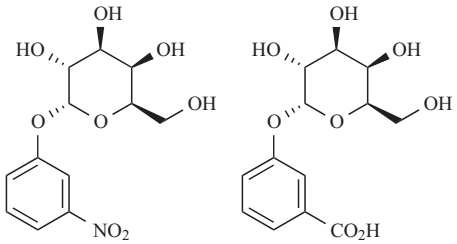
12, R_1= OH, R_2 = H
13, R_1= H, R_2 = OH

Fig. (6). Bernardi's GM1 mimics.

compounds with CTB was evaluated using ELISA assays and it was found that they displayed a similar inhibition of the toxin as the ganglioside GM1. Furthermore, the EC_{50} of GM1-CTB complex, determined using fluorescence titrations [48], was 1.5 μM, and for **10** and **11**, 1.2 μM and 1.3 μM respectively [47c]. Due to the structural and synthetic complexity of **10**-**11**, a second generation of mimics was designed and synthesized, in which the sialic acid residue was substituted with simple α-hydroxy acids, **12** and **13** in (Fig. (**6**)) [49], after conformational analysis studies [50].

The synthesis of ligand **12** is shown in Scheme (**3**) [47b]. Selective etherification of the equatorial hydroxyl group of **14** with Bu_2SnO provided **16** [51], which was then glycosylated at the axial hydroxyl group with donor **17** and in the presence of trimethylsilyl triflate [52]. Removal of the protecting benzyl and acetate esters from the resulting pseudotrisaccharide provided then the desired ligand **12**. From fluorescence titration studies, a K_D = 190 μM was determined for this compound.

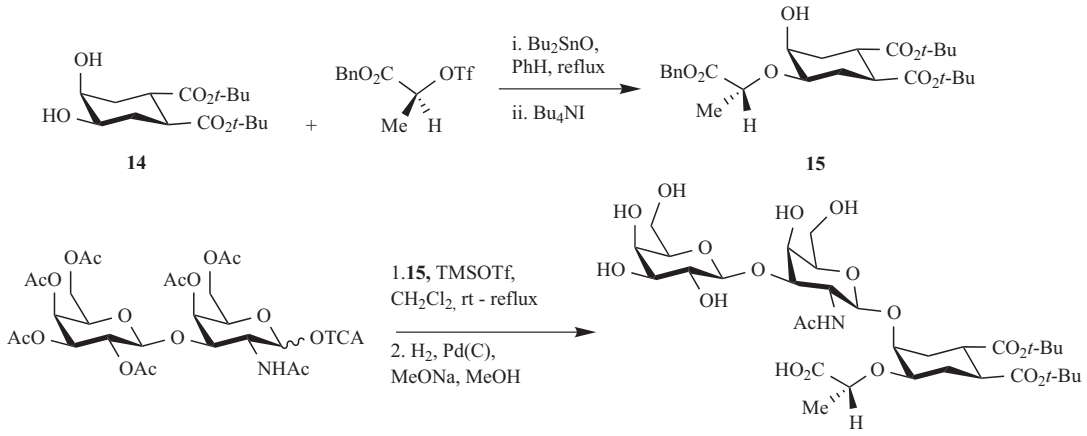
A second, widely used approach to preventing the CTB-GM1 binding consists in using the terminal galactose as an anchor, to which various pharmacophores can be attached. Verlinde and collaborators [53], for instance, screened a number of commercially available galactose derivatives using fluorescence titrations and ELISA assays, in which the compounds' inhibition of CTB binding with the ganglioside GD1b was examined. From ELISA tests it resulted that *m*-nitrophenyl α-D-galactoside (**18**, Fig. (**7**)) was the best inhibitor, with an IC_{50} of 720 μM; its affinity for CTB being 100 times higher than that of galactose. These results where then confirmed by examining the crystal structure of the toxin in the absence and presence of the ligands. In the case of **18**, it seems that the presence of the nitro group in the meta position of the phenyl ring leads to the formation of a strong hydrogen bond between the ligand and the protein. In this case, a conserved water molecule in the crystal is displaced, generating thus an increase in the entropy of the system.



MNPG (**18**) MCPG (**19**)

Fig. (7).

Notably, *m*-carboxyphenyl α-D-galactoside (**19**, Fig. (**7**)) [54] displayed a different binding mode from compound **18**. Significantly, the carboxylate oxygen of **19** did not displace the water molecule as in the case of **18**, and its affinity for LT-I B was ten times lower (Fig. (**8**)).



Scheme (3). Synthesis of the second generation of ligands.

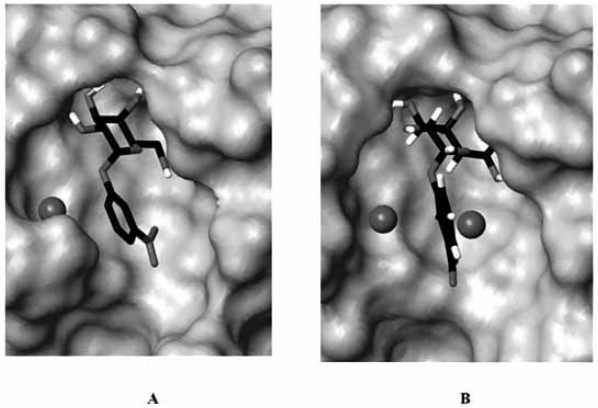


Fig. (8). Placement of MNPG (**A**) and MCPG (**B**) in CTB pocket.

More recently, analyzing the crystal structures of LT and CT with compound **18**, Verlinde and coworkers reached the conclusion that substituents in meta position of the phenyl ring of compound **18** would allow the exploration of different binding site regions [55]. Therefore, a new generation of antagonists was synthesized, in which hydrophobic rigid ring systems were linked to the phenyl ring of compound **18** using short, flexible aliphatic linkers. The synthesis of the library started from the peracetylated galactoside **20**, Scheme (**4**), which was glycosylated with 5-nitro-3-hydroxybenzoic acid, in the presence of $SnCl_4$, to yield **21** in good yield. The carboxylic group of **21** was first activated with cyanuric chloride and then treated with various primary amines, to yield, after deacetylation, the library members, as α/β anomeric mixtures (e.g., **22**).

From the crystallographic studies and electron density calculations, it was revealed that these new ligands display multiple conformational states in the bound form. For instance, in compound **22**, apart from the predicted conformations that replicate the favorable binding mode of compound **18**, the morpholine ring adds conformations from the interaction with different hydrophobic regions of the binding site. Thus, in addition to the interactions displayed by the natural receptor, GM1, in this region, with the side chains of residues Glu11, Tyr12, Lys34 and Arg35, it also presents new favorable interactions with Ile58. It was suggested by the authors that this last interaction plays an important role in the overall affinity of **22** for CTB. Further analysis of the α anomer of **22**, using pulsed ultrafiltration (PUF) and isothermal titration calorimetry (ITC) revealed an average binding constant, K_D, of 12 μM, corresponding to a 14 folds improvement over compound **18**. Notably, these results suggest that addition of relatively flexible appendages to a scaffold used as an 'anchor' into the CTB binding site improve the ligand's affinity for CTB by introducing additional favorable interactions with the targeted regions of CTB pocket.

Pieters and coworkers synthesized monovalent lactose-derived ligands for Cholera Toxin [56]. For **23** (Fig. (**9**)), the attachment of a thioureea moiety and of an aryl group to the parent lactose, enhanced 72-fold the binding of this compound to CTB: K_D = 248 μM for **23** versus K_D = 18 mM for lactose, as determined by fluorescence titration.

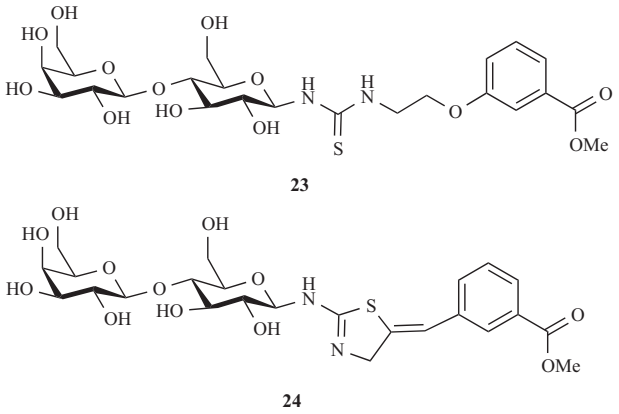
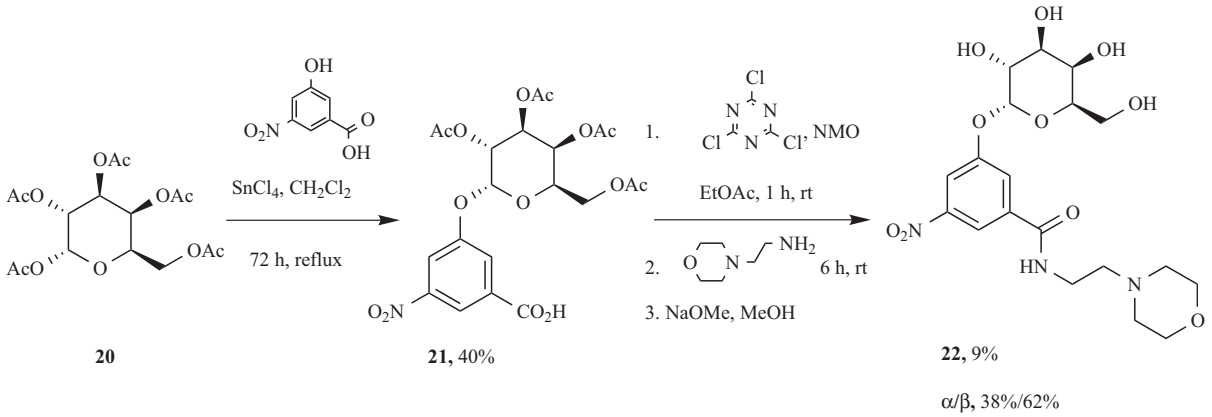


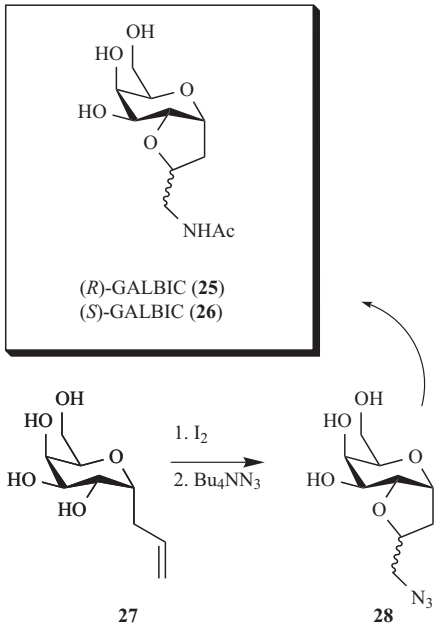
Fig. (9). Pieters' lactose derived ligands.

Compound **24** was then synthesized in an attempt to increase the rigidity of the spacer between the lactose and the aryl group. Fluorescence studies revealed one order of magnitude enhancement in the affinity of **24** (K_D=23 μM) for the cholera toxin B subunit.

Recently, as a result of collaboration within a European Training Network, compounds **25** and **26**, Scheme (**5**), were designed as potential ligands for the cholera toxin B [57].



Scheme (4). Synthesis of a MNPG derivative.

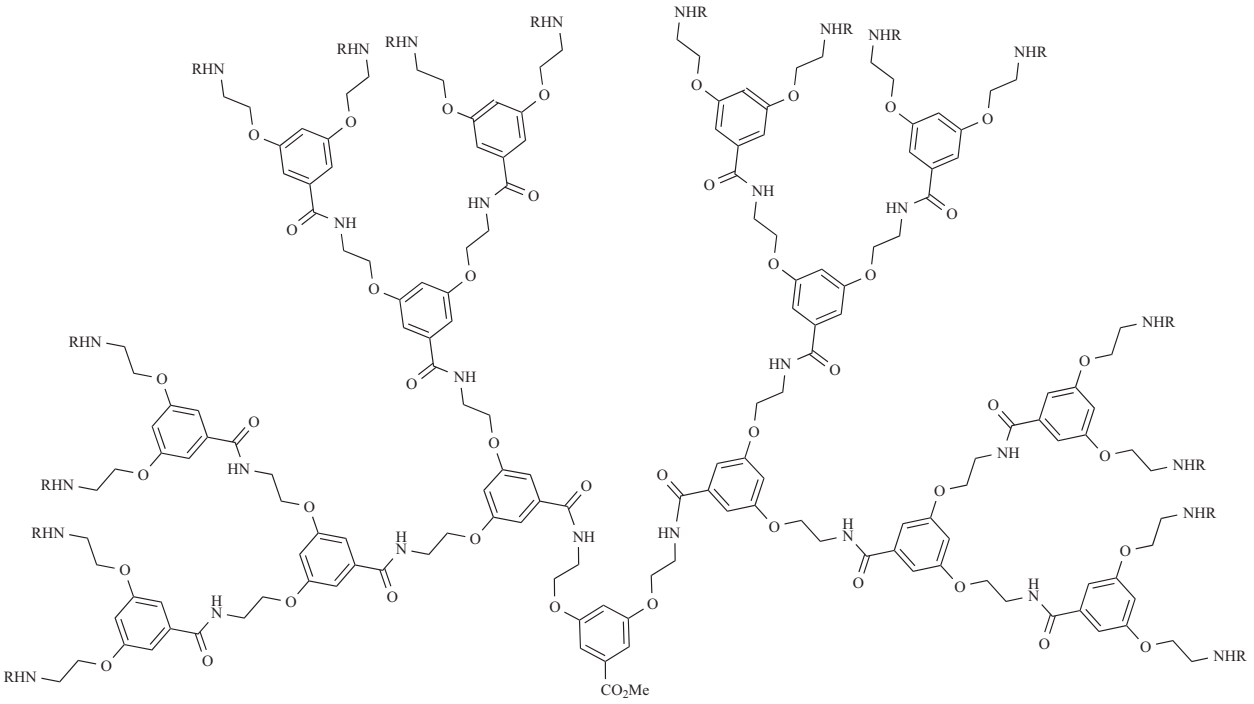


Scheme (5).

The rigid framework and the possibility of functionalization at the appended side-chain made these compounds interesting for further combinatorial development. The synthesis of **25**-**26**, reported in Scheme (**5**), started from unprotected α-*C*-allyl galactoside **27** and, *via* a synthetic route that included iodocyclization, iodine displacement by sodium azide and finally azide reduction in the presence of acetic anhydride. NMR analysis showed, however, that these compounds were more flexible than expected and did not fit in the cholera toxin's binding site.

Multivalency

The use of multivalency as a tool to inhibit the binding of a pathogen to the target cells has been widespread since 1995 [58], when Lee and collaborators [59] during their studies concerning the binding of a lectin to single and multiple saccharides, showed that, while binding to single saccharides is weak, ligands containing multiple saccharides strongly associate with the lectin [60]. Studies on multivalency were, in fact, performed earlier on the Cholera Toxin and *Escheria coli* systems. Thus, starting from the observation made in 1974, that the oligosaccharidic portion of GM1 has a lower affinity for CT than GM1 itself [61], a first attempt on improving o-GM1's affinity using multivalency was made in 1978 [62]. Although the divalent o-GM1 designed and synthesized was a better ligand for Cholera Toxin than o-GM1, its affinity for CT was still lower than that of the natural receptor More recently, Schengrund and coworkers prepared highly active multivalent o-GM1 ligands, by linking o-GM1 to poly-L-lysine [63] or to an octa(propyleneimine)dendrimer [64]. In general, the multivalent ligands are formed from a core, usually a dendrimer or a polyamine, to which a monovalent ligand is linked.



29, R = H
30, R = **33,** from "Fig. (**11**)"

Fig. (10). Pieters' dendrimers.

For instance, Pieters and coworkers [65] used dendrimers derived from 3,5-di-(2-aminoethoxy)benzoic acid repeating units, with 2, 4, and 8 end-groups, such as **29** in (Fig. (**10**)) [66], to which lactose isothiocyanate units **33** (Fig. (**11**)) were attached providing thioureea-linked glycodendrimers **30**. The affinity of these compounds for Cholera Toxin was examined using fluorescence titration, and compared to that of the monovalent lactose ligands (**23**, Fig. (**10**), K_D = 248 μM). From data analysis, the K_D values found for **30** (K_D = 89 μM) and **31** (K_D = 33 μM) showed an improvement of an order of magnitude relative to the monovalent ligands. On the other hand however, an increase in the branching of the dendrimer provided only a modest increase in the potency of the ligand. The data also suggested that the multivalent ligands binded to multiple toxin molecules, rather than to the B pentamers of a single one [67].

With the aim of improving the multivalent ligand's affinity for the toxin, Bernardi's monovalent ligand **12** (Fig. (**6**)), was attached to the dendrimer cores [68]. For further improvement, the polysaccharide scaffold was provided with elongated spacer arms (**32**, Fig. (**11**)). In this case, analysis of surface plasmon resonance data revealed an EC_{50}= 0.5 μM for **32**.

An example of structure-based design of multivalent ligands comes from the work of Fan and collaborators [69], who designed a symmetric non-branched pentavalent Cholera Toxin inhibitor **38**, Scheme (**6**), in which five single site

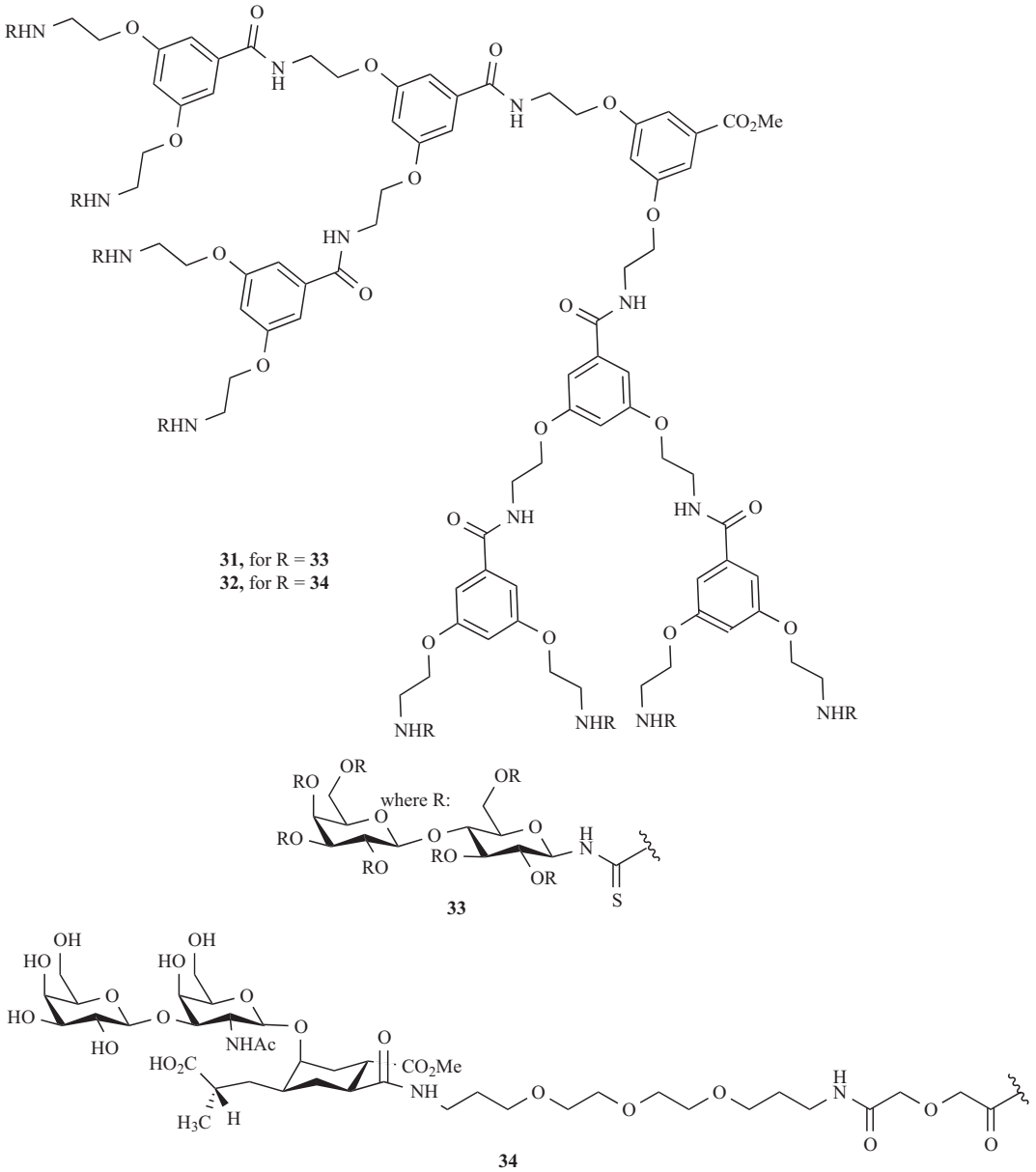


Fig. (11). Pieter's and Bernardi's glycodendrimeric ligands.

antagonists were linked *via* modular linkers to a central pentacyclen core, as shown in (Fig. (**12**)).

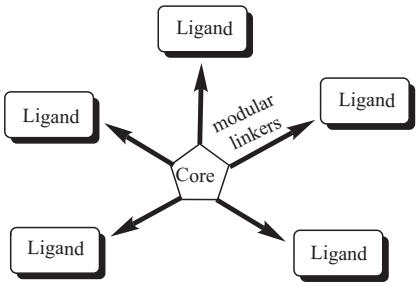


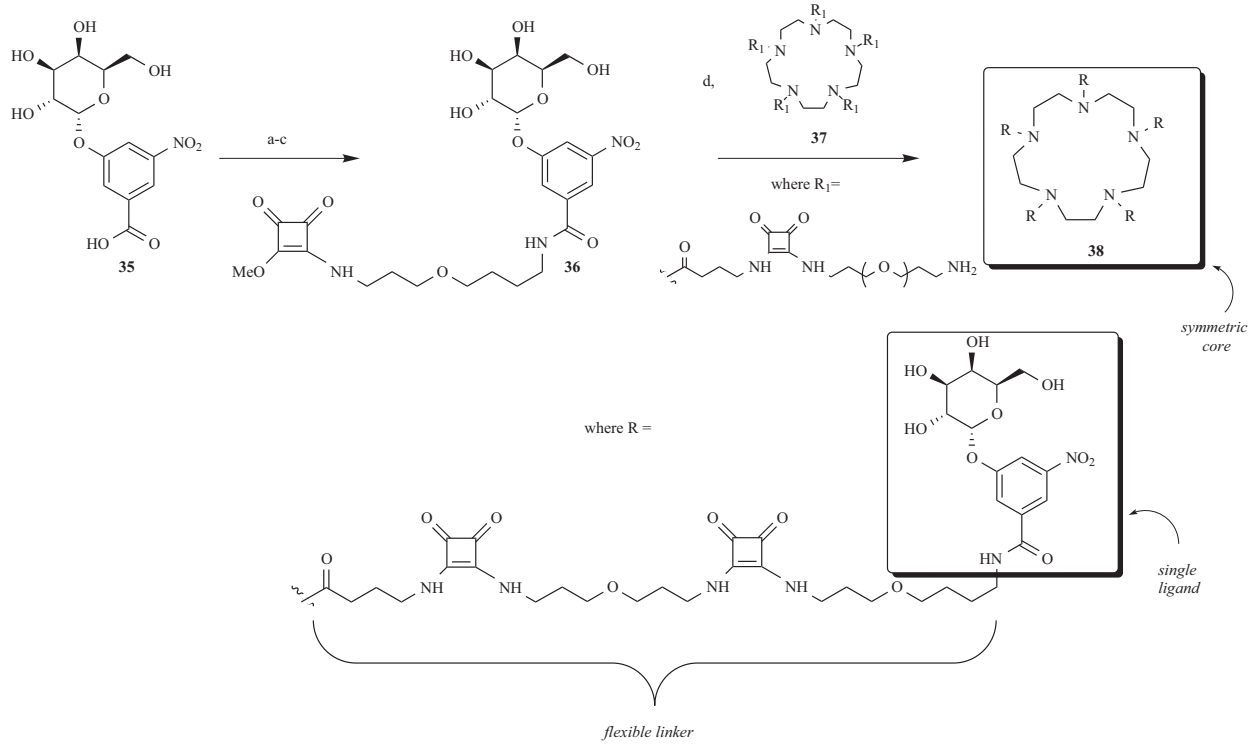
Fig. (12). Fan's structure based design of a pentavalent inhibitor of CT.

The synthesis of the pentavalent ligand started from the previously compound **35** reported in Scheme (**6**). An *N*-Boc protected fragment of the linker was attached to the carboxylic group of **35** *via* amide bond formation. Deprotection of Boc group using TFA provided compound **36**, which was then attached to the pentavalent core **37**, to provide an aqueous solution of the pentavalent ligand **38** in a very good yield, as determined by photometric measurements. The affinity of **38** for CTB was investigated using enzyme-linked adhesion assays, from which an EC_{50} = 0.9 μM was determined. This represents a 263-fold enhancement of activity of **38**, as compared to compound **18**. From dynamic light scattering studies it resulted that a 1:1 CTB/**38** complex was the major mode of association in solution between the ligand and the toxin. In addition, the crystal structure of the **38**:CTB complex was also examined and revealed that each of the five independent receptor binding sites were occupied by one finger of the pentavalent inhibitor. The crystallographic studies brought additional support for a 1:1 association between the ligand and the toxin. Notably, the canonical water is displaced also in this case.

In order to study the binding and the association mode of a branched pentavalent ligand [70], Fan *et al.* designed and synthesized decavalent ligands, **39** (Fig. (**13**)), which were then tested for their affinity for CTB using Elisa tests. It appeared that ligands **39**-**42** were an order of magnitude better than the non-branched ligands. In fact, for **42**, with n = 4, an EC_{50}= 40 nM was found, a value that lies in the same range as the EC_{50} of the natural receptor (50 nM). Dynamic scattering light studies performed on ligand **42** suggested that, depending on the toxin's concentration in solution, both 1:1 and 1:2 association modes are found for the ligand/CTB complex. From DLS competition experiments, performed in the presence of compound **18**, the authors estimated that the EC_{50} of a 1:2 ligand/CTB complex lies in the low hundreds nanomolar range. The drop of affinity in this case is due perhaps to unfavorable entropic effects

An interesting example comes from the recent work of Bernardi *et al.* [71]. In this case, the multivalent ligand **43** (Fig. (**14**)), was designed, in which the GM1 monovalent mimic **12** (Fig. (**6**)) was attached to a calyx [4]arene core [72] using flexible linkers.

Fluorescence titration revealed a very high affinity of **43** for CTB (EC_{50}=48 nm), higher even than that displayed by oligosaccharide GM1 in the same experimental conditions



Scheme (6). Fan's synthesis of the pentavalent ligand **38**.

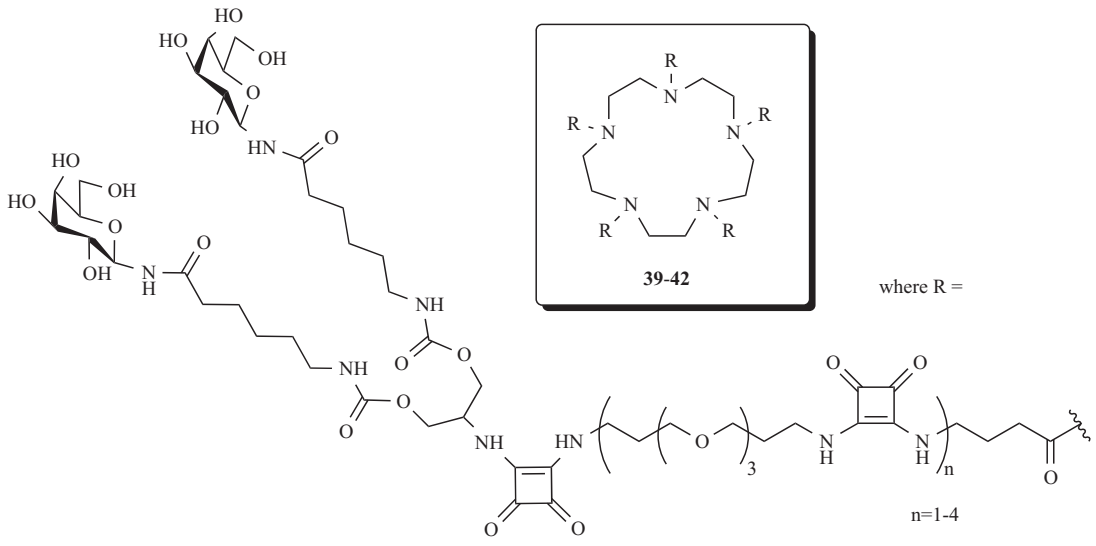


Fig. (13). Branched decavalent ligands.

(EC_{50}=219 nm). Ligand **43** displays thus a 4000-fold enhancement in affinity for CTB relative to that of the monovalent ligand **12**.

CONCLUSIONS

The current preferred strategy for preventing the cholera toxin's entry into the cells, and thus for preventing the disease itself is the design of CTB ligands. The elucidation of the crystal structure of CTB and of CTB-GM1 complex, permits in fact the rational design and synthesis of ganglioside GM1 simplified mimics that maintain the fundamental requirements for this binding. Furthermore, in the recent years there is has been a clear preference for multivalent ligands, due to their capacity either to bind to more than one of the toxin's binding sites or bind to more than one toxin molecule.

ACKNOWLEDGEMENTS

"Work supported in part by the European Community's Human Potential Programme under contract HPRN-CT-2002-00173, [GLYCIDIC SCAFFOLDS]".

REFERENCES

[1] Sack, D. A.; Sack, R. B.; Nair, G. B.; Siddique, A. K. *Lancet,* **2004**, *363*, 223.
[2] Barua, D. *History of Cholera.* In *Cholera*, Barua, D.; Greenough, W.B. Eds.; Plenum, New York, **1992**; 1-35.
[3] Faruque, S. M.; Albert, M. J.; Mekalanos, J. J. *Microbiol. Mol. Biol. Rev.,* **1998**, *62*, 1301.
[4] Koch, R., *Br. Med. J.,* **1884**, *2*, 403.
[5] Swerdlow, D. L.; Ries, A. A. *Lancet,* **1993**, *342*, 382.
[6] Ramamurthy, T.; Grag, S.; Sharma, R. *Lancet,* **1993**, *341*, 703.
[7] Albert, J. B.; Siddique, A. K.; Islam, M. S.; Faruquwe, A. S. G.; Anzarzzaman, M.; Faruque, S. M.; Sack, R. *Lancet,* **1993**, *341*, 704.
[8] Reidl, J.; Klose, K. E. *Microbiol. Rev.,* **2002**, *26*, 125.
[9] Finkelstein, R. A.; Atthasampunna, P.; Chulasamaya, M.; Charunmethee, P. *J. Immunol.,* **1966**, *96*, 440.
[10] De, S. N. *Nature,* **1959**, *18,* 1533.
[11] Lewis, A. C.; Freeman, B. A. *Science,* **1969**, *165*, 808.
[12] Burrows, W. *Ann. Rev. Microbiol.,* **1968**, *22*, 245.
[13] Finkelstein, R. A.; LoSpalluto, J. J. *Science,* **1972**, *175*, 529.
[14] Verwey, W. F.; Finkelstein, R. A. *J. Infect. Dis.,* **1970**, *121*, S63.
[15] Sigler, P. B.; Druyan, M. E.; Kiefer, H. C.; Finkelstein, R. *Science,* **1977**, *197*, 1277.
[16] Spangler, B. D. *Microbiol. Rev.,* **1992**, *56*, 622.
[17] Burnette, W. N. *Structure,* **1994**, *2*, 151.

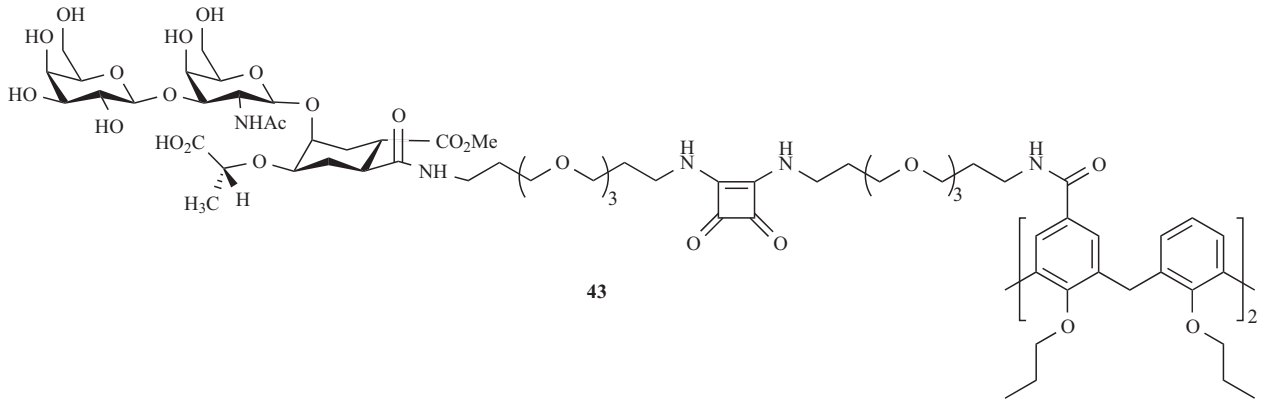


Fig. (14). Bernardi's example of a calixarene core.

[18] Zhang, R. G.; Scott, D. L.; Westbrook, M. L.; Nance, S.; Spangler, B. D.; Shipley, G. G.; Westbrook, E. M. *J. Mol. Biol.,* **1995**, *251*, 563.

[19] Fan, E.; O'Neal, C. J.; Mitchell, D. D.; Robien, M. A.; Zhang, Z.; Pickens, J. C.; Tan, X. J.; Korotkov, K.; Roach, C.; Krumm, B.; Verlinde, C. L. M. J.; Merritt, E. A.; Hol, W. G. J. *Int. J. Med. Microbiol.,* **2004**, *294*, 217.

[20] Tauschek, M.; Gorrell, R. J.; Strugnell, R. A.; Robins-Browne, R. M. *Proc. Natl. Acad. Sci. USA,* **2002**, *99*, 7066.

[21] Sandkvist, M. *Infect. Immun.,* **2001**, *69*, 3523.

[22] Sattler, J.; Schwarzmann, G.; Knack, I.; Rohm, K. H.; Wiegandt, H. *Hoppe-Seyler's Z. Physiol. Chem.,* **1978**, *359*, 719.

[23] Sixma T. K.; Pronk S. E.; Kalk K. H.; Wartna C. E. S.; Van Zanten B. A. M.; Witholt B, Hol W. G. J. *Nature,* **1991**, *351*, 371.

[24] (a) Merritt, E. A.; Hol W. G. J. *Curr. Opin. Struct. Biol.,* **1995**, *5*, 165; (b) Fan, E.; Merritt, E. A.; Verlinde, L. M. J. ; Hol, W. G. J. *Curr. Opin. Struct. Biol.,* **1995**, *10*, 680.

[25] (a) Merritt, E.A.; Sarfaty, S.; van den Akker, F.; L'Hoir, C.L.; Martial, J.A.; Hol W.G.J. *Protein Sci.,* **1994**, *3*, 166; (b) Kuhn, P.; Sarfary, S.; Erbe, J.L.; Holmes, R.K.; Hol, W.G.J. *J. Mol. Biol.,* **1998**, *282*, 1043; (c) Merritt, E.A.; Sarfaty, S.; Feil, I.K.; Hol, W.G.J. *Structure*, **1997**, *5*, 1485; (d) Fan, E.K.; Merritt, E.A.; Zhang, Z.; Pickens, J.C.; Roach, C.; Ahn, M.; Hol, W.G.J. *Acta Crystallogr*., **2001**, *D57*, 201; (e) van den Akker, F.; Steensma, E.; Hol W.G.J. *Protein Sci.*, **1996**, *5*, 118; (f) Pickens, J.C.; Merritt, E.A.; Ahn, M.; Verlinde, C.L.M.J.; Hol, W.G.J.; Fan, E.K. *Chem. Biol.,* **2002**, *9*, 215.

[26] (a) Fukuta, S.; Magnani, J.L.; Twiddy, E.M.; Holmes, R.K.; Ginsburg, V. *Infect. Immun.,* **1988**, *56*, 1748; (b) Ångström, J.; Teneberg, S.; Karlsson, K.-A. *Proc. Natl. Acad. Sci. USA,* **1994**, *91*, 11859; (c) Kuziemko, G.M.; Stroh, M.; Stevens, R. C. *Biochemistry,* **1996**, *35*, 6375; (d) Picking, W. L.; Moon, H.; Wu, H.; Picking, W. D. *Biochim. Biophys. Acta,* **1995**, *1247*, 65; (e) Mertz, J. A.; McCann, J. A.; Picking, W. D. *Biochem. Biophys. Res. Commun.,* **1996**, *226*, 140; (f) Cai, X.-E.; Yang, J. *Biochemistry,* **2003**, *42*, 4028; (g) Masserini, M.; Freire, E.; Palestini, P.; Calappi, E.; Tettamanti, G. *Biochemistry,* **1992**, *31*, 2422; (h) Turnbull, W. B.; Precious, B. L.; Homans, S. W. *J. Am. Chem. Soc.,* **2004**, *126*, 1047.

[27] (a) Merritt, E. A.; Sarfaty., S.; van der Akker, F.; L' Hoir, C. L.; Martial, J. A.; Hol, W. G. J. *Protein Sci.* **1994**, *3*, 166; (b) Merritt, E. A.; Kuhn, P.; Sarfaty, S.; Erbe, J. L., Holmes, R. K.; Hol, W. G. J. *J. Mol. Biol.,* **1998**, *282*, 1043.

[28] Kahn, R. A.; Fu, H.; Roy, C. R. *Trends Biochem. Sci.,* **2002**, *27*, 308.

[29] Lencer, W. I.; Tsai, B. *Trends Biochem. Sci.,* **2003**, *28*, 639.

[30] Saini, S.S.; Gessell-Lee, D.L.; Peterson, J. W. *Inflammation*, **2003**, *27*, 79.

[31] Thiagarajah, J. R.; Verkman, A. S. *Trends Pharmacol. Sci.,* **2005**, *26*, 172.

[32] Other strategies that were investigated include (1) inhibition of the active site of subunit A: (a) Zhou, G.; Parikh, S.L.; Tyler, P. C.; Evans, G. B.; Furneaux, R. H.; Zubkova, O. V.; Benjes, P. A.; Schramm, V. L. *J. Am. Chem. Soc*., **2004**, *126*, 5690; (b) Schramm, V.L. *Acc. Chem. Res*., **2003**, *36*, 588; (2) inhibition of the toxin assembly (a) Hovey, B.T.; Verlinde, C.L.; Merritt, E. A.; Hol, W. G. J. *J. Mol. Biol.,* **1999**, *285*, 1169; (b) Sixma, T.K.; Stein, P.E.; Hol, W. G. J.; Read, R. J. *Biochemistry,* **1993**, *32*, 191; (c) Sixma, T.K.; Kalk, K.; Van Zanten, B. A.; Dauter, Z.; Kingma, J.; Withold, B.; Hol, W. G. J. *J. Mol. Biol.,* **1993**, *230*, 890; (d) Wolfenden, R. *Acc. Chem. Res.,* **1972**, *5*, 10.

[33] Taylor, D. N.; Killeen, K. P.; Hack, D. C.; Kenner, J. R.; Coster, T. S.; Beattie, D. T.; Ezzell, J.; Hyman, T.; Trofa, A.; Sjogren, M. H. *J. Infect. Dis.,* **1994**, *170,* 1518.

[34] Coster, T. S.; Killeen, K. P.; Waldor, M. K.; Beattie, D. T.; Spriggs, D. R.; Kenner, J. R.; Trofa, A.; Sadoff, J. C.; Mekalanos, J. J.; Taylor, D. N. *Lancet,* **1995**, *345*, 949.

[35] Levine, M. M.; Kaper, J. B.; Herrington, D.; Losonsky, G.; Morris, J. G.; Clements, M. L.; Black, R. E.; Tall, B.; Hall, R. *Infect. Immun.,* **1988**, *56*, 161.

[36] Oi, H., Matsuura, D.; Miyake, M.; Ueno, M.; Takai, I.; Yamamoto, T.; Kubo, Joel Moss, M.; Noda, M. *Proc. Natl. Acad. Sci. USA,* **2002**, *99,* 3042.

[37] Saito, T.; Miyake, M.; Toba, M.; Okamatsu, H.; Shimizu, S.; Noda, M. *Microbiol. Immunol.,* **2002**, *46*, 249.

[38] Morinaga, N.; Morinaga, N.; Iwamaru, Y.; Yahiro, K.; Tagashira, M.; Moss, J.; Noda., M. *J. Biol. Chem.,* **2005,** *280,* 23303.

[39] Politi, M.; Alvaro-Blanco, J.; Groves, P.; Prieto, A.; Leal, J.A.; Cañada, F.J.; Jiménez-Barbero, J. *Eur. J. Org. Chem.,* **2006**, 2067.

[40] Toda, M.; Okubo, S.; Ikigai, H.; Suzuki, T.; Suzuki, Y.; Hara, Y.; Shimamura, T. *Microbiol. Immunol.,* **1992,** *36*, 999.

[41] Hor, M.; Rimpler, H.; Heinrich, M. *Planta Medica,* **1995**, *61*, 208.

[42] Dixon, R.A.; Xie, D.Y.; Sharma, S.B. *New Phytol*., **2005**, *165*, 9.

[43] Meyer, B.; Peters, T. *Angew. Chem. Int. Ed.,* **2003**, *42*, 864.

[44] Vijaya, K.; Ananthan, S. *J. Altern. Complement Med*., **1997**, *3*, 13.

[45] Arifkhodzhaev, A.O. *Chem. Nat. Com.,* **2000**, *36*, 229.

[46] (a) Sugimoto, M.; Numata, M.; Koike, K.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res*., **1986**, *15*, 156; (b) Hasegawa, A.; Nagahama, T.; Kiso, M. *Carbohydr. Res*., **1992**, *235*, C13; (c) Schengrund, C. L.; Kovac, P. *J. Lipid Res.,* **1999**, *40*, 160; (d) Ito, H.; Ishida, H.; Kiso, M. *J. Carbohydr. Chem*., **2001**, *20*, 207.

[47] (a) Bernardi, A.; Checchia, A.; Brocca, P.; Sonnino, S.; Zuccotto, F. *J. Am. Chem. Soc*., **1999**, *121*, 2032; (b) Bernardi, A.; Carrettoni, L.; Ciponte, A. G.; Monti, D.; Sonnino, S. *Bioorg. Med. Chem. Lett*., **2000**, *10*, 2197; (c) Bernardi, A.; Arosio, D.; Manzoni, L.; Monti, D.; Posteri, H.; Potenza, D.; Mari, S.; Barbero, J. J. *Org. Biomol. Chem.,* **2003**, *1*, 785.

[48] Mertz, J.; McCann, J. A.; Picking, W. D. *Biochem. Biophys. Res. Commun.,* **1996**, *226*, 140.

[49] Kolb, H. C.; Ernst, B. *Chem. Eur. J.,* **1997**, *3*, 1571.

[50] Bernardi, A.; Raimondi, L.; Zuccotto, F. *J. Med. Chem.,* **1997**, *40*, 1855.

[51] David, S.; Hanessian, S. *Tetrahedron,* **1985**, *41*, 643.

[52] Bernardi, A.; Boschin, G.; Checchia, A.; Lattanzio, M.; Manzoni, L.; Potenza, D.; Scolastico, C. *Eur. J. Org. Chem.,* **1999**, 1311.

[53] Minke, W. E.; Roach, C.; Hol, W. G. J.; Verlinde C. L. *Biochemistry,* **1999**, *38*, 5684.

[54] (a) Minke, W. E.; Pickens, J.; Merritt, E. A.; Verlinde, C. L. M. J.; Hol, W. G. J. *Acta Cryst*., **2000**, *D56*, 795; (b) Fan, E.; Merritt, E. A.; Verlinde, C. L. M. J.; Hol, W. G. J. *Curr. Opin. Struct. Biol*., **2000**, *10*, 680.

[55] Pickens, J. C.; Merritt, E. A.; Ahn, M.; Verlinde, C. L. M. J.; Hol, W. G. J.; Fan, E. *Chem. Biol.,* **2002**, *9*, 215

[56] (a) Vrasidas, I.; de Mol, N. J.; Liskamp, R. M. J.; Pieters, R. J. *Eur. J. Org. Chem*., **2001**, 4685; (b) Vrasidas, I.; Kemmink, R. M.; Liskamp, R. M. J.; Pieters, R. J. *Org. Lett*., **2002**, *4*, 1807.

[57] Velter, A. I.; Nicotra, F.; La Ferla, B.; Bernardi, A.; Marcou, G.; Motto, I.; Mari, S.; Cañada, F. J.; Jiménez-Barbero, J. *Chem. Eur. J.,* **2006,** in press.

[58] Schengrund, C.–L. *Biochem. Pharmacol.,* **2003**, *65*, 699.

[59] Lee, Y. C.; Lee, R. T. *Acc. Chem. Res*., **1995**, *28*, 321.

[60] Lee, R. T., Lee Y. C. *Glycoconj. J.,* **2000**, *17*, 543.

[61] (a) Holmgren, J.; Mansson, J. –E.; Svennerholm, L. *Med. Biol.,* **1974**, *52*, 229; (b) Staerk, J.; Ronnenberger, H.; Wiegandt, H.; Ziegler, W. *Eur. J. Biochem.,* **1974**, *48*, 103.

[62] Schwarzmann, G.; Mraz, W.; Sattler, J.; Schindler, R.; Wiegandt, H. *Hoppe Seylers Z. Physiol. Chem.,* **1978**, *359*, 1277.

[63] Schengrund, C.–L.; Ringler, N. J. *J. Biol. Chem.,* **1989**, *264*, 13233.

[64] Thompson, J. P.; Schengrund, C.–L. *Biochem. Pharmacol*., **1998**, *56*, 591.

[65] Vrasidas, I.; de Mol, N. J.; Liskamp, R. M. J.; Pieters, R. J. *Eur. J. Org. Chem*., **2001**, 4685.

[66] For dendrimer preparation, see: (a) Mulders, S. J. E.; Brouwer, A. J.; van der Meer, P. G. J.; Liskamp, R. M. J. *Tetrahedron Lett*., **1997**, *38*, 631; (b) Mulders, S. J. E.; Brouwer, A. J.; Liskamp, R. M. J. *Tetrahedron Lett*., **1997**, *38*, 3085; (c) Brouwer, A. J.; Mulders, S. J. E.; Liskamp, R. M. J. *Eur. J. Org. Chem*., **2001**, 1903.

[67] A similar bridging model was proposed for the biding of a divalent ligand to Shiga toxin: Lundquist, J. J.; Debenham, S. D.; Toone, E. J. *J. Org. Chem*., **2000**, *65,* 8245.

[68] Arosio, D.; Vrasidas, I.; Valentini, P.; Liskamp, R. M. J.; Pieters, R. J. Bernardi, A. *Org. Biomol. Chem.* **2004**, *2*, 2113.

[69] (a) Fan, E.; Zhang, Z.; Minke, W. E.; Hou, Z.; Verlinde, C. L. M. J.; Hol, W. G. J. *J. Am. Chem. Soc.,* **2000**, *122*, 2663; (b) Merritt, E.; Zhang, Z.; Pickens, J. C.; Hol, W. G. J. Fan, E. *J. Am. Chem. Soc*., **2002**, *124*, 8818.

[70] Bundle and coworkers reported a 1:2 complex for a pentavalent ligand for Shiga toxin. Kitov, P. I.; Sadowska, J. M.; Mulvey, G.; Armstrong, G. D.; Ling, H.; Pannu N. S.; Read, R. J.; Bundle, D. R. *Nature,* **2000**, *403*, 669.

[71] Arosio, D.; Fontanella, M.; Baldini, L.; Mauri, L.; Bernardi, A.; Casnati, A.; Sansone, F.; Ungaro, R. *J. Am. Chem. Soc*., **2005**, *127*, 3660.

[72] For the use of calixarenes in the synthesis of neoglycoconjugates, see also: (a) Dondoni, A.; Mara, A.; Scherrmann, M. C.; Casnatti, A.; Sansone, F.; Ungaro, R. *Chem. Eur. J.,* **1997**, *3*, 1774; (b) Sansone, F.; Chierici, E.; Casnati, A.; Ungaro, R. *Org. Biomol. Chem*., **2003**, *1*, 1802; (c) Roy, R.; Kim, J. M.; *Angew. Chem. Int. Ed.,* **1999**, *38*, 369.

Received: 30 August, 2006 Revised: 16 November, 2006 Accepted: 29 November, 2006