Prospects for Preventing Serious Systemic Toxemic Complications of Shiga Toxin–Producing *Escherichia coli* Infections Using Shiga Toxin Receptor Analogues

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(See the article by Watanabe et al., on pages 360–8.)

Shiga toxin (Stx)–producing *Escherichia coli* (STEC), also known as "verocytotoxin-producing *E. coli*" [1], are zoonotic pathogens that cause potentially fatal and often epidemic food- or waterborne illness [2–4] with a clinical spectrum that includes diarrhea, hemorrhagic colitis [5], and the hemolytic-uremic syndrome (HUS) [6, 7]. HUS, which is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, is the leading and a potentially fatal cause of acute renal failure in children [8–11]. There are no specific therapies for HUS or vaccines to prevent it. Histologically, HUS is characterized by widespread thrombotic microvascular lesions in the renal glomeruli, the gastrointestinal tract, and other organs, such as the brain, the pancreas, and the lungs [12–14]. These lesions probably result from primary Stx-mediated damage to microvascular endothelial cells [3, 4, 15–17]. The mechanism of this injury involves Stx binding to a specific receptor, globotriaosylceramide (Gb_3) [18], on the surface of endothelial cells [19] and toxin internalization by a receptor-mediated endocytic process [20], followed by toxin interaction with subcellular components that results in protein synthesis inhibition [19] or apoptosis [21].

STEC produce 2 major, serologically distinct Stx types, Stx1 and Stx2, which are AB_5 subunit toxins with 1 enzymatically active A subunit and 5 identical copies of the B subunit [22]. The B subunit pentamer is a doughnut-shaped structure composed of the individual subunits arranged symmetrically around a central α-helix–lined pore, over 1 face of which sits the A subunit [23]. The opposite face contains the receptor-binding domains of the B pentamer [24]. Stx2, which is found more often than Stx1 in the commonest STEC serotype, O157:H7 [25, 26], is reported to have a greater cytotoxic effect on human glomerular endothelial cells than does Stx1 in vitro [27] and thus may have a greater propensity than Stx1 to initiate HUS. Although the endothelial cell appears to be the main target of Stx action, there is evidence that these toxins also mediate biological effects on other cell types, such as renal tubular cells, mesangial cells, monocytes [16, 28], and platelets [16, 29]. Circulating proinflammatory cytokines, especially tumor necrosis factor (TNF)–α and interleukin (IL)–1β, stimulated by direct toxin action on monocytes [30], potentiate the action of Stx on endothelial cells by inducing expression of the receptor Gb_3 [31]. Binding of Stx to its target cells presumably initiates a complex chain of events, including coagulation and proinflammatory processes, that results in HUS [16, 28]. Blocking of Stx binding to endothelial cells to halt these events and prevent the development of HUS might be achieved by generation of specific Stx antibodies by active [32, 33] or passive [34–36] immunization or by the use of synthetic Gb_3 receptor analogues that competitively block toxin binding to the endothelial cell receptor, as discussed in the article by Watanabe et al. [37] in this issue of *The Journal of Infectious Diseases.*

Gb_3 (Pk blood group antigen) is composed of a sphingosine base to which are attached a fatty acid side chain and a terminal trisaccharide, Galα(1-4)-Galβ(1-4)-Glcβ1-, which binds to the Stx B pentamer. Although both Stx1 and Stx2 (including subtypes 2c and 2d) bind to Gb_3, their affinity for the molecule is variable, which probably reflects the fact that the amino acid sequence homology between the toxin-binding B subunits of the 2 toxins is only 57% [38]. Affinity is influenced

Received 16 October 2003; accepted 17 October 2003; electronically published 21 January 2004.

The views expressed in this article are solely those of the author and not necessarily those of Health Canada.

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The Journal of Infectious Diseases 2004;189:355–9

0022-1899/2004/18903-0001$15.00

by difference in binding sites for Gb_3 in the toxins [39–41] and by the orientation of the terminal trisaccharide [42], which may be affected by a number of factors, including the length of the fatty acid side chain [41]. These considerations are important in designing synthetic receptor analogues, which, ideally, should bind both toxins with high affinity.

Theoretically, receptor analogues could be administered orally to block Stx produced in the gut from reaching the circulation or parenterally to block circulating Stx from binding to target endothelial cells. Although the precise timing of these events is not known, grounds for reasonable speculation may be based on clues from current knowledge of the natural history and pathophysiology of STEC-associated disease.

The initial symptoms of STEC infection, typically abdominal cramps and nonbloody diarrhea, manifest after an incubation period of ~3–5 days [2]. The mechanism of this initial diarrhea is not known, and there is no evidence that it is related to the action of Stxs. Approximately one-third of patients develop hemorrhagic colitis [2], which probably results from Stx interaction with endothelial cells lining the microvasculature of the gut lamina propria [43]. HUS occurs in approximately one-tenth to one-fourth of cases [2–4] and manifests ~1 week after the onset of diarrhea [2, 44]. Diagnostic studies of the detection of Stxs and STEC in stool samples from patients indicate that STEC bacterial counts and Stx titers in stool are probably already at their peak when the initial gastrointestinal symptoms appear, and the levels decrease thereafter [7, 44–46]. This is consistent with the occurrence of logarithmic growth, resulting in peak STEC bacterial counts and maximal toxin production, during the incubation period, a concept that is supported by observations of STEC growth in laboratory broth media that show maximal toxin titers during the STEC logarithmic growth phase and cessation of toxin production in the stationary phase [47]. Thus, Stx translocation from the gut to the circulation must occur at some point between the incubation period, when Stx is being actively produced, to ~1 week after the start of the illness, when HUS, the end result of toxin action, becomes clinically evident. Evidence based on elevated levels of plasma markers of thrombin generation, intravascular fibrin accretion, and fibrinolysis inhibition [48, 49] indicates that vascular activation has probably already occurred within 4 days after the onset of diarrhea [49], which suggests that toxin binding to its target cells must have occurred even earlier, perhaps by 2–3 days after the onset of symptoms. Thus, for optimal therapeutic effect, a receptor analogue would have to be administered at some point between the incubation period, before symptoms start, and a point within ~2–3 days after the onset of diarrhea. Presumably, the earlier the better. Unfortunately, experimental animal models of HUS have not been developed in which tests can be made to determine which therapies produce optimal effects. Consequently, investigators in Canada [50–52], Japan [53, 54], and Australia [55, 56] have actively pursued the development of potential therapeutic receptor analogue compounds.

The initial work was done in Canada, where Armstrong and colleagues [50, 52] found that Stx1 and Stx2 bound with high affinity to Pk trisaccharide covalently coupled to Chromosorb P (Synsorb; Johns-Manville Products), a calcinated, diatomaceous material used in gas-liquid chromatography. However, when Synsorb-Pk (R. Ippolito, Alberta Research Council, Edmonton, Alberta, Canada) was coincubated with Vero cells, it was much more efficient in neutralizing the Vero cell cytotoxicity of Stx 1 than that of Stx2 [50]. After a successful phase 1 clinical study of orally administered Synsorb-Pk [51], Armstrong et al. [52] implemented a multicenter, randomized, double-blind, placebo-controlled phase 2 clinical trial in children with diarrhea and confirmed STEC infection, close contact with an individual with HUS or STEC infection, or evidence of early signs of HUS. The results were inconclusive. A phase 3 trial that included individuals who had no evidence of HUS, who had experienced ≤4 days of diarrhea, and who had received a diagnosis of STEC infection confirmed by rapid tests was initiated in 1996, but this trial was prematurely terminated for reasons that were not publicized. Another clinical trial in Japan, which used historical controls, was inconclusive [57].

Trachtman et al. [58] postulated that the severity of diarrhea-associated HUS might be related to ongoing intestinal Stx production and systemic absorption and argued that administering Synsorb-Pk to patients after the onset of HUS might reduce the severity of disease. A multicenter, randomized, double-blind, placebo-controlled trial of Synsorb-Pk in 145 children with diarrhea-associated HUS that lasted from 1997 through 2001 failed to show that this compound could diminish the severity of HUS [58].

The terminal Gb_3 trisaccharide, Galα(1-4)-Galβ(1-4)-Glcβ1-, is one of the alternative structures for the outer core of the lipooligosaccharide surface coat of pathogenic *Neisseria* and *Haemophilus* species [59], an observation that prompted Paton et al. [55] to genetically engineer a nonpathogenic *E. coli* strain that expressed this structure on its surface as a potential "probiotic" agent against STEC infection. The recombinant bacterium CWG308:pJCP-Gb3 absorbed and neutralized Stx1, Stx2, Stx2c, and Stx2d very efficiently, and oral administration completely protected streptomycin-treated mice against an otherwise 100% fatal dose of B2F1, the most mouse-virulent strain used in this model [55]. Synsorb-Pk did not offer protection [60]. These protective effects in mice were confirmed using formaldehyde-killed CWG308:pJCP-Gb3 bacteria [55].

Although oral administration of a receptor blocker has the theoretic advantage of neutralizing toxin before the toxin translocates systemically, investigators in Japan [53] and Canada [61, 62] also investigated the feasibility of administering soluble compounds parenterally. Nishikawa et al. [53] developed a series of dendritic (branched, treelike) polymers, referred to as "SUPER TWIGS," in which several Gb_3 trisaccharide molecules are present in different orientations. A SUPER TWIG (1)6 molecule with 6 trisaccharides had a high affinity for and in vitro neutralizing activity against both Stx1 and Stx2. The compound was tested in a mouse model in which mice were rendered highly susceptible to *E. coli* O157:H7 infection and to its systemic Stx toxemic complications by protein calorie malnutrition (PCM), which lasted for ~2 weeks before intragastric challenge with *E. coli* O157:H7. In this model, which was pioneered by Kurioka et al. [63], PCM mice (but not well-nourished mice) develop Stx-related neurologic symptoms 5 days after infection, and approximately three-fourths of the mice die by 10 days after infection. Kurioka et al. [63] found that inoculated *E. coli* O157:H7 multiplied in the intestines between day 2 after infection and day 4, when growth had reached its peak, an incubation period that is similar in duration to that in humans [2]. Stx was detectable in stool at day 2 after infection, and maximal titers were reached by day 4, paralleling the time of postulated peak toxin titers in humans. Stx was detected in blood only from day 3 to day 5 after infection. *E. coli* O157 strains producing Stx1, Stx2, or both were used, and 10-day mortality tended to be highest among mice challenged with strains that produced both toxins. When Nishikawa et al. [53] administered Stx2 intravenously with SUPER TWIG (1)6, the mice were completely protected from Stx2-induced fatality. When mice infected intragastrically with *E. coli* O157:H7 (producing Stx1 and Stx2) were given SUPER TWIG (1)6 intravenously twice a day from day 3 through day 6 after infection, a majority of treated mice survived, compared with untreated controls, none of which survived. Tailored multivalent soluble Gb_3 analogues constructed for intravenous use have also been developed in Canada. One of these compounds, "Starfish," which has an affinity for Stx1 and Stx2 that is several orders of magnitude higher than that of Synsorb-Pk [61], protected mice when it was injected subcutaneously in combination with a lethal dose of Stx1, but not when it was injected in combination with Stx2 [62]. On the other hand, a modified version of Starfish, called "Daisy" [62], protected mice against both Stx1 and Stx2 when toxin-inhibitor mixtures were administered parenterally. Furthermore, Daisy also protected streptomycin-treated mice from a fatal dose of the Stx2d-producing strain B2F1 [62].

The paper by Watanabe et al. [37] represents an investigation of the potential therapeutic efficacy of a new oral synthetic receptor analogue compound with a configuration different from that of SUPER TWIG, referred to as "Gb_3 polymer," which has an affinity for both Stx1 and Stx2 that is much higher than that exhibited by both parenterally administered SUPER TWIG (1)6 and orally administered Synsorb-Pk. This innovative compound was developed as a synthetic oral alternative to the recombinant CWG308: pJCP-Gb3 bacterium developed by Paton and colleagues [55, 56]. It is derived from linear polymers of acrylamide made with different densities of the Gb_3 trisaccharide Galα(1-4)-Galβ(1-4)-Glcβ1- molecules that are attached to the core acrylamide structure by a spacer and thus differs from the dendritic design of the SUPER TWIG compounds. The trisaccharide densities used were not critical for Stx1 binding, whereas high-affinity Stx2 binding was only observed in compounds with highly clustered trisaccharides.

Watanabe et al. [37] investigated the protective effect of intragastrically administered Gb_3 polymer against the systemic toxemic complications of *E. coli* O157:H7 using a protocol in PCM mice similar to that used earlier by Nishikawa et al. [53] to investigate the efficacy of intravenously administered SUPER TWIG molecules. Compared with saline control, Gb_3 polymer, administered intragastrically on days 3–5 after infection (corresponding to the time during the incubation period when gut toxin levels were in the exponential growth phase and Stx was detectable in the blood), exhibited a highly significant protective effect that was associated with a reduction of Stx2 titers (Stx1 was not tested, because Stx2 was considered more relevant clinically) in both blood and stool. The authors estimate that the Stx-binding capacity of Synsorb-Pk is at least 100,000 times lower than that of the Gb_3 polymer and suggest that this is probably because the density of trisaccharide displayed on the surface of Synsorb-Pk is 2000-fold lower than that on the Gb_3 polymer.

The abandonment of the Synsorb-Pk phase 3 clinical trial means we will not know for certain whether Synsorb-Pk would have succeeded clinically in preventing HUS despite its limited ability to neutralize the biological activity of Stx2 in vitro [50] and in the streptomycin-treated mouse model [60]. However, the Gb_3 polymer of Watanabe et al. [37] has clearly overcome the technical limitations associated with Synsorb-Pk. Gb_3 polymer has high binding affinity for both Stx1 and Stx2, is able to neutralize the biological activities of both toxins in cell culture, and is protective against otherwise fatal infection with *E. coli* O157 when the polymer is administered intragastrically in the PCM mouse model 3 days after infection has been initiated but while it is still in the incubation phase. Experimental evidence suggests that Gb_3 polymer has a substantially higher affinity for both Stx1 and Stx2 (Stx2c and Stx2d were not evaluated) than does Synsorb-Pk. Theoretically, therefore, Gb_3 polymer should have greater clinical efficacy than Synsorb-Pk. The challenge is to discover when in the course of infection an agent such as Gb_3 polymer should be administered to reap the desired benefit in reducing the risk that HUS will develop. This depends on whether toxin production in the gut and toxin translocation are prolonged processes that offer a wide window of opportunity for therapeutic intervention to ameliorate disease severity or whether they are of limited duration and thus offer only a narrow window of opportunity. Clinical laboratory observations [7, 44–46] supported by studies of STEC growth in the test tube [47] indicate that peak STEC growth rates and toxin production occur in the incubation period and that the levels decrease thereafter, an observation further corroborated in the PCM mouse model [53, 63]. This argues against continuous toxin production after the onset of symptoms. Observations from studies of the natural history and pathogenesis of STEC infection, supported by observations in the PCM mouse model [53, 63], suggest that a narrow window of therapeutic opportunity probably exists during the incubation period and might perhaps extend to a period of 2–3 days after the onset of diarrhea. However, initiation of therapy after the appearance of symptoms may not be practical, given that the average interval between the onset of diarrhea and the initial visit to a physician is estimated to be 2.5–3.0 days [44]. On the other hand, it may be possible to prevent HUS if the Gb_3 analogue is administered, as suggested by Paton et al. [56], to contacts of patients with STEC infection, or to individuals in a suspected outbreak, before they have developed symptoms and while they may still be incubating the infection, a situation analogous to the use of prophylactic antibiotics in contacts of persons with meningococcal infection. Antibiotics are a risk factor for HUS, and their use is contraindicated in patients with STEC infection [64]. Uncertainty about prophylactic antibiotic use in contacts of STEC-infected persons strengthens the case for considering oral Gb_3 analogue prophylaxis in this setting. Similar arguments can be made for the prophylactic use of humanized monoclonal antibodies in contacts to prevent HUS [34–36]. However, if oral Gb_3 analogues and humanized monoclonal antibodies were found to be equally efficacious in preventing systemic toxemic complications of STEC infection, the oral agent would be preferred over its parenterally administered counterpart.

References

1. Konowalchuk J, Speirs JI, Stavric S. Vero response to a cytotoxin of *Escherichia coli*. Infect Immun 1977; 18:775–9.
2. Griffin PM. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL, eds. Infections of the gastrointestinal tract. New York: Raven Press, 1995:739–61.
3. Karmali MA. Infection by verocytotoxin-producing *Escherichia coli*. Clin Microbiol Rev 1989; 2:15–38.
4. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 1998; 11:142–201.
5. Riley LW, Remis RS, Helgerson SD, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 1983; 308:681–5.
6. Karmali MA, Petric M, Steele BT, Lim C. Sporadic cases of hemolytic uremic syndrome associated with fecal cytotoxin and cytotoxin-producing *Escherichia coli*. Lancet 1983; 1: 619–20.
7. Karmali MA, Petric M, Lim C, Fleming PC, Arbus GS, Lior H. The association between hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J Infect Dis 1985; 151:775–82.
8. Fitzpatrick MM, Shah V, Trompeter RS, Dillon MJ, Barratt TM. Long term renal outcome of childhood haemolytic uraemic syndrome. BMJ 1991; 303:489–92.
9. Loirat C, Sonsino E, Moreno A, et al. Hemolytic uremic syndrome: an analysis of the natural history and prognostic features. Acta Paediatr Scand 1984; 73:505–14.
10. Siegler RL, Milligan MK, Burningham TH, Christofferson RD, Chang S-Y, Jorde LB. Long-term outcome and prognostic indicators in the hemolytic uremic syndrome. J Pediatr 1991; 118:195–200.
11. Trompeter RS, Schwartz R, Chantler C, et al. Haemolytic uraemic syndrome: an analysis of prognostic features. Arch Dis Child 1983; 58: 101–5.
12. Fong JSC, de Chadarevian JP, Kaplan BS. Hemolytic uremic syndrome: current concepts and management. Pediatr Clin North Am 1982; 29:835–56.
13. Richardson SE, Karmali MA, Becker LE, Smith CR. The histopathology of the hemolytic uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infections. Hum Pathol 1988; 19:1102–8.
14. Upadhyaya K, Barwick K, Fishaut M, Kashgarian M, Segal NJ. The importance of nonrenal involvement in hemolytic uremic syndrome. Pediatrics 1980; 65:115–20.
15. Obrig TG, Louise CB, Lingwood CA, et al. Shiga toxin–endothelial cell interaction. In: Karmali MA, Goglio A, eds. Recent advances in verocytotoxin-producing *Escherichia coli* infections. Amsterdam: Elsevier Science, 1994: 317–24.
16. Taylor CM, Monnens LA. Advances in haemolytic uraemic syndrome. Arch Dis Child 1998; 78:190–3.
17. Vitsky BH, Suzuki Y, Strauss L, Churg J. The hemolytic uremic syndrome: a study of renal pathologic alternations. Am J Pathol 1969; 57: 627.
18. Lingwood CA, Law H, Richardson SE, Petric M, Brunton JL, de Grandis S, Karmali M. Glycolipid binding of natural and recombinant *Escherichia coli* produced verotoxin *in vitro*. J Biol Chem 1987; 262:8834–9.
19. Obrig T. Interaction of Shiga toxins with endothelial cells. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology Press, 1998:303–11.
20. Sandvig K, van Deurs B. Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. Physiol Rev 1996; 76: 949–66.
21. te Loo DM, Monnens LA, van den Heuvel LP, Gubler MC, Kockx MM. Detection of apoptosis in kidney biopsies of patients with D+ hemolytic uremic syndrome. Pediatr Res 2001; 49:413–6.
22. O'Brien AD, Tesh VL, Donohue-Rolfe A, et al. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr Top Microbiol Immunol 1992; 180:65–94.
23. Stein PE, Boodhoo A, Tyrell GJ, Brunton JL, Read RJ. Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. Nature 1992; 355:748–50.
24. Fraser ME, Chernaia MM, Kozlov YV, James MN. Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 A resolution. Nat Struct Biol 1994; 1:59–64.
25. Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JB, Johnson RP, Gyles CL. Associations between virulence factors of Shiga toxin–producing *Escherichia coli* and disease in humans. J Clin Microbiol 1999; 37:497–503.
26. Ostroff SM, Tarr PI, Neill MA, Lewis JH, Hargrett-Bean N, Kobayashi JM. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J Infect Dis 1989; 160:994–8.
27. Louise CB, Obrig T. Specific interaction of *Escherichia coli* O157:H7–derived Shiga-like toxin II with human renal endothelial cells. J Infect Dis 1995; 172:1397–401.
28. Monnens L, Savage CO, Taylor CM. Pathophysiology of hemolytic-uremic syndrome. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology Press, 1998:287–92.
29. Karpman D, Papadopoulou D, Nilsson K, Sjogren AC, Mikaelsson C, Lethagen S. Platelet activation by Shiga toxin and circulatory factors as a pathogenetic mechanism in the he-

molytic uremic syndrome. Blood 2001;97:3100–8.

30. van Setten PA, Monnens LA, Verstraten RG, van den Heuvel LP, van Hinsbergh VW. Effects of verocytotoxin-1 on nonadherent human monocytes: binding characteristics, protein synthesis, and induction of cytokine release. Blood 1996;88:174–83.
31. van de Kar NCAJ, Monnens LAH, Karmali MA, van Hinsbergh VWM. Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosyl ceramide on human endothelial cells: implications for the pathogenesis of the hemolytic uremic syndrome. Blood 1992;80:2755–64.
32. Bielaszewska M, Clarke I, Karmali MA, Petric M. Localization of intravenously administered verocytotoxins (Shiga-like toxins) 1 and 2 in rabbits immunized with homologous and heterologous toxoids and toxin subunits. Infect Immun 1997;65:2509–16.
33. Capozzo AVE, Creydt VP, Dran G, et al. Development of DNA vaccines against hemolytic uremic syndrome in a murine model. Infect Immun 2003;71:3971–8.
34. Mukherjee J, Chios K, Fishwild D, et al. Human Stx2-specific monoclonal antibodies prevent systemic complications of *Escherichia coli* O157:H7 infection. Infect Immun 2002;70:612–9.
35. National Institute of Allergy and Infectious Diseases (NIAID). Report of an expert panel. Prevention of hemolytic uremic syndrome (HUS) caused by infection with Shiga toxin–producing *Escherichia coli* (STEC) with monoclonal antibody therapy. Bethesda, MD: NIAID, 2002. Available at: http://www.niaid.nih.gov/dmid/enteric/hus_prevent.htm. Accessed on 9 January 2004.
36. Yamagami S, Motoki M, Kimura T, et al. Efficacy of postinfection treatment with anti-Shiga toxin (stx) 2 humanized monoclonal antibody TMA-15 in mice lethally challenged with stx-producing *Escherichia coli*. J Infect Dis 2001;184:738–42.
37. Watanabe M, Matsuoka K, Kita E, et al. Oral therapeutic agents with highly clustered globotriose for treatment of Shiga toxigenic *Escherichia coli* infections. J Infect Dis 2004;189:360–8 (in this issue).
38. Jackson MP, Neill RJ, O'Brien AD, Holmes RK, Newland JW. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933. FEMS Microbiol Lett 1987;44:109–14.
39. Itoh K, Tezuka T, Inoue K, Tada H, Suzuki T. Different binding property of verotoxin-1 and verotoxin-2 against their glycolipid receptor, globotriaosyl ceramide. Tohoku J Exp Med 2001;195:237–43.
40. Nyholm PG, Magnusson G, Zheng Z, Norel R, Binnington-Boyd B, Lingwood CA. Two distinct binding sites for globotriaosyl ceramide on verotoxins: identification by molecular modelling and confirmation using deoxy analogues and a new glycolipid receptor for all verotoxins. Chem Biol 1996;3:263–75.
41. Lingwood CA, Mylvaganam M, Arab S, et al. Shiga toxin (Verotoxin) binding to its receptor glycolipid. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology Press, 1998:129–39.
42. Stromberg N, Nyholm PG, Pascher I, Normark S. Saccharide orientation at the cell surface affects glycolipid receptor function. Proc Nat Acad Sci USA 1991;88:9340–4.
43. Richardson SE, Rotman TA, Jay V, et al. Experimental verocytotoxemia in rabbits. Infect Immun 1992;60:4154–67.
44. Tarr PI. Shiga toxin–producing *Escherichia coli* infections: challenges and opportunities. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology Press, 1998:393–401.
45. Tarr PI, Neill MA, Clausen CR, Watkins L, Christie DL, Hickman RO. *Escherichia coli* O157:H7 and the hemolytic uremic syndrome: importance of early cultures in establishing the etiology. J Infect Dis 1990;162:553–6.
46. Tarr PI. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin Infect Dis 1995;20:1–8.
47. Karmali MA, Petric M, Lim C, Cheung R, Arbus GS. Sensitive method for detecting low numbers of verotoxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymyxin extraction of verotoxin. J Clin Microbiol 1985;22:614–9.
48. Proulx F, Seidma EG, Karpman D. Pathogenesis of Shiga toxin–associated hemolytic uremic syndrome. Pediatr Res 2001;50:163–71.
49. Tarr PI. Basic fibroblast growth factor and Shiga toxin–O157:H7–associated hemolytic uremic syndrome. J Am Soc Nephrol 2002;13:817–20.
50. Armstrong GD, Fodor E, Vanmaele R. Investigation of Shiga-like toxin binding to chemically synthesized oligosaccharide sequences. J Infect Dis 1991;164:1160–7.
51. Armstrong GD, Rowe PC, Goodyear P, et al. A phase-1 study of chemically-synthesized verotoxin (Shiga-like toxin) Pk-trisaccharide receptors attached to chromosorb for preventing hemolytic uremic syndrome. J Infect Dis 1995;171:1042–5.
52. Armstrong GD, McLaine PN, Rowe PC. Clinical trials of synsorb Pk in preventing hemolytic uremic syndrome. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology Press, 1998:374–84.
53. Nishikawa K, Koji M, Kita E, et al. A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin–producing *Escherichia coli* O157:H7. Proc Natl Acad Sci USA 2002;99:7669–74.
54. Takeda T, Yoshino K, Adachi E, Sato Y, Yamagata K. In vitro assessment of a chemically synthesized Shiga toxin receptor analog attached to chromosorb P (synsorb Pk) as a specific absorbing agent of Shiga toxin 1 and 2. Microbiol Immunol 1999;43:331–7.
55. Paton AW, Morona R, Paton JC. A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. Nat Med 2000;6:265–70.
56. Paton JC, Rogers T, Morona R, Paton AW. Oral administration of formaldehyde-killed recombinant bacteria expressing a mimic of the Shiga toxin receptor protects mice from fatal challenge with Shiga-toxigenic *Escherichia coli*. Infect Immun 2001;69:1389–93.
57. Ito H, Takeda T, Honda M, et al. Preventive effect of TAK-751S on complications of hemorrhagic colitis (results of clinical study of TAK-751S). Jpn J Antibiot 2002;55:203–27.
58. Trachtman H, Cnaan A, Christen E, et al. Effect of an oral Shiga toxin–binding agent on diarrhea-associated hemolytic uremic syndrome in children. JAMA 2003;290:1337–44.
59. Mandrell RE, Apicella MA. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host modifications of LOS. Immunobiology 1993;187:382–402.
60. Rogers ME, Armstrong G, O'Brien AD. Therapeutic value of stx-specific antibodies or synsorb in streptomycin (Str)–treated mice orally infected with shiga toxin–producing *Escherichia coli* (STEC) [abstract v149]. Presented at: 3rd International Symposium and Workshop on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* (STEC) Infections, Baltimore, 22–26 June 1997. Available at: http://ecoli.bham.ac.uk/vtec/ses7.html.
61. Kitov PI, Sadowska JM, Mulvey G, et al. Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. Nature 2000;403:669–72.
62. Mulvey G, Marcato P, Kitov P, Sadowska JM, Bundle DR, Armstrong GD. Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. J Infect Dis 2003;187:640–9.
63. Kurioka T, Yunou Y, Kita E. Enhancement of susceptibility to Shiga toxin–producing *Escherichia coli* O157:H7 by protein calorie malnutrition in mice. Infect Immun 1998;66:1726–34.
64. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. N Engl J Med 2000;342:1930–6.

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Tetrahedron Letters 45 (2004) 9383–9386

Tetrahedron Letters

Synthesis of a useful lauryl thioglycoside of sialic acid and its application

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Received 1 October 2004; revised 18 October 2004; accepted 20 October 2004
Available online 5 November 2004

Abstract—An efficient synthesis of a useful thioglycosyl donor **2** was accomplished directly from known peracetylated sialic acid methyl ester and 1-dodecanethiol (lauryl mercaptan) in the presence of BF_3–OEt_2. The reactivities of the lauryl glycosides for glycosidation by means of TMSOTf as a convenient promoter were investigated, and the lauryl thioglycoside showed satisfactory activities. Further transformation of the lauryl glycoside was also attempted to give a 5-azide analogue **14** of the sialic acid, which was also reacted with a secondary alcohol in the presence of TMSOTf to give known glycoside **15** in high yield.

Oligosaccharide chains of glycoconjugates take important biological events such as fertilization, differentiation, aging, malignant alteration, and so on.[1] Sialylated oligosaccharides have various oligosaccharide structures and play roles in cell recognition and signaling.[2] Since sialic acid usually exists at the nonreducing ends of oligosaccharide chains of glycoconjugates on the cell surface, it seems that there are many opportunities for interaction between carbohydrate and receptor protein. In order to investigate the significance and mechanisms of those ligand—receptor interactions, a method for synthesizing sialooligosaccharide is needed.[3] Glycosidation to form sialoside by using various glycosyl donors derived from sialic acid has been extensively investigated.[4] Although those donors are useful for assembly of sialic acid moiety into oligosaccharide chains, an improvement of the preparation of sialyl donors is ongoing. In the chemical syntheses of sialo-oligosaccharides, the thioglycoside methodology is frequently used for such objective, despite the fact that a large number of sialyl donors have been prepared.[4] For making thioglycoside donors of sialic acid, a variety of volatile thiols or those stinking TMS derivatives are generally utilized.[5] Recently, Sakairi and co-workers reported thioglycosides having a lauryl moiety in order to avoid such undesired factors, and the lauryl thioglycosides showed efficient ability for the glycoside syntheses.[6] In this letter, we describe an efficient synthesis of novel thioglycoside **2** of sialic acid having a lauryl moiety from known fully protected sialic acid derivative **1** and its applications, including possible utilization as a glycosyl donor and further transformation of **2** into its 5-azide analogue **14**.

For preparation of methyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonate **1** as a starting material, we selected Sinaÿ's protocol[7] to obtain anomeric mixtures of **1** in high yield. Although preparation of a thioglycoside of sialic acid is usually carried out using a TMS derivative of the thiol in the presence of Lewis acid as a catalyst, direct conversion of **1** into its thioglycoside was tested, since a TMS derivative of l-dodecanthiol is not available. Acetate **1** was treated with 1-dodecanthiol (4 M excess) in the presence of BF_3–OEt_2[8] (3 M excess) at 0 °C and then at rt, and the reaction was monitored by TLC until disappearance of **1**. The usual work-up of the reactant gave an anomeric mixture of **2**, which was separated by means of silica gel chromatography into anomers[†], **2α** (26.2%), $[\alpha]_D^{28}$ +26 (*c* 1.09, $CHCl_3$) and **2β** (59.2%), $[\alpha]_D^{28}$

Keywords: Thioglycosides; Lauryl mercaptan; Sialic acid; Glycosidation; Carbohydrates.
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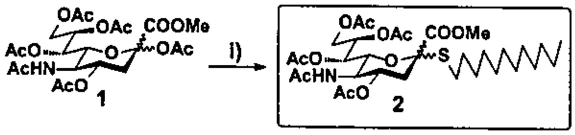
[†] All new compounds with specific rotation data gave satisfactory results of elemental analyses or high resolution mass spectra.

0040-4039/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2004.10.105



Scheme 1. Reagents and conditions: (i) dodecanthiol (4M excess), BF_3–OEt_2 (3M excess), CH_2Cl_2, 0°C → rt, 3.5h.

Table 1. Selected chemical shifts and J values related to sialic acid moieties

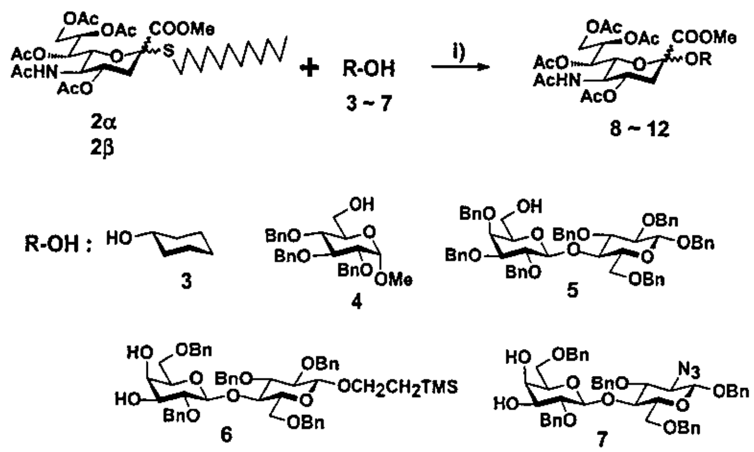
Compound	H-3eq (ppm)	H-4 (ppm)	$J_{7,8}$ (Hz)	Δδ|H-9a-H-9b| (ppm)
2α	2.71	4.86	8.1	0.20
2β	2.52	5.27	2.68	0.63
8α[a]	2.60	4.83	8.0	0.20
8β[a]	2.44	5.18	2.4	0.81
9α[b]	2.65	4.86	9.1	0.28
9β[b]	2.48	5.17	ND[e]	0.97
10α	2.53	4.84	ND[e]	0.25
10β	2.33	4.82	ND[e]	0.60
11[c]	2.50	4.85	7.9	0.36
12	2.51	4.86	8.0	0.33
14	2.66	5.29	1.6	0.55
15α[d]	2.66	5.28	5.8	0.48
16	5.97[f]	5.56	6.1	0.34

[a] Lit. Ref. 12.
[b] Lit. Ref. 13.
[c] Lit. Ref. 14.
[d] Lit. Ref. 16.
[e] ND means not determined due to overlapping of other protons.
[f] H-3 proton of the glycal.

−67 (c 0.91, $CHCl_3$). The total yield of **2** was 85.4% after isolation. The structure of each anomer was confirmed by the results of ^1H NMR[9] and the results are summarized in Table 1 (see Scheme 1).

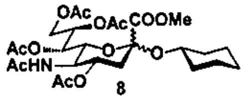
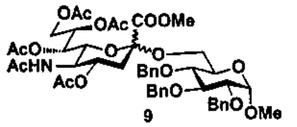
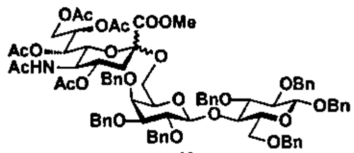
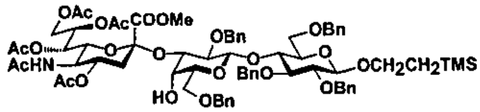
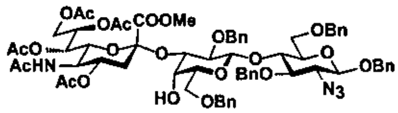
Given the success of the preparation of a thioglycoside of sialic acid having a lauryl moiety, we next turned our attention to the potency of **2** as a glycosyl donor for application to sialoside synthesis. A large number of methods for activation of thioglycosides have been developed and are summarized in review papers.[5] Since the activation of thioglycoside is similar to that of the n-pentenyl glycoside, initially introduced by Fraser-Raid and co-workers.[10] Huchel and Schmidt[11] reported a convenient system for activation of a thioglycosyl donor by using NIS–TMSOTf as mediators instead of NIS–TfOH, IDCP, NIS–TESOTf, as well as DMTST. Because of the low cost, easy handling, and availability of TMSOTf, we investigated the usefulness of a TMSOTf–NIS system for activating the lauryl thioglycoside **2**, and the results are summarized in Scheme 2 and Table 1. In the preliminary glycosidation, the thioglycoside **2α** and secondary alcohol **3** as a typical control were condensed by using NIS–TMSOTf in acetonitrile at −35°C for 3h. When TLC showed complete disappearance of **2α**, the reaction mixture was filtered on a pad of Celite. The usual work-up gave **8α**[12] in 46.0% yield and **8β** in 32.9% yield (α:β = 58:42) after chromatographic separation, **8α**, $[\alpha]_D^{24}$ −20 (c 0.60, $CHCl_3$). In the case of **2β**, the same treatment with alcohol **3** in the presence of TMSOTf gave **8α** in 52.6% yield and **8β** in 39.5% yield (α:β = 57:43) after isolation. Glycosidation of **2** with primary alcohols, **4** and **5**, was performed in same manner as that described for alcohol **3** to afford known **9**[13] and **10** in high yields with similar stereoselectivity, **10**, $[\alpha]_D^{37}$ −13 (c 0.21, $CHCl_3$). As for secondary alcohols, **6**[14] and **7**[15], the glycosidation also proceeded smoothly to give known **11**[14] and **12**, respectively, in high yields, **12**, $[\alpha]_D^{34}$ −6.3 (c 1.00, $CHCl_3$). These results suggested that the lauryl glycoside **2** underwent TMSOTf-promoted glycosidation with various alcohols, the anomeric ratio of the newly formed glycosidic bonds was dependent on the anomeric configuration of the donor, and the α-selectivity of **2α** in the glycosidation reaction was slightly higher than that of **2β**. However, using β-lauryl thioglycoside **2β** as a donor for the glycosidation gives a higher yield than that of **2α** even though α-selectivity is lower (see Table 2).

In our ongoing synthetic study of sialyl oligosaccharides, we previously reported the synthesis and reactivity of a 5-azido analogue of sialic acid.[16] 5-Azido analogues



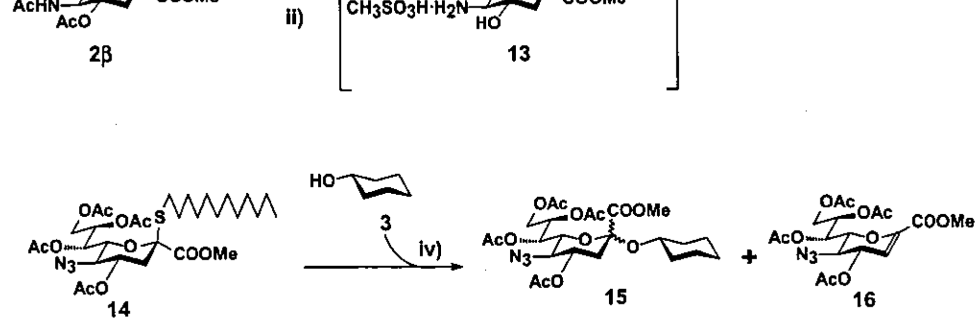
Scheme 2. Reagents and conditions: (i) NIS (2M excess), TMSOTf (0.2M excess), R–OH (0.5M excess), MS3A, CH_3CN, −35°C, 3h.

Table 2. Results of glycosidation of **2** with alcohols

Donor	Acceptor R–OH	Product	Yield[a] (%)	Ratio[b] (α:β)
2α **2β**	**3**	 **8**	79 92	58:42 57:43
2α **2β**	**4**	 **9**	87 93	71:29 65:35
2α **2β**	**5**	 **10**	70 83	63:37 57:43
2α **2β**	**6**	 **11**	48 55	1:0[c] 1:0[c]
2α and **2β**	**6**	**11**	50	1:0[c]
2α and **2β**	**7**	 **12**	66	1:0[c]

[a] Isolated yield based on alcohol.
[b] Mol/mol ratio after isolation.
[c] Isolation of β-anomer was not conducted.

have been prepared by several groups,[17] and 5-azido analogues of sialic acid are, therefore, of great importance for developing *N*-substituted sialooligosaccharide. Therefore, conversion of **2β** into its 5-azido analogue **14** was attempted by the previously reported method shown in Scheme 3. In brief, de-*O*-acetylation of **2β** followed by acid hydrolysis of the acetamide[18] gave corresponding ammonium salt **13**, which was treated with TfN_3 followed by usual acetylation to give pure **14** in 89.6% yield after silica gel chromatographic purification, $[\alpha]_D^{31}$ −90 (*c* 1.31, $CHCl_3$), IR (KBr) 2114 ($\nu_{N=N=N}$) and 1748 ($\nu_{C=O}$) cm^{-1}, ^1H NMR ($CDCl_3$) δ 3.21 (t, 1 H, $J_{4,5} = J_{5,6}$ 10.2 Hz, H-5). The azide **14** was quantitatively condensed with an alcohol **3** by TMSOTf-mediated glycosidation as described above to yield known **15α** (48.9%) and **15β** (51.1%) (α:β = 49:51) after isolation. The reaction also gave glycal product **16** in 43.4% yield based on **14**. In contrast to our previous study of 5-azido



Scheme 3. Reagents and conditions: (i) NaOMe, MeOH, rt, 2 h; (ii) CH_3SO_3H (2 M excess), MeOH, 60 °C, 19 h; (iii) TfN_3 (4.5 M excess), DMAP (3 M excess), MeOH, rt, overnight, then, Ac_2O–Pyr, rt; (iv) NIS (2 M excess), TMSOTf (0.2 M excess), **3** (0.5 M excess), MS3A, CH_3CN, −35 °C, 3 h.

sialic acid,[16] the yield of α glycoside from **14** was unfortunately lower than that from phenyl thioglycoside (60.7%).

In conclusion, an efficient synthesis of thiolauryl glycoside **2** was accomplished using nonstinking thiol, and TMSOTf-mediated glycosidation of both anomers was tested and showed excellent reactivities. Further transformation of thioglycoside **2β** into corresponding azido analogue **14** was performed, and **14** also underwent TMSOTf-mediated glycosidation to give a known glycoside in high yield. This methodology is applicable for our synthetic studies[19] of 'Glyco-Silicon Functional Materials', including assembly of sialyl lactose[19c] and sialyl lactosamine,[19d] and the results will be reported in the near future.

Acknowledgements

This work was partly supported by a grant from NEDO [New Energy and Industrial Technology Development Organization (Glycocluster project)]. We are grateful to Snow Brand Milk Products Co., Ltd, for providing the sialic acid used in this study.

References and notes

1. For example, see: Varki, A.; Cummings, R.; Esco, J.; Freeze, H.; Hart, G.; Marth, J. *Essentials of Glycobiology*; Cold Spring Harbor: New York, 1999, and references cited therein.
2. For example, see: Hakomori, S.-I.; Igarashi, Y. *J. Biochem.* **1995**, *118*, 1091–1103, and references cited therein.
3. For example, see: Inoue, Y.; Lee, Y. C.; Troy, F. A., II. *Sialobiology and other novel forms of glycosylation*; Gakushin Publishing Co.: Osaka, Japan, 1999, and references cited therein.
4. Boons, G.-J.; Demchenko, A. V. *Chem. Rev.* **2000**, *100*, 4539–4565.
5. Garegg, P. *Adv. Carbohydr. Chem. Biochem.* **1997**, *52*, 179–205.
6. Matsui, H.; Furukawa, J.-I.; Awano, T.; Nishi, N.; Sakairi, N. *Chem. Lett.* **2000**, 326–327.
7. Marra, A.; Sinaÿ, P. *Carbohydr. Res.* **1989**, *190*, 317–322.
8. (a) Hasegawa, A.; Murase, T.; Ogawa, M.; Ishida, H.; Kiso, M. *J. Carbohydr. Chem.* **1990**, *9*, 415–428; (b) Xue, J.; Pan, Y.; Guo, Z. *Tetrahedron Lett.* **2002**, *43*, 1599–1602.
9. (a) Dabrowski, U.; Friebolin, H.; Brossmer, R.; Supp, M. *Tetrahedron Lett.* **1979**, *20*, 4637–4640; (b) van der Vleugel, D. J. M.; van Heeswijk, W. A. R.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1982**, *102*, 121–130; (c) van der Vleugel, D. J. M.; Wassenburg, F. R.; Zwikker, J. W.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1982**, *104*, 221–233.
10. Konradsson, P.; Udodong, U. E.; Fraser-Reid, B. *Tetrahedron Lett.* **1990**, *31*, 4313–4316.
11. Huchel, U.; Schmidt, R. R. *Tetrahedron Lett.* **1998**, *39*, 7693–7694.
12. Kanie, O.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1988**, *7*, 501–506.
13. Okamoto, K.; Kondo, T.; Goto, T. *Tetrahedron* **1987**, *43*, 5909–5918.
14. Hasegawa, A.; Nagahama, T.; Ohki, H.; Kiso, M. *J. Carbohydr. Chem.* **1992**, *11*, 699–714.
15. Paulsen, H.; Steiger, K.-M. *Carbohydr. Res.* **1987**, *169*, 105–125.
16. Matsuoka, K.; Oka, H.; Terunuma, D.; Kuzuhara, H. *Carbohydr. Lett.* **2001**, *4*, 123–130.
17. (a) Schneider, R.; Freyhardt, C. C.; Schmidt, R. R. *Eur. J. Org. Chem.* **2001**, 1655–1661; (b) Yu, C.-S.; Niikura, K.; Lin, C.-C.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2001**, *40*, 2900–2903; (c) Lu, K.-C.; Tseng, S.-Y.; Lin, C.-C. *Carbohydr. Res.* **2002**, *337*, 755–760.
18. Komba, S.; Galustian, C.; Ishida, H.; Feizi, T.; Kannagi, R.; Kiso, M. *Angew. Chem., Int. Ed.* **1999**, *38*, 1131–1133.
19. (a) Matsuoka, K.; Terabatake, M.; Esumi, Y.; Terunuma, D.; Kuzuhara, H. *Tetrahedron Lett.* **1999**, *40*, 7839–7842; (b) Matsuoka, K.; Kurosawa, H.; Esumi, Y.; Terunuma, D.; Kuzuhara, H. *Carbohydr. Res.* **2000**, *329*, 765–772; (c) Matsuoka, K.; Oka, H.; Koyama, T.; Esumi, Y.; Terunuma, D. *Tetrahedron Lett.* **2001**, *42*, 3327–3330; (d) Matsuoka, K.; Ohtawa, T.; Hinou, H.; Koyama, T.; Esumi, Y.; Nishimura, S.-I.; Hatano, K.; Terunuma, D. *Tetrahedron Lett.* **2003**, *44*, 3617–3620.

MAJOR ARTICLE

Oral Therapeutic Agents with Highly Clustered Globotriose for Treatment of Shiga Toxigenic *Escherichia coli* Infections

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(See the editorial commentary by Karmali, on pages 355–9.)

Shiga toxin (Stx) is a major virulence factor in infection with Stx-producing *Escherichia coli* (STEC). We developed a series of linear polymers of acrylamide, each with a different density of trisaccharide of globotriaosylceramide (Gb_3), which is a receptor for Stx, and identified Gb_3 polymers with highly clustered trisaccharides as Stx adsorbents functioning in the gut. The Gb_3 polymers specifically bound to both Stx1 and Stx2 with high affinity and markedly inhibited the cytotoxic activities of these toxins. Oral administration of the Gb_3 polymers protected mice after administration of a fatal dose of *E. coli* O157:H7, even when the polymers were administered after the infection had been established. In these mice, the serum level of Stx was markedly reduced and fatal brain damage was substantially suppressed, which suggests that the Gb_3 polymers entrap Stx in the gut and prevent its entrance into the circulation. These results indicate that the Gb_3 polymers can be used as oral therapeutic agents that function in the gut against STEC infections.

Shiga toxin (Stx)–producing *Escherichia coli* (STEC), including *E. coli* serotype O157:H7, causes gastrointestinal diseases in humans that are often followed by potentially fatal systemic complications, such as acute encephalopathy and hemolytic-uremic syndrome (HUS) [1–4]. During infection, STEC colonizes the gut and releases Stx into the gut lumen. The toxin is then absorbed into the circulation and causes vascular damage in specific target tissues, such as the brain and kidney, resulting in systemic complications. Therefore, the development of an effective Stx adsorbent that functions in the gut or an Stx neutralizer that functions in the circulation would be a promising approach to finding a viable therapy.

Stx is classified into 2 closely related subgroups, Stx1 and Stx2. Epidemiologic and experimental studies have suggested that Stx2 has greater clinical significance than does Stx1. Stx2-producing STEC strains are associated with the development of HUS in humans more frequently than are Stx1-producing strains [5], and Stx2-producing strains were found to cause more-severe neurologic symptoms in an experimental study of STEC-infected piglets [6]. Both Stx1 and Stx2 consist of a catalytic A subunit that has RNA *N*-glycosidase activity and inhibits eukaryotic protein synthesis and a pentameric B subunit that recognizes and binds to the functional cell-surface receptor globotriaosylceramide [Gb_3; Galα(1-4)-Galβ(1-4)-Glcβ1-ceramide] [4, 7, 8]. Because multiple interactions of the B subunit pentamer with the trisaccharide moiety of Gb_3 are known to be essential to high-affinity binding of Stx to its receptor, several Stx neutralizers containing the trisaccharide

Received 14 May 2003; accepted 1 July 2003; electronically published 21 January 2004.

Presented in part: 37th Joint Conference on Cholera and Other Bacterial Enteric Infections Panel, Okinawa, Japan, 17–19 December 2002.

Financial support: Ministry of Health, Labor and Welfare, Japan (Health Sciences Research Grant on Emerging and Re-emerging Infectious Diseases H12-E-25 and Grant for International Health Cooperation Research 14-K-10).

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0022-1899/2004/18903-0002$15.00

in multiple configurations have been developed [9–12].

Recently, we developed a series of carbosilane dendrimers carrying various numbers of the trisaccharides (referred to as "SUPER TWIG") and identified a SUPER TWIG with 6 trisaccharides [SUPER TWIG (1)6] as an Stx neutralizer functioning in the circulation [13]. Intravenous administration of SUPER TWIG (1)6 protected STEC-challenged mice, even when SUPER TWIG (1)6 was administered after the infection had been established, which indicates that SUPER TWIG (1)6 is a promising therapeutic agent for use against STEC infection in humans [13]. On the other hand, development of an Stx adsorbent that functions in the gut is important, because oral administration of this type of agent can be widely applicable not only to treatment of individuals with STEC infection, but also to treatment of those at risk of such infections. Recently, oral administration of a genetically manipulated bacterium expressing these trisaccharides on its surface was reported to protect mice after challenge with a fatal dose of STEC [12, 14]. However, no synthetic compound has previously been developed that effectively adsorbs Stx, especially Stx2, present in the gut.

In this study, we used a series of linear polymers of acrylamide with different numbers of the trisaccharide of Gb_3 to develop Stx adsorbents that would function in the gut. We found that Gb_3 polymers with highly clustered trisaccharides specifically bound to both Stx1 and Stx2 with high affinity and markedly inhibited the cytotoxic activities of these toxins. The K_d values of the most active Gb_3 polymer to the B subunits of Stx1 and Stx2 were even lower than those of SUPER TWIG (1)6, which indicates that this Gb_3 polymer binds to the B subunits more strongly than does SUPER TWIG (1)6. Finally, oral administration of the Gb_3 polymers protected mice after challenge with a fatal dose of *E. coli* O157:H7, which suggests that the Gb_3 polymer could be used as an oral therapeutic agent to treat STEC infections in humans.

MATERIALS AND METHODS

Materials. Polymers with carbohydrates used in this study were synthesized as described elsewhere (K.M., A.M., T.W., and D.T., unpublished data), and were characterized by ^1H nuclear magnetic resonance spectroscopy to confirm their structures. In brief, globotriaosyl derivatives with a polymerizable aglycon were prepared from D-galactose and D-lactose by a slight modification of the method of Matsuoka et al. [15]. Elongation of the aglycon as a spacer arm was performed by a radical addition of aminoethanethiol to the C=C double bond, followed by acryloylation to produce the acrylamide-type carbohydrate monomer. These water-soluble monomers were polymerized by a standard radical polymerization protocol [16] to produce white, powdery glycopolymers of high molecular weight after lyophilization. The molar ratio of oligosaccharide to acrylamide of each polymer was determined by ^1H nuclear magnetic resonance spectroscopy. The average molecular weights of the polymers were estimated by size-exclusion chromatography in water using a Shodex Asahipak GS-510 7E column. Calibration curves were obtained using pullulan standards (5.8, 12.2, 23.7, 48, 100, and 186 kDa; Shodex Standard P-82). Free trisaccharide was kindly provided by Kyowa Hakko Kogyo (Tokyo). Recombinant Stx1 and Stx2 were prepared according to methods described elsewhere [17]. Recombinant histidine-tagged Stx1 B subunit (1B-His) and Stx2 B subunit (2B-His), in which 6 histidine residues were added at the carboxy termini of the B subunits, were prepared as follows: From the pUC118 vector and the pCH283 vector, which contained the complete coding sequences of Stx1 and Stx2, respectively (constructs were kindly provided by S. Yamasaki and T. Hamabata, International Medical Center of Japan, Tokyo) [18], an *NcoI-BamHI* fragment was prepared by polymerase chain reaction with the primers 5'-AGAGCCATGGCGACGCCTGATTGTGTAACT-3' and 5'-AGAGGGATCCGCACGAAAAATAACTTCGCT-3' for Stx1 and 5'-AGAGCCATGGATTGTGCTAAAGGTAAAATT-3' and 5'-AGAGGGATCCGCGTCATTATTAAACTG-3' for Stx2. The fragments obtained were ligated into the *NcoI-BamHI* site of the pET-28a vector (Novagen). Competent *E. coli* BL21DE(3) cells (Novagen) were then transformed with these vectors. The transformed BL21DE(3) cells were cultured in 1 L of Luria-Bertani broth (Difco) supplemented with 30 μg/mL kanamycin (Nacalai Tesque) at 37°C to midexponential phase. The cultures were subsequently treated with 1.0 mmol/L isopropyl β-D(−)-thiogalactopyranoside (Wako Pure Industries) for 4 h at 37°C. Collected cell pellets were lysed in 10 mL of PBS containing 6000 U/mL polymyxin B (Sigma). After centrifugation, the resulting supernatants were incubated with 100 μL of Ni^{2+}-charged resin (Novagen) for 2 h at 4°C. After extensive washing of the beads, soluble 1B-His and 2B-His were eluted from the beads by incubation with elution buffer (1 mol/L imidazole, 500 mmol/L NaCl, and 80 mmol/L Tris-HCl; pH 7.9) for 5 min at 25°C. Phospholipid vesicles containing either Gb_3 or globotetraosylceramide (Gb_4) were prepared using phosphatidylcholine and either glycolipid (molar ratio, 24:1), as described elsewhere (X. T. Zeng, K. Nishikawa, and Y. Natori, unpublished data). ^{125}I-labeled Stx1 (^{125}I-Stx1) and ^{125}I-Stx2 were prepared by the iodine monochloride method, as described elsewhere [19].

Cells. Vero cells were maintained in DMEM supplemented with 10% fetal calf serum. Cells were seeded in 24- and 96-well plastic microplates for binding and cytotoxicity assays, respectively.

Kinetic analysis of Gb_3 polymer binding to immobilized 1B-His and 2B-His. Gb_3 polymer binding to immobilized 1B-His and 2B-His was quantified using a BIAcore instrument [20]. Ni^{2+} was fixed on a nitrilotriacetic acid sensor chip (BIAcore), and recombinant 1B-His or 2B-His (10 μg/mL) was injected into the system, where it was immobilized on the chip. Various concentrations of compounds were injected (time 0) over the immobilized 1B-His or 2B-His at a flow rate of 20 μL/min for at least 3 min to reach plateau at 25°C. The resonance unit (RU) is an arbitrary unit used by the BIAcore system. The RU value obtained without recombinant protein was subtracted from the data obtained from immobilized 1B-His or 2B-His to correct for the background. The binding kinetics were analyzed by Scatchard plot, using BIAevaluation software, version 3.0 (BIAcore).

^{125}I-Stx binding assay. For the binding assay, Vero cells were treated with 1 μg/mL ^{125}I-Stx1 or ^{125}I-Stx2 (7×10^6 or 3.8×10^6 cpm/μg of protein, respectively) in the absence or presence of the desired amount of a given compound or with unlabeled Stx1 or Stx2 (50 μg/mL) for 30 min at 4°C. After extensive washing, the cells were dissolved in lysis solution (0.1 mol/L NaOH and 0.5% SDS). Recovered radioactivity was measured by a γ-counter (Packard). Specific binding of these radiolabeled Stxs was confirmed by the complete inhibition of the unlabeled Stxs (data not shown).

Cytotoxicity assay. For the cytotoxicity assay, subconfluent Vero cells in a 96-well plate were treated with Stx1 or Stx2 (10 pg/mL) in the absence or presence of the desired amount of a given compound for 72 h. The relative number of living cells was determined by using a WST-1 Cell Counting Kit (Wako Pure Industries).

Mouse infection protocol. Specific pathogen–free, 3-week-old female C57BL/6 mice that had been weaned were purchased from Charles River Laboratories. The animals were fed a low-protein diet (5% protein) for 2 weeks to achieve protein calorie malnutrition [21]. At 5 weeks of age, mice were infected intragastrically with 2×10^6 cfu of *E. coli* O157:H7 strain N-9, which produces both Stx1 and Stx2, as described elsewhere [21]. The animals were fed the low-protein diet even after the start of the infection. Seven or 8 infected mice received Gb_3 polymers (25 μg/g of body weight) dissolved in 0.1 mL of saline twice a day intragastrically; this treatment was initiated on day 3 after infection and continued until day 5. Fifteen infected mice were treated with 0.1 mL of saline as a vehicle control by the same protocol used for treatment with Gb_3 polymers. At day 4 after infection, 3 mice from each group were killed, and the Stx content in their blood was determined. The animal experimentation guidelines of Nara Medical University (Kashihara, Nara, Japan) were followed. Data were analyzed using the Kaplan-Meier survival analysis or, when no mice had died by the end of the observation, Fisher's exact test.

Measurement of Stx2 in blood and stool. Blood was obtained from the ophthalmic arteries or by cardiac puncture of infected mice. Serum was separated from clotted blood by centrifugation. Quantification of Stx2 in blood and stool samples was performed by ELISA using a commercially available kit (Bio-Rad Laboratories), as described elsewhere [21], after a standard curve had been constructed with purified Stx2 incorporated into stool or serum from normal mice. With this kit, the limit of detection for Stx2 was 12 pg/mL of stool and 18 pg/mL of serum.

Stool samples were homogenized in PBS at a concentration of 50 mg/mL and then diluted 5-fold with the dilution buffer supplied by the manufacturer. A 100-μL volume of homogenate was assayed with the ELISA kit. Serum samples were concentrated 20-fold by ultrafiltration, 20 μL of each concentrated sample was mixed with 4 volumes of the sample dilution buffer, and 100 μL of each mixture was assayed with the ELISA kit.

Histological and immunohistochemical examination. For histological and immunohistochemical examination of the brain, 5 mice that had been treated with Gb_3 polymer or saline, as described above, were used. At day 4 (for saline-treated mice) or day 30 (for Gb_3 polymer–treated mice) after infection, the mice were killed, and their brains were immediately fixed in 10% formalin. For histological examination, some of the paraffin-embedded sections were stained with hematoxylin-eosin, Alcian blue (pH 2.5)–periodic acid–Schiff stain, or Luxol fast blue. Immunohistological localization of Stx2 was detected by a monoclonal anti-Stx2 antibody (IgG; 1 μg/mL; Toxin Technology), as described elsewhere [21].

RESULTS

Direct, high-affinity binding of Gb_3 polymers to the Stx B subunit. We developed a series of linear polymers of acrylamide, each with a different density of the trisaccharide of Gb_3 [Galα(1-4)-Galβ(1-4)-Glcβ1-] or lactose (Lac) [Galβ(1-4)-Glcβ1-], through a spacer that binds the sugar group to the core structure (figure 1). The molar ratio of oligosaccharide to acrylamide was varied as shown in table 1. Polymers were indicated as X:Y, in which X and Y represent the number of carbohydrate-assembled and carbohydrate-free acrylamide units, respectively. Because the molar content of oligosaccharide per weight differed among these polymers (table 1), the concentration of each polymer was given as micromolar concentrations of trisaccharide or Lac in the following experiments, which enables direct comparison of their activities on a per-oligosaccharide basis.

With 1B-His and 2B-His immobilized on a BIAcore sensor chip, K_d for each polymer for the B subunit pentamer of Stx1 or Stx2 was determined by Scatchard plot analysis. Gb_3 polymer 1:0, which had the most densely clustered trisaccharides, directly bound to both 1B-His and 2B-His with very high affinity (figure 2). The K_d values determined by Scatchard plot analysis were 0.34 and 0.68 μmol/L, respectively (table 2), both of which are one-half of those for SUPER TWIG (1)6 (0.72 and 1.3 μmol/L), when comparison is made on a per-trisaccharide basis

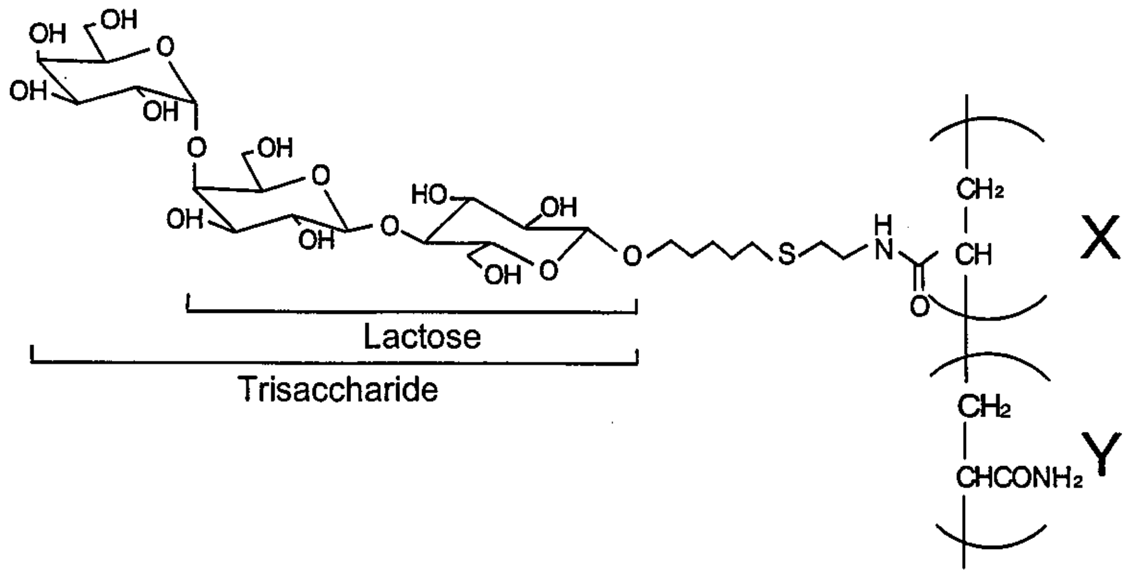

Figure 1. Structures of globotriaosylceramide (Gb_3) and lactose polymers. Linear polymers of acrylamide with trisaccharide of Gb_3 [Galα(1-4)-Galβ(1-4)-Glcβ1-] or lactose [Galβ(1-4)-Glcβ1-] are shown.

(K. Nishikawa, K. Matsuoka, K. Hino, K. Igai, D. Terunuma, and Y. Natori, unpublished data), which indicates that the Gb_3 polymer binds to the B subunits more strongly than does SUPER TWIG (1)6. Under the same conditions, phospholipid vesicles containing Gb_3, but not Gb_4, at a molar ratio of 25:1 specifically bound these recombinant B subunits, confirming their specific recognition of the trisaccharide of Gb_3 (data not shown). In contrast, Lac polymer 1:0, which has a structure that is almost the same as that of Gb_3 polymer 1:0, except for the terminal sugars, bound to neither 1B-His nor 2B-His (figure 2), which suggests that the terminal galactose of the trisaccharide is strictly required for high-affinity binding to the B subunits. Interestingly, the K_d values of Gb_3 polymers 2:17, 1:11, and 1:12 for the Stx2 B subunit were 2, 6, and 10 times higher, respectively, than the value of Gb_3 polymer 1:0, whereas the K_d values of all of these Gb_3 polymers for the Stx1 B subunit were in a similar range (figure 2 and table 2). These results indicate that more highly clustered trisaccharides in the Gb_3 polymers are required for high-affinity binding to the Stx2 B subunit, clearly demonstrating a different sugar-clustering effect in the recognition of trisaccharide between Stx1 and Stx2.

Inhibition of the biological activities of Stx by Gb_3 polymers. All of the Gb_3 polymers markedly inhibited the binding of ^{125}I-Stx1 and ^{125}I-Stx2 to Vero cells, one of the cell types most sensitive to Stx (figure 3*A*). The IC_{50} values of Gb_3 polymer 1:0 for ^{125}I-Stx1 and ^{125}I-Stx2 binding were 0.33 and 0.34 μmol/L (table 3), which are similar to and 10 times lower than those of SUPER TWIG (1)6 (0.33 and 3.5 μmol/L, respectively), when comparison is made on a per-trisaccharide basis. These results indicate that the inhibitory effect of Gb_3 polymer 1:0 is superior to that of SUPER TWIG (1)6. In contrast, no inhibitory effect was observed with Lac polymer 1:0 or free trisaccharide, even at a concentration of 100 μmol/L (figure 3*A*).

The Gb_3 polymers effectively inhibited the cytotoxic activity of Stx1 and, to a lesser extent, Stx2 (figure 3*B*). The IC_{50} value of Gb_3 polymer 2:17 for Stx2 was 18.8 μmol/L, which is 23 times higher than that of Gb_3 polymer 1:0 (0.82 μmol/L), whereas the value for Stx1 was 0.16 μmol/L, or 3 times higher than that of Gb_3 polymer 1:0 (0.049 μmol/L) (table 3). These results indicate that the dependency of the inhibitory effect on the trisaccharide density of each polymer was more clearly observed for Stx2, which further supports the hypothesis that the sugar-clustering effect in the recognition of trisaccharide for Stx1 is different from that for Stx2. No inhibitory effect was observed with Lac polymer 1:0 or free trisaccharide, even at a

Table 1. Molar content of the trisaccharide of globotriaosylceramide (Gb_3) or lactose (Lac) in linear polymers of acrylamides.

Polymer, X:Y[a]	Density, mol × 10^3/g
Gb_3	
1:0	1.4
2:17	0.75
1:11	0.66
1:12	0.63
Lac 1:0	1.7

[a] The molar ratio of oligosaccharide to acrylamides was determined by ^{1}H nuclear magnetic resonance spectroscopy and described as X:Y, in which X and Y represent the nos. of carbohydrate-assembled and carbohydrate-free acrylamide units, respectively.

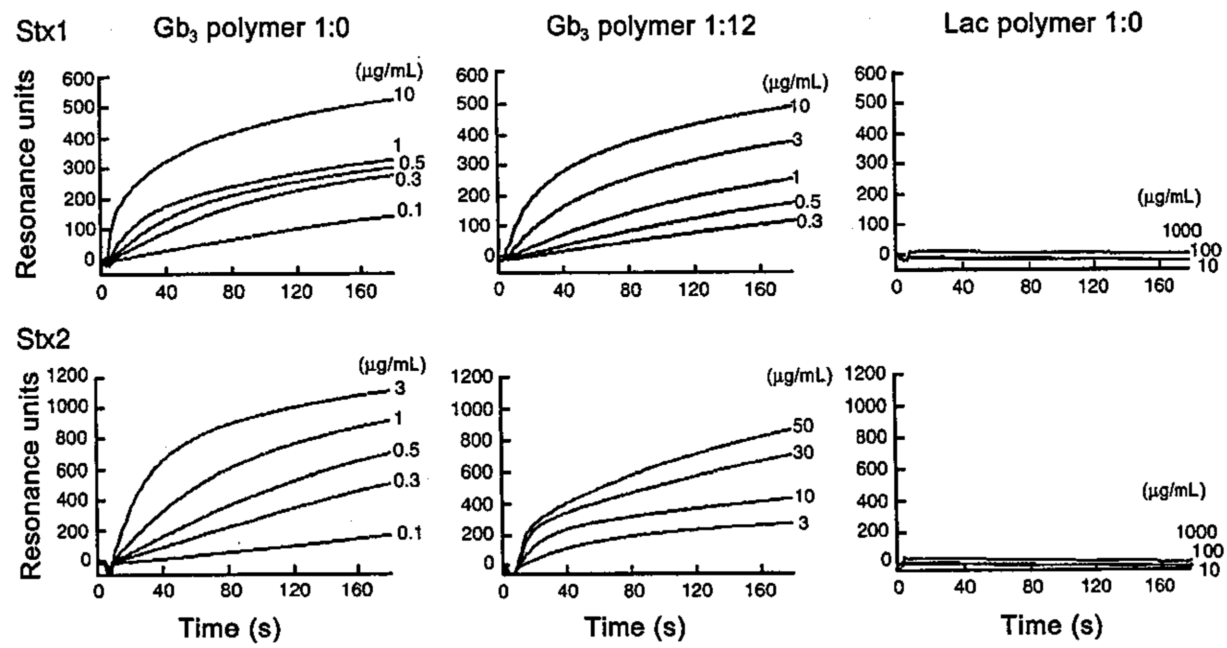

Figure 2. Kinetic analysis of globotriaosylceramide (Gb_3) polymer binding to immobilized Shiga toxin (Stx) B subunits, using a BIAcore system. Recombinant histidine-tagged Stx1 and Stx2 B subunits were immobilized on an nitrilotriacetic acid sensor chip (BIAcore), and the indicated amount of each compound (in μg/mL)—Gb_3 polymer 1:0, Gb_3 polymer 1:12, or lactose (Lac) polymer 1:0—was injected at time 0 over the immobilized B subunits at a flow rate of 20 μL/min for 3 min to reach plateau.

concentration of 100 μmol/L (figure 3*B*). Each polymer itself did not affect the cell viability (data not shown). These results demonstrate that Gb_3 polymers with highly clustered trisaccharides effectively inhibited the biological activities of not only Stx1 but also Stx2 against the target cells, which is consistent with direct and high-affinity binding of the polymers to the Stx B subunits, as described above.

Effect of Gb_3 polymers in vivo. Next, we investigated the inhibitory effects of Gb_3 polymers on the lethality of Stx-producing *E. coli* O157:H7 infections in mice. We used mice with protein calorie malnutrition, which are very susceptible to infection with *E. coli* O157:H7 [21]. In this model, the establishment of infection can be diagnosed by the detection of Stx both in stool on day 2 and in serum on day 3 after intragastric injection of *E. coli* O157:H7 [21]. We administered Gb_3 polymers intragastrically twice a day for 3 consecutive days (days 3–5). All the control animals developed neurologic symptoms after postinfection day 5 and succumbed to the infection by day 12 (figure 4). In contrast, all 5 of the mice treated with Gb_3 polymer 1:0 and 3 of the 4 mice treated with Gb_3 polymer 1:12 survived ($P<.001$ and $P<.01$, respectively) for >30 days without any neurologic symptoms (figure 4). Treatment with other Gb_3 polymers (2:17 and 1:11) also reduced lethality (2 of 2 mice survived in each group; figure 4). These results clearly indicate that the Gb_3 polymers can protect mice after challenge with a fatal dose of *E. coli* O157:H7, even when the polymers are administered after the infection has been established.

Table 2. Results of kinetic analysis of the binding of globotriaosylceramide (Gb_3) polymers to His-tagged Shiga toxin (Stx) B subunits, using a BIAcore system.

	Stx1 B subunit		Stx2 B subunit	
Gb_3 polymer	K_d, mean μmol/L ± SE	RU_{max}, mean ± SE	K_d, mean μmol/L ± SE	RU_{max}, mean ± SE
1:0	0.34 ± 0.05	468 ± 28	0.68 ± 0.05	1340 ± 67
2:17	0.44 ± 0.11	614 ± 60	1.4 ± 0.26	803 ± 50
1:11	0.43 ± 0.12	604 ± 80	4.2 ± 0.57	1380 ± 56
1:12	0.60 ± 0.06	560 ± 6	7.1 ± 0.64	961 ± 73

NOTE. RU_{max}, maximal resonance unit.

Stx2 content, which is more closely related to the lethality of *E. coli* O157:H7 infections than Stx1 content in this mouse model, was measured in serum and stool samples. The Stx2 content in serum from mice treated with Gb_3 polymers 1:0 and 1:12 decreased to an undetectable level by day 4, when the serum level of Stx2 had reached its maximum without treatment (table 4) [21]. Interestingly, the Stx2 content in stool was also substantially reduced by treatment with Gb_3 polymers 1:0 and 1:12, to one-half of and less than the control value, respectively (table 4). In an in vitro test, a high concentration of Gb_3 polymer 1:0 (≤0.5 mg/mL) did not affect the results of ELISA for detection of Stx2, even in the presence of serum or stool (data not shown), which confirms that there is a sub-

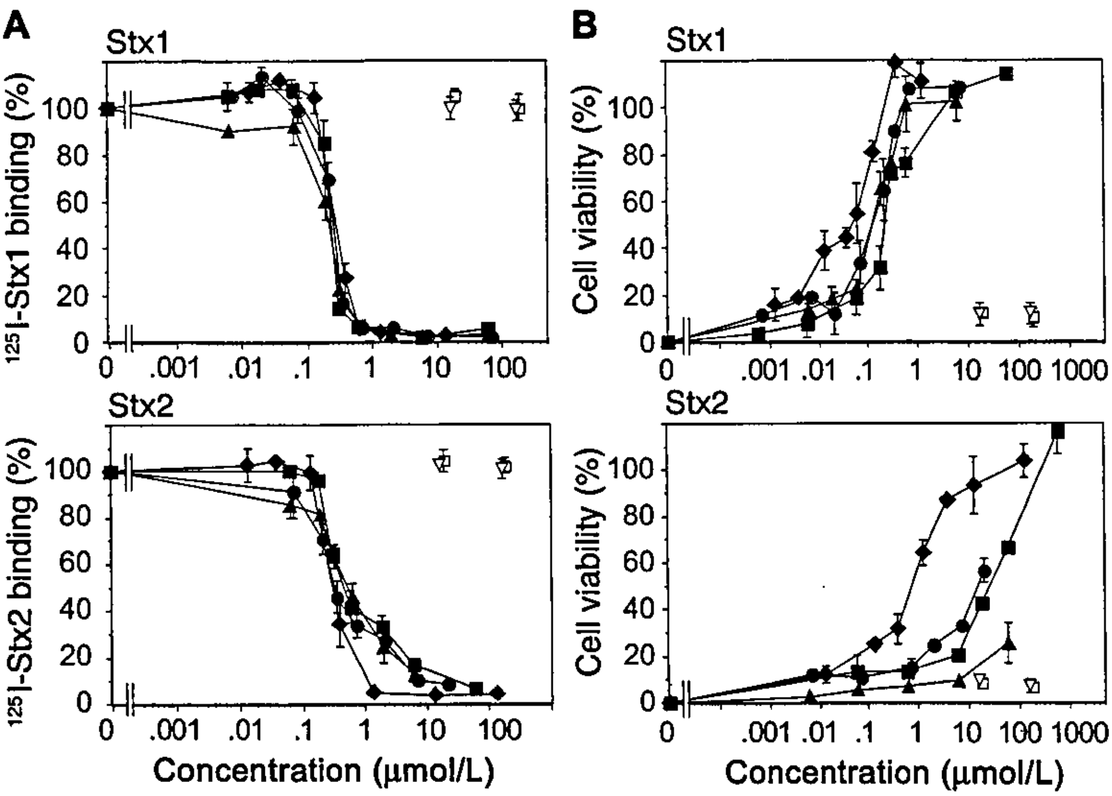

Figure 3. Inhibitory effects of globotriaosylceramide (Gb_3) polymers on the biological activities of Shiga toxin (Stx) in Vero cells. *A,* Results of ^{125}I-labeled Stx1 (^{125}I-Stx1) and ^{125}I-Stx2 binding assay. Data are presented as the percentage of activity in the absence of polymers (mean ± SE; n = 3 or 4). *B,* Results of cytotoxicity assay using Vero cells. Data are presented as the percentage of cell viability in the absence of Stxs (mean ± SE; n = 3). *Filled diamonds,* Gb_3 polymer 1:0; *filled circles,* Gb_3 polymer 2:17; *filled triangles,* Gb_3 polymer 1:11; *filled rectangles,* Gb_3 polymer 1:12; *open rectangles,* lactose polymer 1:0; *open triangles,* free trisaccharide.

stantial mass reduction of Stx2 by Gb_3 polymer 1:0 treatment in vivo.

Because *E. coli* O157:H7 infection causes severe brain damage in a mouse model in which protein calorie malnutrition was used, pathological changes in cerebral blood vessels, such cell infiltration and hemorrhage, were investigated in Gb_3 polymer 1:0–treated and untreated mice. In untreated control mice, cell infiltration (figure 5*A*) and hemorrhage (figure 5*B*) were observed in the cerebral cortex on day 5 after infection. Demyelinated nerve fibers were not noticed at the brain stems of the control animals, despite marked cell infiltration (figure 5*C*), which is consistent with findings we have published elsewhere [21]. In contrast, no histological changes were observed in the brains of Gb_3 polymer 1:0–treated mice, even at day 30 after infection (data not shown). In the hippocampus of untreated mice, immunoreactions for Stx2 were detected (figure 5*D*); those reactions were absent in the brain of Gb_3 polymer 1:0–treated mice (figure 5*E*). These results suggest that Gb_3 polymer 1:0 suppressed the lethality of *E. coli* O157:H7 infection by reducing the serum level of Stx2 and subsequent Stx2-associated fatal brain damage.

DISCUSSION

In this study, we used a series of linear polymers of acrylamide, each with a different density of the trisaccharide of Gb_3, to develop an Stx adsorbent that functions in the gut. We found that the Gb_3 polymers with highly clustered trisaccharides specifically bound to both Stx1 and Stx2 with high affinity and markedly inhibited the biological activities, such as binding activity and cytotoxic activity toward the target cells, of these

Table 3. IC_{50} of globotriaosylceramide (Gb_3) polymers for the biological activities of Shiga toxin (Stx) toward Vero cells.

	IC_{50}, mean μmol/L ± SE			
	In binding assay		In cytotoxicity assay	
Gb_3 polymer	Stx1 (n = 4)	Stx2 (n = 3)	Stx1 (n = 3)	Stx2 (n = 3)
1:0	0.33 ± 0.04	0.34 ± 0.05	0.05 ±0.004	0.82 ± 0.16
2:17	0.33 ± 0.04	0.38 ± 0.07	0.16 ± 0.05	18.8 ± 4.6
1:11	0.25 ± 0.03	0.60 ± 0.13	0.14 ± 0.01	Not determined
1:12	0.33 ± 0.04	0.55 ± 0.13	0.30 ± 0.06	26.6 ± 3

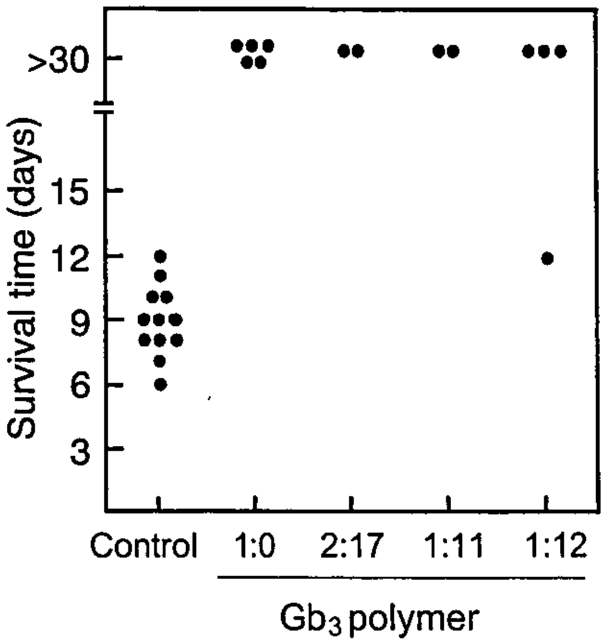

Figure 4. Inhibitory effect of globotriaosylceramide (Gb_3) polymers on the lethality of infection with *Escherichia coli* O157:H7 in mice. Mice with protein calorie malnutrition were infected intragastrically with *E. coli* O157:H7 strain N-9 (2×10^6 cfu) on day 0. Gb_3 polymer (25 μg/g of body weight) or saline alone was administered intragastrically to the mice (control group, $n = 12$; Gb_3 polymer 1:0, $n = 5$; Gb_3 polymer 2:17, $n = 2$; Gb_3 polymer 1:11, $n = 2$; Gb_3 polymer 1:12, $n = 4$) twice a day on days 3–5 after infection. Data are the survival time of each mouse. The data were analyzed by Kaplan-Meier survival analysis or, when no mice had died by the end of the observation, by Fisher's exact test.

toxins. The K_d values of the Gb_3 polymer 1:0, the most active Gb_3 polymer, for the B subunits of Stx1 and Stx2 were even lower than those of SUPER TWIG (1)6, which indicates that the Gb_3 polymer binds to both Stxs more strongly than does SUPER TWIG (1)6. Interestingly, we found that the sugar-clustering effect in the recognition of trisaccharide for Stx1 was different from that for Stx2 and that more highly clustered trisaccharides in the Gb_3 polymers are required for high-affinity binding to Stx2. This observation provides an important insight into the development of Stx adsorbents, especially against Stx2, which has greater clinical importance than Stx1.

Recently, Dohi et al. [22] reported a linear polymer of acrylamide that contained the trisaccharide attached to a spacer of a phenyl group. This compound substantially inhibited the cytotoxic activity of Stx1 toward human renal adenocarcinoma ACHN cells, another cell type that is very sensitive to Stx, but did not show any inhibitory effect on Stx2 at concentrations $\leq$100 μmol/L on a per-trisaccharide basis. The major difference between this compound and the Gb_3 polymers developed in the present study is the length of the spacer arm through which the trisaccharide group binds to each core structure. On the other hand, it is generally accepted that the fatty acid chain length of Gb_3 can have an important effect on the extent to which Stx1 and Stx2 bind to Gb_3 [23]. When these data are considered with the results of our present study, it is highly possible that not only the high density of trisaccharides, but also the long alkyl spacer present in the Gb_3 polymers is required for high-affinity binding to Stx2. In a recent report, in which self-assembled monolayers of Gb_3 mimics that contain the trisaccharide with alkyl chains of different lengths were used, it was demonstrated that Stx2, but not Stx1, preferred a longer alkyl chain for high-affinity binding [24], which further supports our contention.

We found that oral administration of the Gb_3 polymers protected mice against a fatal dose of *E. coli* O157:H7 and that Stx2 content in serum samples from such mice was substantially reduced, compared with levels in serum from untreated mice. Although these Gb_3 polymers are heterogeneous in their molecular size, the average molecular sizes of Gb_3 polymers 1:0 and 1:12 were determined to be 36 and 73 kDa, respectively, by gel permeation chromatography (data not shown), both of which can be calculated to contain ~50 trisaccharides/molecule of these compounds. Judging by all of these findings, it is highly possible that Gb_3 polymers bind to Stx2 in multiple ways to form large complexes in the gut, thereby inhibiting the entrance of Stx2 into the circulation and resulting in a reduction in the serum level of Stx2. Interestingly, we found that the Stx2 content in stool samples was also reduced by treatment with Gb_3 polymers. Although the precise mechanism of this reduction remains to be elucidated, this phenomenon may reflect another aspect of the mechanism by which the Gb_3 polymers effectively function as oral therapeutic agents in the gut.

In a previous report, it was shown that oral administration of another chemically synthesized Stx adsorbent (Synsorb-Pk; Synsorb Biotech), which consists of globotrisaccharide covalently linked to silica particles [9, 25], did not protect mice against oral challenge with STEC, although it delayed time to

Table 4. Quantification of Shiga toxin (Stx) 2 on day 4 after infection with *Escherichia coli* O157:H7 in stool and serum samples from mice treated with globotriaosylceramide (Gb_3) polymers or saline.

	Mean concentration of Stx2 ± SE[a]	
Treatment	In stool, pg/mg ($n = 3$)	In serum, pg/mL ($n = 3$)
Gb_3 polymer		
1:0	25 ± 6	—
1:12	33 ± 5	—
Saline	71 ± 6	41 ± 6

NOTE. —, lower than the limit of detection.

[a] The limits of detection were 12 pg/mg of stool and 18 pg/mL of serum, respectively.

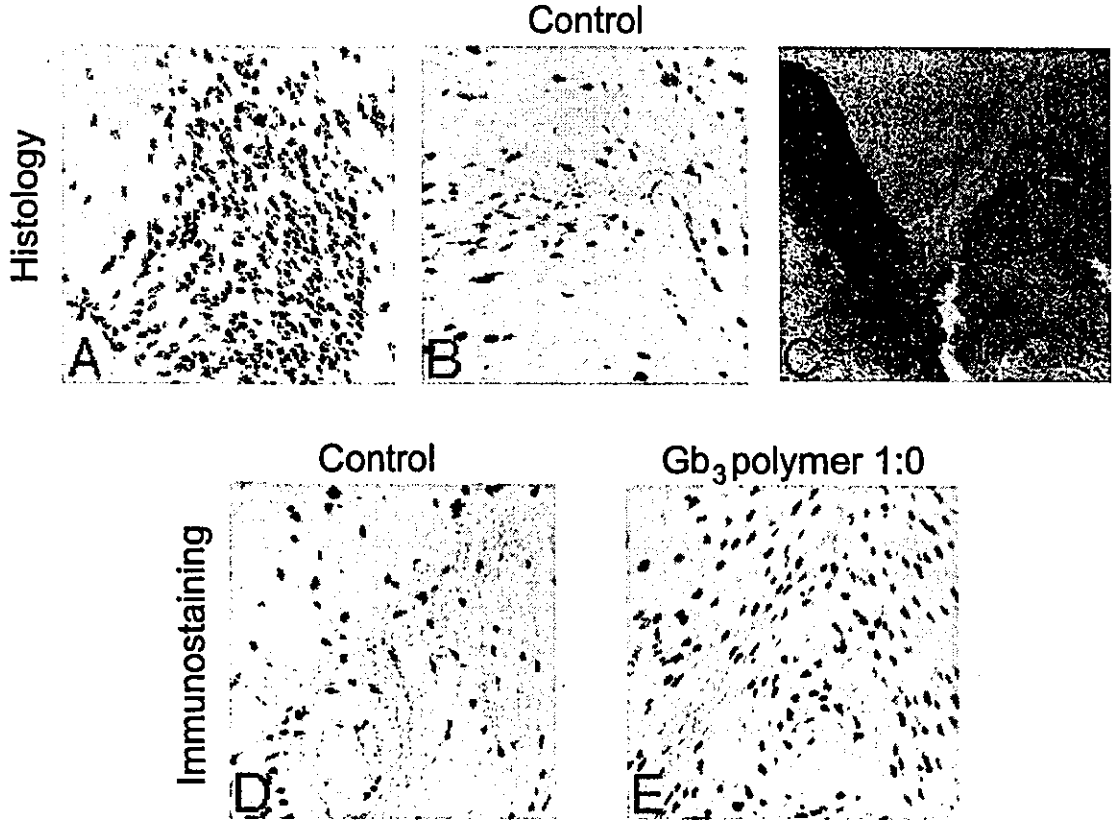

Figure 5. Histological examination and immunostaining of Shiga toxin (Stx) 2 in the brains of mice treated with globotriaosylceramide (Gb_3) polymer 1:0 *(E)* or not treated *(A–D)* and infected with *Escherichia coli* O157:H7. Sections of the cerebral cortex were used for histological examination. The sections were stained with hematoxylin-eosin (*A* and *B;* original magnification, ×450) or Luxol fast blue (*C;* original magnification, ×150). Stx2 present in sections of the hippocampus was detected using specific antibody against Stx2 (*D* and *E;* original magnification, ×450). The sections were stained afterward with hematoxylin.

death by 1 day [26]. The Stx-binding capacity of Synsorb-Pk is at least 100,000 times lower than that of the Gb_3 polymers estimated from our present results (table 3 and figure 3*A*); this is probably the result of the low density of trisaccharide displayed on the surface of Synsorb-Pk, which is ~2000 times lower than that of the Gb_3 polymers [9]. Therefore, the marked inhibitory effect of the Gb_3 polymers on the lethality of STEC infections may be mainly attributed to the superiority of the capacity of Gb_3 polymers to bind toxin.

All of the results of our present study indicate that Gb_3 polymers can be used as an oral therapeutic agent to treat STEC infections in humans. This type of agent is expected to have significant therapeutic advantages, because it can be widely applicable not only to individuals with STEC infection, but also to those at risk for such infections.

Acknowledgments

We thank Shinji Yamasaki and Takashi Hamabata for providing us with the pUC118 vector and the pCH283 vector that contained the complete coding sequences for Shiga toxins 1 and 2, respectively.

References

1. Karmali MA, Steele BT, Petric M, Lim C. Sporadic cases of hemolytic uremic syndrome associated with fecal cytotoxin and cytotoxin-producing *Escherichia coli.* Lancet 1983; 1:619–20.
2. Riley LW, Remis RS, Helgerson SD, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 1983; 308:681–5.
3. O'Brien AD, Holmes RK. Shiga and Shiga-like toxins. Microbiol Rev 1987; 51:206–20.
4. Paton JC, Paton AW. Pathogenesis and diagnosis of Shiga toxin–producing *Escherichia coli* infections. Clin Microbiol Rev 1998; 11: 450–79.
5. Ostroff SM, Tarr PI, Neill MA, Lewis JH, Hargrett-Bean N, Kobayashi JM. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J Infect Dis 1989; 160: 994–8.
6. Tesh VL, Burris JA, Owens JW, et al. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect Immun 1993; 61:3392–402.
7. Karmali MA, Petric M, Lim C, Fleming PC, Arbus GS, Lior H. The association between idiopathic hemolytic uremic syndrome and infec-

tion by verotoxin-producing *Escherichia coli*. J Infect Dis 1985; 151: 775–82.

8. Melton-Celsa AR, O'Brien AD. Structure, biology, and relative toxicity of Shiga toxin family members for cells and animals. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology Press, 1998:121–8.
9. Armstrong GD, Fodor E, Vanmaele R. Investigation of Shiga-like toxin binding to chemically synthesized oligosaccharide sequences. J Infect Dis 1991; 164:1160–7.
10. Kitov PI, Sadowska JM, Mulvey G, et al. Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. Nature 2000; 403: 669–72.
11. Dohi H, Nishida Y, Mizuno M, et al. Synthesis of an artificial glycoconjugate polymer carrying Pk-antigenic trisaccharide and its potent neutralization activity against Shiga-like toxin. Bioorg Med Chem 1999; 7:2053–62.
12. Paton AW, Morona R, Paton JC. A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. Nat Med 2000; 6:265–70.
13. Nishikawa K, Matsuoka K, Kita E, et al. A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin–producing *Escherichia coli* O157:H7. Proc Natl Acad Sci USA 2002; 99: 7669–74.
14. Paton JC, Rogers TJ, Morona R, Paton AW. Oral administration of formaldehyde-killed recombinant bacteria expressing a mimic of the Shiga toxin receptor protects mice from fatal challenge with Shiga-toxigenic *Escherichia coli*. Infect Immun 2001; 69:1389–93.
15. Matsuoka K, Terabatake M, Esumi Y, Terunuma D, Kuzuhara H. Synthetic assembly of trisaccharide moieties of globotriaosyl ceramide using carbosilane dendrimers as cores: a new type of functional glycomaterial. Tetrahedron Lett 1999; 40:7839–42.
16. Matsuoka K, Nishimura S-I. Synthetic glycoconjugates. 5. Polymeric sugar ligands available for determining the binding specificity of lectins. Macromolecules 1995; 28:2961–8.
17. Noda M, Yutsudo T, Nakabayashi N, Hirayama T, Takeda Y. Purification and some properties of Shiga-like toxin from *Escherichia coli* O157:H7 that is immunologically identical to Shiga toxin. Microb Pathog 1987; 2:339–49.
18. Kurazono H, Sasakawa C, Yoshikawa M, Takeda Y. Cloning of a Vero toxin (VT1, Shiga-like toxin I) gene from a VT1-converting phage isolated from *Escherichia coli* O157:H7. FEMS Microbiol Lett 1987; 44: 23–6.
19. Nishikawa K, Arai H, Inoue K. Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. J Biol Chem 1990; 265:5226–31.
20. Plant AL, Brigham-Burke M, Petrella EC, O'Shannessy DJ. Phospholipid/alkanethiol bilayers for cell-surface receptor studies by surface plasmon resonance. Anal Biochem 1995; 226:342–8.
21. Kurioka T, Yunou Y, Kita E. Enhancement of susceptibility to Shiga toxin–producing *Escherichia coli* O157:H7 by protein calorie malnutrition in mice. Infect Immun 1998; 66:1726–34.
22. Dohi H, Nishida Y, Takeda T, Kobayashi K. Convenient use of non-malodorous thioglycosyl donors for the assembly of multivalent globo- and isoglobosyl trisaccharides. Carbohydr Res 2002; 337:983–9.
23. Lingwood CA, Mylvaganam M, Arab S, et al. Shiga toxin (verotoxin) binding to its receptor glycolipid. In: Kaper JB, O'Brien AD, eds. *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* strains. Washington, DC: American Society for Microbiology Press, 1998: 129–39.
24. Miura Y, Sasao Y, Dohi H, Nishida Y, Kobayashi K. Self-assembled monolayers of globotriaosylceramide (Gb3) mimics: surface-specific affinity with shiga toxins. Anal Biochem 2002; 310:27–35.
25. Takeda T, Yoshino K, Adachi E, Sato Y, Yamagata K. In vivo assessment of a chemically synthesized Shiga toxin receptor analog attached to chromosorb P (Synsorb Pk) as a specific absorbing agent of Shiga toxin 1 and 2. Microbiol Immunol 1999; 43:331–7.
26. Rogers JE, Armstrong G, O'Brien AD. Therapeutic value of Stx-specific antibodies or synsorb in streptomycin (STR)–treated mice orally infected with Shiga toxin–producing *Escherichia coli* (STEC) [abstract V149/VII]. In: Program and abstracts of the 3rd International Symposium and Workshop on Shiga Toxin (Verotoxin)–Producing *Escherichia coli* Infections (Baltimore). Melville, NY: Lois Joy Galler Foundation for Hemolytic Uremic Syndrome, 1997:114.

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Tetrahedron Letters 44 (2003) 3617–3620

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LETTERS

Synthesis of a useful anomeric thioacetate of an *N*-acetyllactosamine derivative and its application☆

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Received 25 February 2003; revised 13 March 2003; accepted 14 March 2003

Abstract—A novel anomeric β-thioacetate of an *N*-acetyllactosamine derivative was efficiently synthesized in high yield from known 2-azido glycosyl chloride using thioacetic acid as a convenient reagent. The synthesis involved not only an S_N2 replacen of the chloride by a carbothiolate anion but also a reductive acetamidation of the azide group. Applications of the thioacetate glycosidation were demonstrated to provide both *O*- and *S*-glycosides in high yields. Furthermore, both intermediates gave a class of glycoclusters that included thioglycosidic linkages.

N-Acetyllactosamine (LacNAc; Galβ1→4GlcNAc) is known as an extremely valuable core structure of glycoconjugates such as glycoproteins and glycolipids.[1] In order to use oligosaccharides for elucidating their functional roles in biological systems, synthetic construction of oligosaccharides, including LacNAc, has been reported by several groups.[2] Unfortunately, LacNAc or its precursor to be used as the starting material for such a purpose has not been readily obtained. Lemieux et al. introduced an azidonitoration reaction followed by treatment of the products using Et_4NCl for peracetylated D-lactal to provide crystalline *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-3,6-di-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl chloride **1** as the precursor of *N*-acetlylactosamine.[3] The chloride, however, has not been widely used for glycosciences. In this study, we therefore investigate further manipulation of this unused chloride to obtain useful derivatives of *N*-acetyllactosamine. In this communication, we describe a stereospecific synthesis of the novel peracetylated *N*-acetyllactosaminyl 1-β-thioacetate **2** by modified Lemieux's protocol and the applicability of **2** a glycosyl donor.

Since Rosen et al. initially reported a convenient highly chemoselective reduction of azides to provide corresponding acetamide in good yield,[4] we exami the use of a slight modification of this procedure[5] preparation of *N*-acetyllactosaminyl chloride. Fre: prepared 2-azido-1-α-chloride **1** was treated with a l: amount of thioacetic acid in the presence of pyridin rt. The reaction proceeded smoothly on TLC to gi major product after purification by silica gel cl matography. In order to elucidate the structure of product, the IR spectrum of the compound was measured and showed absorptions at 1753, 1703, 1(and 1541 cm^{-1} and the disappearance of absorptio: around 2100 cm^{-1} due to the azide functional gr ($\nu_{N=N=N}$). The absorptions at 1753, 1674, and 1541 c were assigned to an ester ($\nu_{C=O}$), an amide I ($\nu_{C=O}$), an amide II (δ_{N-H}), respectively. There was still unknown absorption at 1703 cm^{-1}. A flame reactio: a compound on the copper metal reminded us of I stein test to detect a halide atom in the compoı Consequently, Beilstein test was performed on product, and the results showed a negative color, i cating vanishment of the chloride atom in the prod Our attention was then focused on replacement of chloride atom at the anomeric center. A ^{1}H N spectrum of the product revealed a signal assignabl H-1 at δ 5.09 as a doublet with $J_{1,2}$ 10.9 Hz and SA

Keywords: *N*-acetyllactosamine; glycosides; thioacetic acid; thioacetates; glycoclusters.

☆ Glyco-Silicon Functional Materials. Part 6. For Part 5, see Ref. 13.

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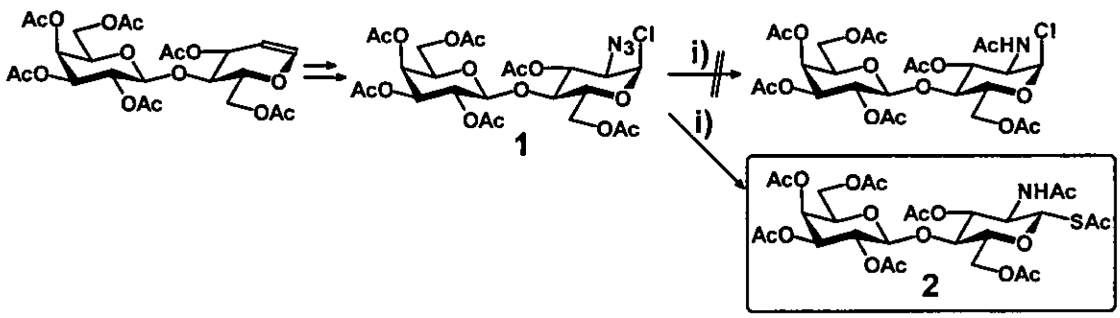
0040-4039/03/$ - see front matter
doi:10.1016/S0040-4039(03)00697-X

δ 2.35 ppm as a singlet. Accordingly, the structure was confirmed to be a β-thioacetate **2** of *N*-acetyllactosamine, and the yield was estimated to be 71.6%,‡ $[\alpha]_D^{24}$ +7.3° (*c* 0.15, methanol), IR (KBr) 1703 cm^{-1} [$\nu_{C=O}$ (SAc)], ^{1}H NMR ($CDCl_3$) δ 4.49 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1′) and 5.76 (d, 1H, $J_{2,NH}$ 9.8 Hz, NH). We propose that the simultaneous reactions of thioacetic acid with 2-azido-1-α-chloride **1** include a usual reduction followed by acetylation at the azido functional group and a nucleophilic replacement by the carbothiolate anion from the β face at the anomeric carbon to provide **2** as the sole product (Scheme 1).

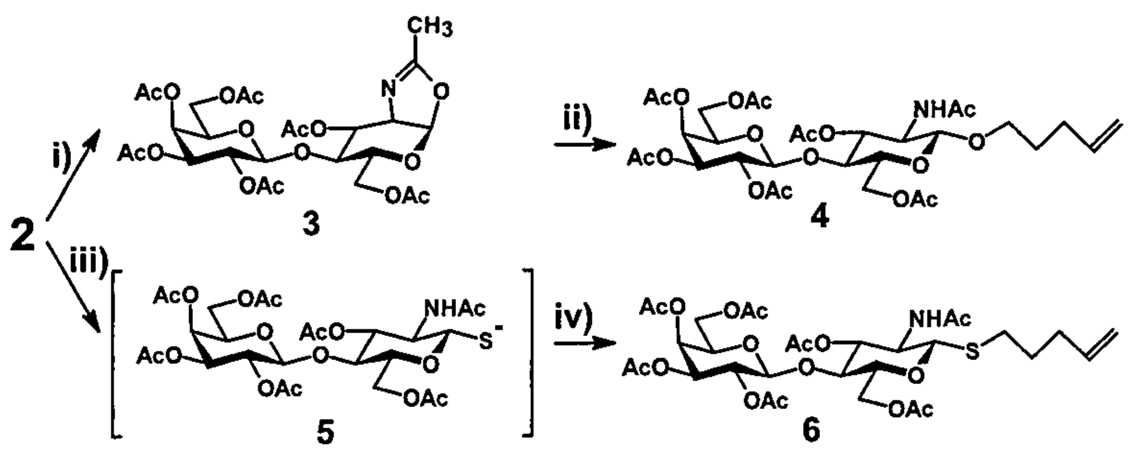
Since the preparation and the structural assignment of **2** had been accomplished, we turned our attention to the potency of **2** as a glycosyl donor. To the best of our knowledge, no direct activation of thioacetate as a donor for glycoside synthesis has been reported.[6] Thus, thioacetate **2** was treated with NIS–TfOH as a typical glycosidation protocol using thioglycoside as a donor. Interestingly, the reaction proceeded smoothly to give the oxazoline derivative **3**[7] (Scheme 2), which is known as a useful precursor for an *O*-glycoside, in 70.0% yield after purification by silica gel chromatography, ^{1}H NMR ($CDCl_3$) δ 3.46 (d, 1H, $J_{4,5}$ 9.4 Hz, H-4), 5.64 (d, 1H, $J_{2,3}$ 9.4 Hz, H-3), and 5.91 (d, 1H, $J_{1,2}$ 7.3 Hz, H-1). The oxazoline **3** was then converted into its *O*-glycoside by the method previously reported[8] to yield the corresponding β-glycoside **4**.

Given the success of the conversion of thioacetate **2** into an *O*-glycoside, we examined the further transformation of thioacetate **2** to a thioglycoside that can be used as a potential inhibitor of glycosidases. The removal of *S*-acetyl of **2** in the presence of sodium methoxide in methanol[9] gave the thiolate anion **5**, which was successively allowed to react with 5-bromo-1-pentene to afford the corresponding β-thioglycoside **6** in 78.9% yield after reacetylation, $[\alpha]_D^{22}$ −32.3° (*c* 0.48, chloroform), ^{1}H NMR ($CDCl_3$) δ 2.13 (m, 2H, SCH_2), 4.42 (d, 1H, $J_{1,2}$ 10.3 Hz, H-1), 4.50 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1′) and 5.58 (d, 1H, $J_{2,NH}$ 9.7 Hz, NH).

In our ongoing synthetic study of artificial glycoconjugates, synthetic assembly of carbohydrate moieties using carbosilane dendrimers has been achieved using β-cyclodextrin,[10] globotriaose,[11] functional monosaccharides,[12] and sialyllactose.[13] Since such carbohydrate moieties were found in our previous investigation to be coupled to carbosilane dendrimers through a spacer arm including a sulfide linkage, the formation of thioglycoside demonstrated here, which is referred to as a model compound without an aglycon, was then exam-



Scheme 1. *Reagents and conditions*: (i) AcSH (large excess), Pyr, rt, 4 h.



Scheme 2. *Reagents and conditions*: (i) NIS (4 molar excess), TfOH (4 molar excess), CH_2ClCH_2Cl, 0°C→rt, 4 h; (ii) 4-penten-1-ol, CSA, CH_2ClCH_2Cl, 90°C; (iii) NaOMe, MeOH, −40°C; (iv) 5-bromo-1-pentene (3 molar excess), 2 h, −40°C, then Ac_2O–Pyr, rt.

‡ All new compounds with the specific rotation data gave satisfactory results of elemental analyses.