Multivalent Galacto-Trehaloses: Design, Synthesis, and Biological Evaluation Under the Concept of Carbohydrate Modules

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Multivalent *Galacto*-trehaloses: Design, Synthesis, and Biological Evaluation under the Concept of Carbohydrate Modules

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Galacto-trehalose (GT) is a novel class of 1,1′-linked nonreducing disaccharide having an α-galactoside epitope. In this study, a pair of α,α- and α,β-GT isomers were prepared in one pot with our α-glycosylation method, converted into vinyl monomers and then subjected to radical copolymerization with a second sugar (4-acrylamidophemyl β-Glc or β-GlcNAc) in the presence of acrylamide. The derived glycopolymers were assayed with α-galactoside-specific proteins (BSI-B_4 lectin and Shiga toxin-1) to show the results that both α,α- and α,β-isomers are recognized by these carbohydrate-binding proteins more strongly in forms of the GT polymers. Moreover, the glycopolymer carrying both α,α-GT and β-GlcNAc along the polymer chain showed an integrated detoxifying activity to the *E. coli* toxin as the result of a "module effect" of the second sugar.

Introduction

Trehalose is a symmetric nonreducing disaccharide in which two α-D-glucoses are linked together by a 1,1′-linkage. It occurs in desert-living plants, insects, brine shrimps, and nematodes and probably serves as a stabilizer of proteins and cell membranes for living under the hot and dry conditions.[1−6] *Galacto*-trehalose is a dissymmetric trehalose, in which one of the α-glucoses is replaced with α-D-galactose (α,α-GT, Figure 1). Such symmetric *galacto*-trehalose is also reported[7] that has a bis-α-D-galactoside 1,1′-linkage. The dissymmetric *galacto*-trehalose was first proposed as a molecular mimic of globosyl disaccharide (Gb2, Figure 1) and prepared from trehalose in a chemical process.[8] Later, enzyme-catalytic methods were proposed,[9,10] and this sugar seems ready for practical applications. In addition to its potential utility as a glycosidase-resistant disaccharide,[8,10] a biochemical or medicinal interest may arise from the α-galactoside epitope, which is widely expressed in mammalian tissues in the form of the cell surface glycolipids or glycoproteins.

Theoretically, the dissymmetric α,α-GT gives rise to other three isomers around the 1,1-linkage. Among them, α,β-GT also possesses the α-galactoside epitope while having a molecular geometry quite different from those of α,α-GT and Gb2 (Figure 1). Nothing is reported on this isomer, and this fact prompted us to compare biological properties between the two GT isomers. In the present study, we tried to develop a chemical route to prepare both α,α- and α,β-GT isomers starting from D-glucose and D-galactose derivatives. In this paper, we report a convenient access by applying our one-pot α-glycosylation methodology.[11,12] Next, we adapted a concept of "carbohydrate module approach"[13,14] for mimetic assembly of Gb3 ceramide in which the derived GT isomers were incorporated in glycosyl copolymers as key carbohydrate modules and then evaluated with α-galactoside-specific BSI-B_4 lectin and Shiga toxin-1. We discuss here the modular approach as well as the results of the bioassays in terms of multivalent binding and module effects of the key carbohydrates constructing the P^K antigenic Gb3 ceramide.

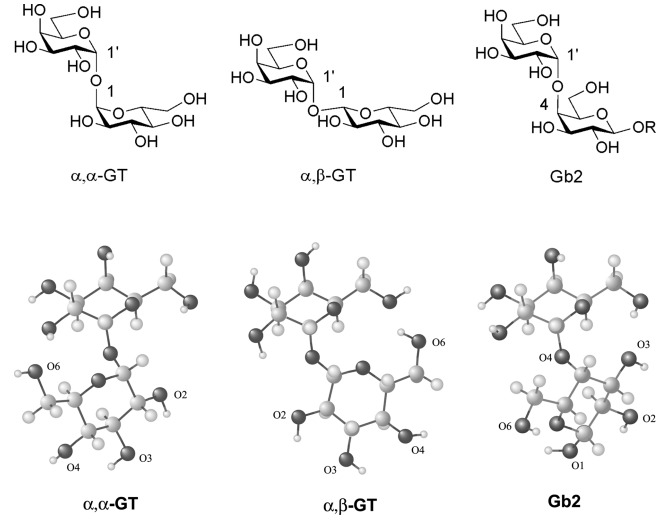

Figure 1. Structures and molecular geometries of α,α- and α,β-*galacto*-trehaloses (GT) in comparison with Gb2. In the ball stick models, α-galactoside is located at the upper position. The conformation around the glycosidic linkage was minimized with a force field calculation (MM-2).

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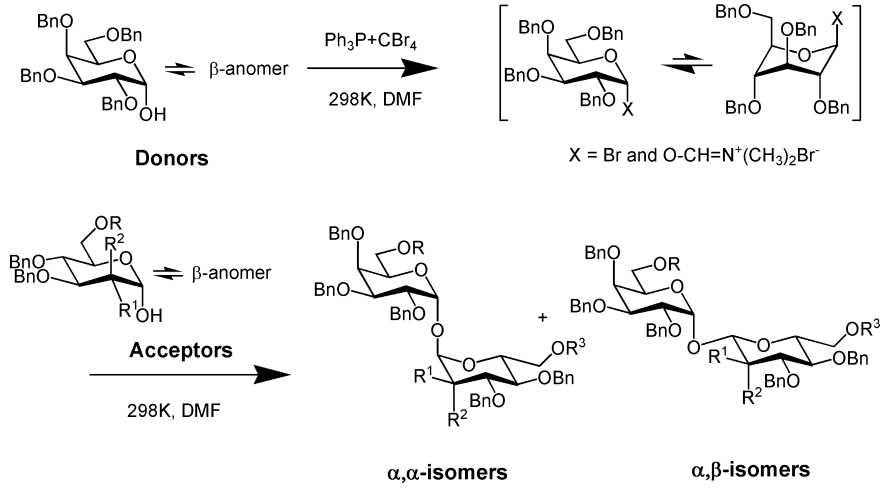
Scheme 1. One-Pot Syntheses of α,α- and α,β-*Galacto*-trehalose Isomers



Experimental Section

General Procedure. Chemical reagents of the highest commercial quality were purchased and used without further purification. All reactions were carried out under a dry nitrogen atmosphere and monitored by thin-layer chromatography (TLC) on E. Merck aluminum roll Silica Gel 60-F_{254} and visualized with UV light and *p*-anisaldehyde-sulfuric acid solution. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova 500 spectrometer and JEOL-JNM-LA-400. Unless otherwise stated, ^{1}H NMR spectra were recorded at 25 °C in $CDCl_3$ using an internal Me_4Si standard at 0 ppm. Optical rotations were measured on JASCO DIP-1000 with a water-jacketed 100 mm cell at 25 °C. IR spectra were recorded on JASCO FT/IR-230 Fourier transform infrared spectrometer in a form of KBr discs. Size exclusion chromatography (SEC) was conducted at 40 °C with JASCO 800 high-performance liquid chromatography with Shodex B804+805 column in phosphate buffer (pH 7.5) and calibrated with pullulan standards.

Typical Procedure for One-Pot α-Glycosylation. All reactions were conducted at ambient temperature (15−25 °C) in a glass vessel closed with a septum cap. Neither molecular sieves nor inert gas were used as long as the vessel was dried at 120 °C in an oven prior to the use. A solution of 1-OH sugar (donors) in DMF was treated with Ph_3P (3 mol equiv) and CBr_4 (3 mol equiv) and stirred for 3 h at room temperature. Then 1-OH sugar (acceptors, 3 mol equiv) was added, and the mixture was stirred at room temperature. The reaction was terminated when the bromide donor was completely consumed, as evidenced by TLC analysis. The mixture was diluted with a mixture of toluene and EtOAc, washed with satd aq $NaHCO_3$ and NaCl solutions, dried over $MgSO_4$, and concentrated. Products were purified by column chromatography on silica gel.

6′-*O*-Acetyl-2′,3′,4′-tri-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside 7a and 6′-*O*-Acetyl-2′,3′,4′-tri-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside 7b. A mixture of **7a** and **7b** was characterized without separation. FABMS: *m*/*z* calcd for $C_{58}H_{62}O_{13}Na$ $[M + Na]^+$, 989; found, 990 (all NMR data are available in Supporting Information).

Table 1. Results of One-Pot α-Galactosylation Reactions with Different Donor and Acceptor Sugars

entry	donors	acceptors	solvents	time (h)	yield[a] (%)	products	α,α/α,β[b]
1	**2**	**4**	DMF	14	51	**7**	25:75
2	**2**	**5**	DMF	21	44	**8**	23:77
3	**3**	**4**	DMF	20	68	**9**	30:70
4	**3**	**6**	DMF	23	58	**10**	82:18

[a] Isolated yields (%) based on the amount of the donor. [b] Determined by ^{1}H NMR spectroscopy.

6′-*O*-Acetyl-2′,3′,4′-tri-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-acetyl-2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside 8a and 6′-*O*-Acetyl-2′,3′,4′-tri-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranoside 8b. A mixture of **8a** and **8b** was characterized without separation. FABMS: *m*/*z* calcd for $C_{63}H_{66}O_{12}Na$ $[M + Na]^+$, 1037; found, 1037.

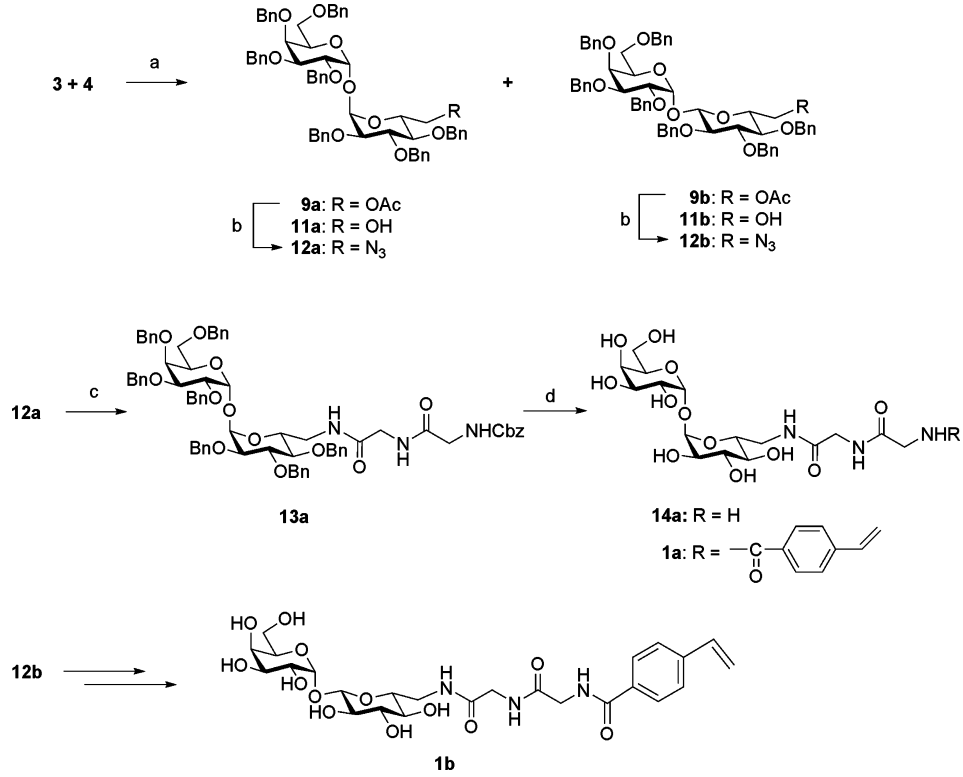
2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside 9a and 2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside 9b. A mixture of **9a** and **9b** was characterized with NMR and MS spectroscopy together with authentic compounds.[12]

2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-mannopyranoside 10a and 2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-mannopyranoside 10b. A mixture of **10a** and **10b** was characterized. FABMS: *m*/*z* calcd for $C_{63}H_{66}O_{12}Na$ $[M + Na]^+$, 1037; found, 1037.

2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-azido-2,3,4-tri-*O*-benzyl-6-deoxy-α-D-glucopyranoside 12a. To a solution of **9a** (644 mg, 0.63 mmol) in a mixture of CH_2Cl_2 (4.5 mL) and MeOH (2.3 mL) was added K_2CO_3 (132 mg, 0.95 mmol), and the mixture was stirred at rt for 2 h. The reaction mixture was neutralized with Amberlyst 15E-ion-exchange resin, and the mixture was filtered and concentrated in vacuo. The residue containing **11a** was dissolved in CH_2Cl_2 (7.5 mL), to the solution was added NEt_3 (1.0 mL) and methanesulfonyl chloride (97.5 μL, 1.3 mmol) gradually at 0 °C, and the mixture was stirred at rt for 11 h. The reaction was quenched by iced water, and to the mixture was added $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was dissolved in DMF (13 mL), and the solution was added NaN_3 (195 mg, 3.0 mmol). The suspension was stirred at 80 °C for 6 h. After cooling the mixture, water and EtOAc were added, and the organic layer was separated. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was purified by column chromatography (silica gel, 10:1 toluene−EtOAc) to give **12a** (602 mg, 96%) as a colorless syrup. $[\alpha]_D^{30}$ +111° (c = 0.61, $CHCl_3$). FABMS: *m*/*z* calcd for $C_{61}H_{63}N_3O_{10}Na$ $[M + Na]^+$, 1020; found, 1020. (All NMR data are available in Supporting Information).

2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-2,3,4-tri-*O*-benzyl-6-*N*-(*N*-carbobenzoxy-glycylglycyl)-6-deoxy-α-D-glucopyranoside 13a. To a solution of **12a** (361 mg, 0.36 mmol) in THF (25 mL) was added 10% $Pd(OH)_2$/C, and the mixture was hydrogenated at rt for 4 h. After filrate was concentrated under diminished pressure. To a solution of the residue in DMF (15 mL) was added *N*-carbobenzoxy-glycylglycine (142 mg, 0.53 mmol), EDC (204 mg, 1.06 mmol), HOBt (81 mg, 0.53 mmol), and 4-*N*,*N*-dimethylaminopyridine (4.4 mg, 36 μmol) at 0 °C, and the reaction mixture was gradually

Scheme 2[a]



[a] Reagents and conditions: (a) Ph_3P, CBr_4, DMF, rt, 68%; (b) (i) K_2CO_3, CH_2Cl_2, MeOH; (ii) MsCl, CH_2Cl_2, NEt_3, 0 °C; (iii) NaN_3, DMF, 80°C, 96% for **12a**, 93% for **12b** (3 steps); (c) (i) H_2, $Pd(OH)_2$/C, THF; (ii) *N*-(benzyloxycarbonyl)-glycylglycine, EDC, HOBt, DMAP, DMF, 66% for **13a**, 94% for **13b**; (d) (i) H_2, Pd/C, HCl, MeOH; (ii) 4-vinylbenzoyl chloride, pyridine, 78% for **1a**, 61% for **1b**.

warmed to rt and stirred for 5 h. Water and EtOAc were added, and the organic layer was washed with brine, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was purified by column chromatography (silica gel, 1:1 hexane−EtOAc) to give **13a** (281 mg, 66% in two steps) as a colorless syrup. $[\alpha]_D^{30}$ +62.2° (c = 1.45, $CHCl_3$). FABMS: m/z calcd for $C_{73}H_{77}O_{14}N_3Na$ $[M + Na]^+$, 1242; found, 1242.

α-D-Galactopyranosyl-(1′→1)-6-deoxy-6-*N*-[*N*-(*p*-vinylbenzoyl-glycylglycyl)]-α-D-glucopyranoside 1a. To a solution of **13a** (270 mg, 0.22 mmol) in MeOH (35 mL) was added 10% Pd/C, and the mixture was hydrogenated at rt for 16 h. The reaction mixture was filtered through a Celite pad, and the filtrate concentrated under diminished pressure to give **14a** as colorless syrup. To a solution of **14a** in pyridine (20 mL) and NEt_3 (360 μL, 2.2 mmol) was gradually added *p*-vinyl benzoylchloride (43 μL, 0.39 mmol) at 0 °C, and the mixture was stirred at rt for 5 h. The reaction was quenched by MeOH, and concentrated under diminished pressure. The residue was purified by column chromatography (ODS silica gel, water−MeOH) to furnish **1a** (103 mg, 78%) as a colorless solid. $[\alpha]_D^{30}$ +50.8° (c = 0.58, H_2O). MALDI-TOF-MS: calcd for $C_{25}H_{35}N_3O_{13}Na$ $[M + Na]^+$, 608; found, 608.

2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-6-azido-2,3,4-tri-*O*-benzyl-6-deoxy-β-D-glucopyranoside 12b. To a solution of **9b** (863 mg, 0.85 mmol) in CH_2Cl_2 (9 mL) and MeOH (4.5 mL) was added K_2CO_3 (185 mg, 1.3 mmol), and the mixture was stirred at rt for 2 h. The mixture was neutralized with Amberlyst 15E-ion-exchange resin, and the mixture was filtered and concentrated under diminished pressure. The residue containing **11b** was dissolved in CH_2Cl_2 (5.4 mL), to the solution was added NEt_3 (1.2 mL, 8.5 mmol) methanesulfonyl chloride (188 μL, 2.42 mmol) gradually at 0 °C, and the mixture was stirred at rt for 12 h. The reaction was quenched by iced water, and to the mixture was added $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was dissolved in DMF (16 mL), and to the solution was added NaN_3 (260 mg, 4.0 mmol). The suspension was heated to 80 °C and stirred for 6 h. After cooling the mixture, water and EtOAc were added, and the organic layer was washed with brine, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was purified by column chromatography (silica gel, 8:1 toluene−EtOAc) to give **12b** (791 mg, 93%) as a colorless syrup. $[\alpha]_D^{24}$ +72.0° (c = 1.0, $CHCl_3$). LRMS (FAB): m/z calcd for $C_{61}H_{63}N_3O_{10}Na$ $[M + Na]^+$, 1020; found, 1020.

2′,3′,4′,6′-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1′→1)-2,3,4-tri-*O*-benzyl-6-*N*-(*N*-benzyloxycarbonyl-glycylglycyl)-6-deoxy-β-D-glucopyranoside 13b. To a solution of the azide compound **12b** (353 mg, 0.35 mmol) in THF (15 mL) was added 10% $Pd(OH)_2$/C, and the mixture was hydrogenated at rt for 12 h. After filtration through a pad of Celite, the filtrate was concentrated under diminished pressure. To a solution of the residue in DMF (10 mL) was added *N*-carbobenzyloxy-glycylglycine (141 mg, 0.53 mmol), EDC (102 mg, 0.53 mmol), HOBt (81 mg, 0.53 mmol), and DMAP (cat.) at 0 °C, and the reaction mixture was gradually warmed to room temperature and stirred for 3 h. Water and EtOAc were added, and the organic layer was washed with brine, dried over $MgSO_4$, and concentrated under diminished pressure. The residue was purified by column chromatography (silica gel, 1:1 toluene−EtOAc) to give **13b** (357 mg, 94%) as a colorless syrup. $[\alpha]_D^{30}$ +58.2° (c = 1.0, $CHCl_3$). LRMS (FAB): m/z calcd for $C_{73}H_{77}O_{14}N_3Na$ $[M + Na]^+$, 1242; found, 1242.

α-D-Galactopyranosyl-(1′→1)-6-*N*-[*N*-(*p*-vinylbenzoyl)-glycylglycyl]-6-deoxy-β-D-glucopyranoside 1b. To a solution of **13b** (347 mg, 0.28 mmol) in MeOH (35 mL) was added 10% Pd/C and HCl (2 drops), and the mixture was hydrogenated at rt for 16 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated under diminished pressure. To a solution of the amine **14b** in pyridine (20 mL) and NEt_3 (557 μL, 2.8 mmol) was gradually added *p*-vinyl benzoylchloride (67 μL, 0.42 mmol) at 0 °C, and the mixture was stirred at rt for 5 h. The reaction was quenched by MeOH and concentrated under diminished pressure. The residue was purified on column chromatography (ODS silica gel, water−MeOH) to give **1b** (98 mg, 61%) as a colorless solid. $[\alpha]_D^{30}$ +24.2° (c = 0.96, H_2O). MALDI-TOF-MS m/z: calcd for $C_{25}H_{35}N_3O_{13}Na$ $[M + Na]^+$, 608; found, 608.

Typical Procedure for Radical Polymerization. Reaction conditions are summarized in Table 2, and key processes were conducted in a following manner. To a solution of sugar monomer(s) (0.02 mmol) and acrylamide in solvent was added AAPD [2,2′-azobis(2-amidinopropane) dihydrochloride; 2 mol % to the monomers] in a glass tube. The mixture was cooled to −78 °C and degassed under reduced pressure. The tube was sealed in vacuo and kept at 60 °C for 18 h. The solution was poured into methanol in a centrifugation tube, and the mixture was centrifuged (10 °C, 300 rpm, 10 min). The precipitate was dissolved in water, dialyzed for 3 days in water (M_w 3500 cut off), and lyophilized to afford glycopolymers as white powders.

Lectin Binding Assay by Fluorescence Spectroscopy. FITC-labeled BSI-B_4 lectin was purchased from Sigma and dissolved in a PBS buffer (pH 7.4). A part of the resulting solution (3 mL, ca. 1×10^{-7} M protein solution) was used for a titration assay with the synthetic glycopolymers. Fluorescence spectra (ex. 490 nm) were measured at 25 °C. Apparent binding constants (K_a per GT-residue) were estimated for the synthetic glycopolymers in a following way. An aliquot of a glycopolymer PBS buffer solution (0.3 μL) was added to the FITC-lection solution, and the maximum fluorescence intensity was measured after 20 min. Observed fluorescence change ($\Delta F/F_0$) was plotted against the mole concentration of GT in polymer [*S*]. The Scatchard plot gave saturation curves as shown in Figure 5. A good linearity ($R^2 > 0.96$) was observed when experimental values of $[S]F_0/\Delta F$ were plotted against [*S*] with the Stern−Volmer equation of

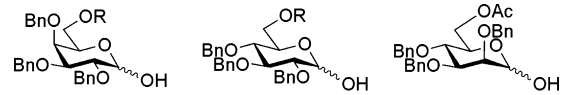
$$\frac{[S]F_0}{\Delta F} = \frac{[S]F_0}{\Delta F_{max}} + \frac{F_0}{\Delta F_{max}K_a} \quad (1)$$

where ΔF is the observed fluorescence change at the mole concentration of GT [*S*], and F_0 is the initial fluorescence intensity of the FITC-lectin solution ($[S] = 0$). The line slope is equal to the value of $F_0/\Delta F_{max}$, while the *Y*-intercept reads the value of $(F_0/\Delta F_{max})/K_a$, thus allowing us to determine the K_a value. The theoretical background of this fluorescence assay is reported in literatures.[15]

Bioassays with Shiga Toxin-1 and HeLa Cells. Stx-1 was prepared from *E. coli* (BL21/pETSTX1) and purified by affinity chromatography using a Gb3 ceramide-immobilized Sepharose column (CL-4B, Amersham Pharmacia Biotech, Uppsala, Sweden) in our established way.[16] HeLa 229 cells were generously provided by Dr. Tomoaki Yoshida (Aichi Medical University, Aichi-prefecture, Japan) and maintained in a Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical) supplemented with 10% heat-inactivated fetal calf serum (ICN Biomedicals, Aurora, O., U.S.A.), 100 U/mL penicillin G, and 0.1 mg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. The toxin neutralizing activity was assayed for each of the synthetic glycopolymers using HeLa cells in a previously reported way.[17]

Results and Discussion

Synthesis of α,α- and α,*β*-*Galacto*-trehaloses from Monosaccharides. For the chemical synthesis of α,*β*-GT, α-galactosylation at the anomeric-OH position in *β*-glucose becomes a key reaction. Here, our one-pot α-glycosylation methodology[11,12] was applied for this purpose. The method utilizes 2-*O*-benzyl-1-hydroxyl sugars as glycosyl donors, a reagent combination of CBr_4 and Ph_3P as activator and DMF as solvent. Here, two 2-*O*-benzyl-1-hydroxyl galactoses (**2** and **3**, Figure 2) were tested as the donors and two 2-*O*-benzyl-1-hydroxyl glucoses (**4** and **5**) as the acceptors. As another acceptor, a mannose-1-OH derivative **6** was also tested. Here it should be noticed that the donors and acceptors are 2-*O*-benzylated 1-hydroxyl sugars. They can be discriminated in an established procedure as follows: first, the donor 1-hydroxyl sugar is activated with CBr_4 and Ph_3P in DMF in forms of 1-bromo and cationic imidate intermediates (Scheme 1). Then, the mixture is treated with the acceptor 1-hydroxyl sugar (3 mol equiv to the donor; Table 1). Here, it should also be noticed that the acceptor sugars allow equilibrium between α- and *β*-anomers (α/*β* = ca. 1:1 in the case of **4** and **5** and ca. 7:3 in **6** as judged by ^{1}H NMR spectroscopy). Therefore, the one-pot α-glycosylation reaction may yield two isomers if we assume α-selective galactosylation on each of the anomers.



2: R = Ac
3: R = Bn

4: R = Ac
5: R = Bn

6

Figure 2. Structures of glycosyl donors and acceptors.

The results in Table 1 show that every glycosylation reaction gave a mixture of two products that were identified as the α,α- and α,*β*-1,1-linked isomers. No *β*-galactosylated product was detected in these reactions, indicating that the galactosylation was α-selective with respect to the galactosyl donor. Entries 1−3 show that the α,*β*-GT isomer was the derived major product, while entry 4 shows that the α,α-isomer became a major product in the case of the mannose **6**. The α,α-configuration of the major product was determined by ^{1}H NMR spectroscopy for a mixture of **10** with assistance from the data of **6**; the H-3 and H-5 signals of α-mannose are deshielded in comparison with the mannose *β*-isomers by about +0.2−0.3 ppm. The downfield shift is due to anisotropy from the anomeric axial oxygen (O-1) of α-mannose in 1,3-*syn*-relation with the axial H-3 and H-5. The result indicates that 1,2-*trans*-configurated anomeric OH is more reactive than 1,2-*cis* one. In the case of the *galacto*-trehalose synthesis, the α,*β*-GT isomer is preferentially derived from the D-glucose derivative (Scheme 1).

Assembly of Gb3 Ceramide-mimetic Polymers by Copolymerization of Key Carbohydrate Modules. Next, we prepared a series of glycopolymers carrying each of the GT isomers in clusters along polyacrylamide backbone. This kind of synthetic glycopolymers was introduced by Kobayashi et al.[18] and now widely accepted as neoglycoconjugates.[19,20] They usually show species-specific and integrated affinities to a target carbohydrate-binding protein as the result of multivalent binding and carbohydrate cluster effects. This fact indicates that the biological potential of simple sugars can be integrated in form of glycopolymers. Previously, we proposed the concept of "carbohydrate module approach" in studies on mimetic assembly of sialyl Lewis^X and 6-sulfo-sialyl Lewis^X tetrasaccharides.[13,14] This approach involves dissection of a target oligosaccharide ligand into simpler sugars followed by separately incorporation into a polymeric chain by radical copolymerization. This process may create such surrogate ligands in which the original molecular geometry is brought together by virtue of polymeric "fused" presentation. This approach seems effective when the target oligosaccharide is branched and the gross structure recognized by the receptor protein. In the present study, the modular approach was directed to the P^K-antigenic Gb3 ceramide (Figure 3) serving as a ligand of Shiga toxins and other α-galactoside-binding proteins. There, the GT-isomers are employed as key modules instead of using the native Gb2 disaccharide labile to enzymatic hydrolysis.[8]

First, the Gb3 ceramide structure was dissected into Gb2, *β*-Glc, and ceramide, as shown in Figure 3A. The Gb2 segment should be of the essence for interactions with Shiga toxins and other receptor proteins. The ceramide moiety is thought to play a key role for constructing a self-assembled Gb3 microdomain

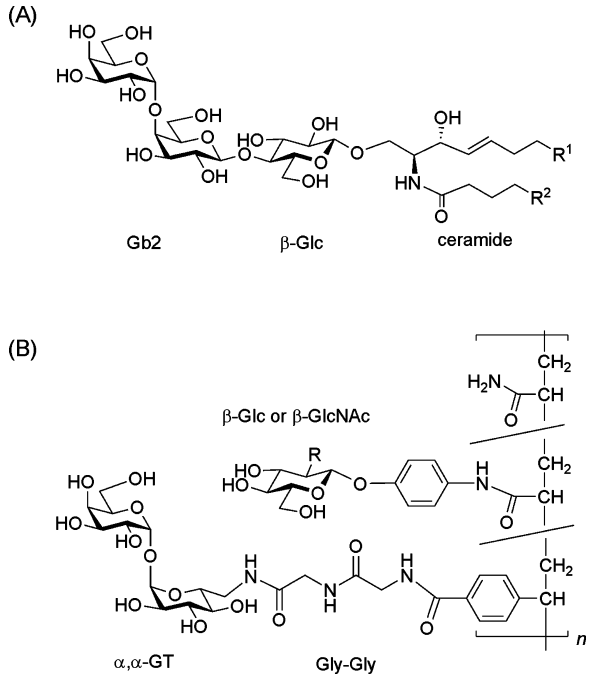


Figure 3. Design of Gb3 ceramide-mimetic polymer based on the concept of carbohydrate modules.

in cell membranes. The resultant Gb3-cluster serves as an actual ligand of such carbohydrate-binding proteins that organize a symmetric multiple-subunits structure. For example, Shiga toxin-1 has more than ten carbohydrate-binding sites in its C_5-symmetric AB_5 structure.[21] The carbohydrate−protein interaction is spread out into two dimension on the host cell, indicating that the design of multivalent ligands is inevitable for tackling the toxin to the host Gb3 interaction.[22−25]

A remaining β-Glc moiety may serve as a hydrophilic spacer and may or may not be involved essentially in interactions with receptor proteins. Under these assumptions, we designed such a Gb3 ceramide mimetic which carries α,α-GT (or α,β-GT) and β-Glc (or β-GlcNAc; Figure 3B) along a polymer chain. The *galacto*-trehalose is linked to the polymer chain with a dipeptide (Gly-Gly) linker. The 2-acetamide sugar (β-GlcNAc) was also selected as the second sugar as an attempt.

Styrene-type GT monomers (**1a** and **1b**, Scheme 2) were prepared by chemical manipulations starting from a mixture of **9a** and **9b** derived from the one-pot α-glycosylation between **3** and **4** (entry 3 in Table 1). After deacetylation at the 6-*O* position, the products (**11a** and **11b**) could be easily separated on silica gel. Each isomer was converted into the styrene monomer (**1a** and **1b**) via introduction of amino group at the glucose C-6 position followed by coupling with *N*-(benzyloxycarbonyl)-glycylglycine and then with 4-vinylbenzoyl chloride.

A series of GT polymers were prepared by copolymerization of the glycosyl monomers in the presence of acrylamide, which involve α,α- and α,β-GT copolymers (**Ia** and **Ib**) and *ter*-polymers (**IIa**, **IIIa**, **IIb**, **IIIb**) carrying also β-Glc or β-GlcNAc (Figure 4).

As references, β-Glc (**IV**) and β-GlcNAc glycopolymers (**V**) without GT were also prepared from 4-acylamidophenyl β-glycosides in our reported way.[13,14] The radical copolymerization was performed in the presence of acrylamide using 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPD, 2 mol % to the monomer) as initiator (Table 2). The feed mole ratios were set to be 1:4 (GT/acrylamide) in the case of **Ia** and **Ib**, and 1:1:2 (GT/second sugar/acrylamide) in the case of **IIa**, **IIb**, **IIIa**, and **IIIb**. Because of poor water solubility of 4-acrylamidophenyl β-Glc, some of the radical copolymerization reactions were conducted in DMSO. The glycopolymers (**IIa**, **IIb**, and **IV**) derived in DMSO were determined to have comparable number average molecular weights (M_n) with the polymers derived in water but lower GT contents. The *ter*-polymers **IIIa** and **IIIb** have larger weights than **Ia** and **Ib** but have comparable GT contents. The number average molecular weights (M_n) were estimated by SEC analysis to be in a range of 3.5∼4.0 × 10^4, which amounts to the presence of 40∼45 GT-residues in the derived polymer. The molecular weight dispersion (M_w/M_n) was in a range of 1.5∼1.6. Though we tried to prepare glycopolymers having the molar ratios and GT contents as close as possible to each other, these deviations could not be avoided easily. These deviations were taken into account when biological activities are compared among the glycopolymers.

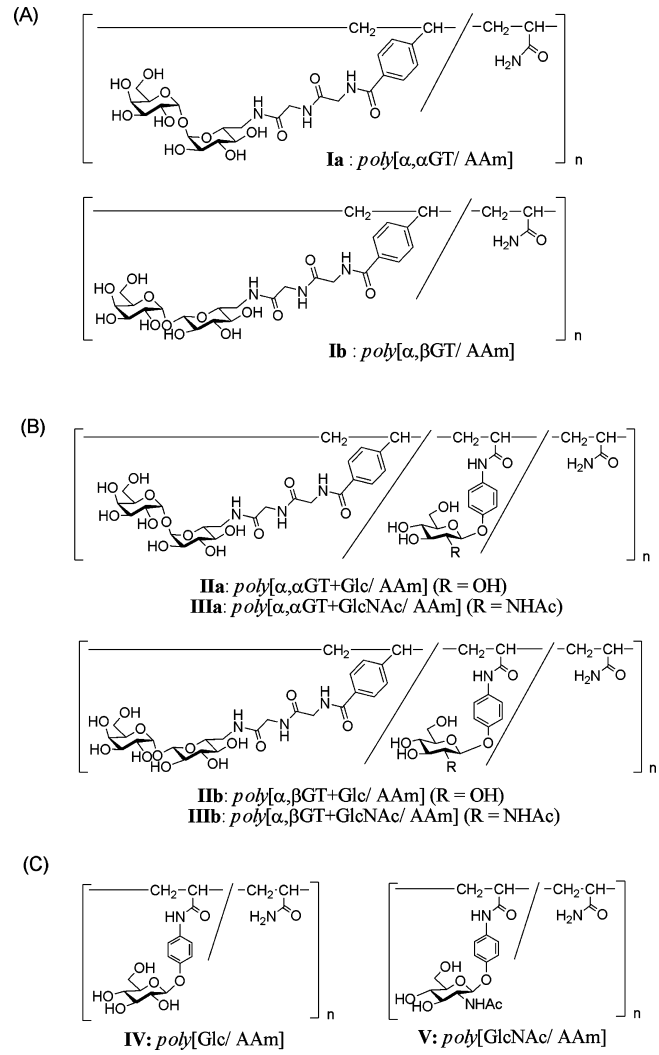


Figure 4

Bioassay Using FITC-Labeled Lectin (BSI-B_4). The derived glycopolymers were assayed with BSI-B_4 lectin (*Bandeiraea simplicifolia*), which is specific to α-galactoside-carrying oligosaccharides including Gb3 ceramide and human blood B determinants.[26] In this assay, we wanted to check first whether this lectin recognizes the GT polymers carrying the α-1,1-linked galactoside.

When an aliquot of the buffer solution of **Ia** was added to the same buffer solution of FITC-labeled lectin, the initial fluorescence intensity decreased as shown in Figure 5A. The

Table 2. Results of Copolymerization in the Presence of Acrylamide (AAm)[a]

polymer	monomer	solvent	molar ratio of GT/Glc or GlcNAc/AAm (feed ratio)[b]	yield (%)	M_n[c]	avg number of GT residues
Ia	α,α-GT (**1a**)	H_2O	18:0:82 (20:0:80)	65	3.8×10^4	42
Ib	α,β-GT (**1b**)	H_2O	23:0:77 (20:0:80)	62	3.7×10^4	45
IIa	**1a** and β-Glc	DMSO	18:32:50 (20:20:60)	78	3.6×10^4	27
IIb	**1b** and β-Glc	DMSO	22:26:52 (20:20:60)	80	3.2×10^4	28
IIIa	**1a** and β-GlcNAc	H_2O	18:24:58 (20:20:60)	63	4.7×10^4	37
IIIb	**1b** and β-GlcNAc	H_2O	22:24:54 (20:20:60)	85	4.3×10^4	37
IV	β-Glc	DMSO	0:19:81 (0:20:80)	69	2.7×10^4	
V	β-GlcNAc	H_2O	0:20:80 (0:20:80)	74	3.1×10^4	

[a] Copolymerization was conducted under diminished pressure with a degassed solution of glycosyl monomer (0.02 mmol) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPD, 2 mol % to the monomer) as initiator. [b] Determined with ^{1}H NMR spectroscopy. [c] The observed number average molecular weights (M_n) and weight dispersions (M_w/M_n = 1.5−1.6) were estimated by size exclusion HPLC (SEC).

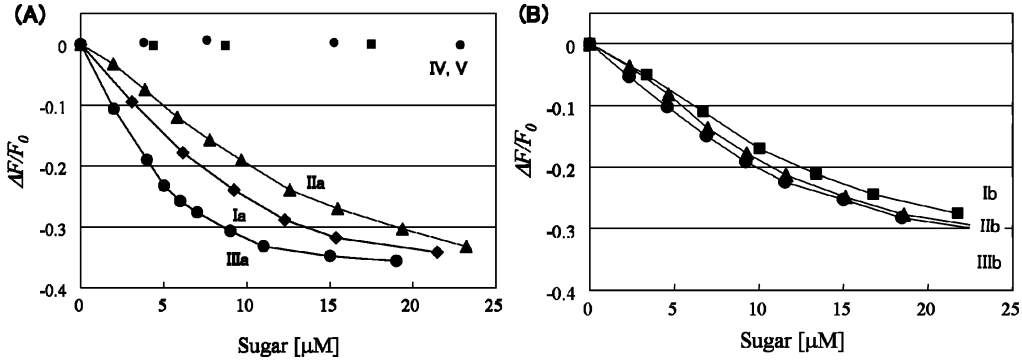


Figure 5. Schatchard plots for the fluorescence change (ex. 490 nm) of FITC-labeled BSI-B4 lectin solutions versus the mole concentrations of GT residue in glycopolymers. In the case of **IV** and **V**, the mole concentrations of β-Glc and β-GlcNAc are shown. F_0 = initial fluorescence intensity of the FITC-lectin solution before addition of polymers. ΔF = observed change in the fluorescence intensity.

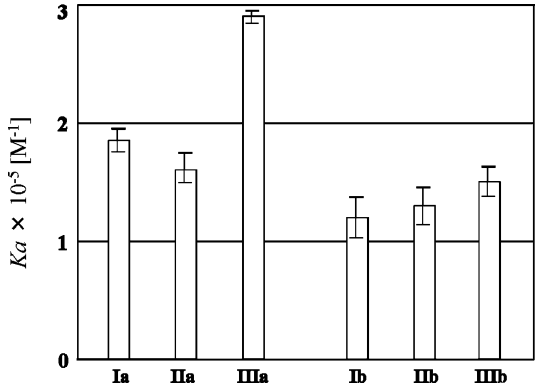


Figure 6. Comparison of apparent affinity constants to the FITC-labeled BSI-B4 lectin (The K_a values are based on the per mole faction of GT-residue in each polymer: **Ia**, 1.8×10^5; **IIa**, 1.6×10^5; **IIIa**, 2.9×10^5; **Ib**, 1.2×10^5; **IIb**, 1.3×10^5; **IIIb**, 1.5×10^5 M^{-1}).

change increased with the polymer concentrations and reached saturation around 30 μM (mole concentration of GT residue). No such fluorescence change was induced by the GT-monomers (**1a** and **1b**) and β-Glc and β-GlcNAc polymers (**IV** and **V**) at concentrations between 0 and 50 μM (mole concentration of the glycoside). These results support that both α,α- and α,β-GT-polymers have binding activity with this lectin, and the binding activity is integrated as the result of multivalent binding and/or carbohydrate cluster effects.

From the observed fluorescence change, the apparent association constants (K_a) per GT-residue were calculated to be 1.8×10^5 M^{-1} (**Ia**) and 1.2×10^5 M^{-1} (**Ib**; Figure 6), showing that α,α-GT polymer is more favorably recognized by this lectin than α,β-GT polymer, indicating that the recognition of this lectin is extended from the key α-galactoside epitope in GT into the internal 1,1-linked glucose residue.

Next, we examined the effect of β-Glc and β-GlcNAc residues added as second sugars. The data given in Figures 5 and 6 indicate the α,α-GT *ter*-polymer **IIIa** carrying both α,α-GT and β-GlcNAc has the strongest affinity to this lectin (K_a = 2.9×10^5 per GT mole fraction). Judging from the result that the β-GlcNAc polymer **V** showed no affinity to this lectin under the examined conditions (Figure 5A), the observed enhancement may be explained in terms of "module effect" by the second sugar. A similar phenomenon was observed between α,β-GT polymers (**Ib** and **IIIb**), though the enhancement by GlcNAc was smaller than the case between **Ia** and **IIIa**. The β-Glc-residue in **IIa** and **IIb** brought about no positive effect (Figures 5 and 6), and the affinity of **IIa** was lower than that of **Ia**. This may be partly because the GT contents in **IIa** were less than the case of **Ia**. But the effect of β-Glc was obviously different from the enhancement by β-GlcNAc.

In Vitro Cell Assay with Shiga Toxin-1. The synthetic glycopolymers were evaluated also with Shiga toxin-1 and HeLa 229 cells. In this assay, a Gb2 copolymer (M_n = ca. 400 KDa, Gb2/acrylamide = ca. 1:4) was used as a positive reference having notable activity to this toxin.[16] If the α,α- or α,β-GT isomer could serve as a good Gb2 mimetic, the GT-polymers would also show comparable activity. The data in Figure 7 show that the Gb2 copolymer exhibited neutralizing activity at 1.0 μM (mole concentration of Gb2 residue). At this concentration, however, these GT-polymers showed no activity. The α,α-GT copolymer **Ia** showed no activity even at 50 μM, while the α,β-GT copolymer **Ib** showed activity at this concentration. Obviously, the neutralization activity of these GT-polymers is much weaker than that of the Gb2 polymer. This may be partly because the number average molecular weight (M_n) of the Gb2 copolymer used for this test is nearly 10 times bigger than those of **Ia** and **Ib** and mainly because this toxin recognizes the molecular geometry of Gb2 very strictly.[16,27]

The α,α-GT *ter*-polymer **IIIa** turned to showing clear activity at concentrations between 20 and 50 μM (per GT residue),

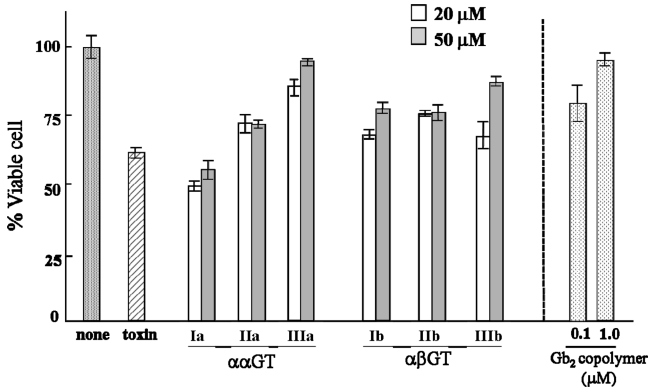


Figure 7. Detoxifying activity of GT-polymers against Shiga toxin-1 Concentrations (μM) are based on the GT mole fraction of each polymer. In this assay, the number of viable HeLa cells was suppressed to ca. 50−65% level when treated with this toxin. After preincubation of this toxin with GT polymers, the number of the viable cells was recovered.

showing that the β-GlcNAc residue embedded in the polymer can enhance the neutralizing activity. A similar effect was observed for the α,β-GT *ter*-polymer **IIIb**, though the enhancement was smaller. The β-Glc residue in **IIa** showed a similar effect though the effect was ambiguous in comparison with the case of β-GlcNAc in **IIIa**.

Interestingly, these results were in good accord with what we observed in the preceding lectin assay. In both cases, the combination of α,α-GT and β-GlcNAc in **IIIa** has brought about the highest activity to these α-galactoside-binding proteins.

Conclusions

In the literature, both symmetric and dissymmetric trehaloses have been designed and prepared by chemical or enzymatic approaches.[8−10,28−37] In this paper, we proposed a chemical pathway to a pair of α,α- and α,β-*galacto*-trehaloses starting from D-glucose and D-galactose derivatives, in which our one-pot α-glycosylation method was effectively employed. A series of glycopolymers were assembled from the *galacto*-trehaloses under the concept of "carbohydrate module approach" and evaluated with BSI-B4 lectin and Shiga toxin-1. In both assays, both α,α- and α,β-GT polymers showed activity to these α-galactoside-binding proteins, indicating the high biological potential of the *galacto*-trehaloses.

The α,α-GT polymer carrying also β-GlcNAc showed superior activity for detoxifying Shiga toxin-1 in comparison with the glycopolymer without this second sugar. The observed "module effect" suggests a notable role of β-GlcNAc for enhancing interactions with the carbohydrate binding proteins in a supplementary way. Though it seems difficult to ascertain the module effect, the clear difference was observed in the effect between β-Glc and β-GlcNAc. It is hard to imagine such a molecular packing geometry in which the α,α-GT and GlcNAc residues are fused along a polymer chain to make a Gb3 ceramide mimetic. Instead, we may be allowed to speculate such a universal action of the 2-acetamide sugar that assists carbohydrate−protein interactions. The 2-acetamide group is made up of -CH_3 (hydrophobic group), -NH- (H donor in hydrogen bonding), and -C═O (hydrogen acceptor), and these elements can be involved in carbohydrate−protein interactions. For example, a bovine β1,4-galactosyltrasnferase can discriminate between Glc and GlcNAc as the acceptor substrates by a NHAc group binding site.[38] In the human blood-type determinant system, α-GalNAc serves as the A-type blood determinant being discriminated from α-Gal in the B-type blood determinant. Moreover, the post-translational modification of proteins by GlcNAc is crucial in the cellular processes including signaling, cell cycle, and transcription. The GlcNAc residue is thought to affect protein−protein interactions, activity, stability, and gene expression.[39−43] When these natural roles are considered, the module effect of the GlcNAc may not be surprising.

We and others disclosed previously that synthetic Gb2 and Gb3 polymers[16,17,22,25,44] and other multivalent models[23,24] are able to trap and detoxify Shiga toxins. The notable activity of the Gb3 polymers was verified with mice.[16,44,45] Although the activity of the α,α- and α,β-GT glycopolymers was not strong enough for testing with mouse, the α-1,1-linkage tolerable to enzymatic hydrolysis[8,10] may have a practical meaning in a future study. The study along this line is being conducted in our group and will be reported elsewhere.

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Supporting Information Available. NMR data and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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