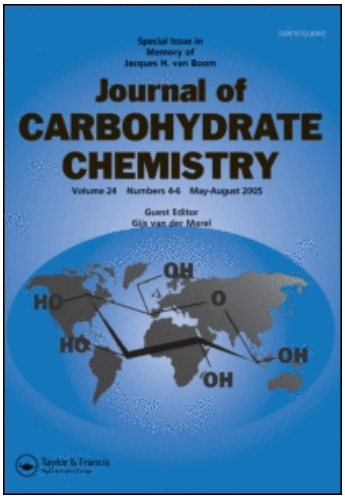

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Synthesis of Immunosuppressive Neoglycoproteins: Bovine Serum Albumin Coupled with 8-(Hydrazino-Carbonyl)Octyl 4- Or 6-*O*-α-D-Mannopyranosyl-α-D-Mannopyranoside

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SYNTHESIS OF IMMUNOSUPPRESSIVE NEOGLYCOPROTEINS: BOVINE SERUM ALBUMIN COUPLED WITH 8-(HYDRAZINOCARBONYL)OCTYL 4- OR 6-*O*-α-D-MANNOPYRANOSYL-α-D-MANNOPYRANOSIDE

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ABSTRACT

Recombinant cytokines generated by bacteria, especially *E.coli*, are nonglycosylated. To investigate the effects of carbohydrates on their activities, we attempted to develop new cytokines by introduction of carbohydrates. As a model we synthesized neoglycoproteins in which potential immunoregulatory carbohydrates were coupled to bovine serum albumin(BSA). Mannose dimers with C9 spacer, Manα1-6Man, which is reported to be immunosuppressive, and a reference substance Manα1-4Man were synthesized as follows. Benzylidenation of 8-(methoxycarbonyl)octyl α-D-mannopyranoside (**10**), followed by acetylation and cleavage of the benzylidene acetal, gave a glycosyl acceptor (**13**) with a free hydroxyl group in the C-4 position. Glycosylation of **13** with acetobromomannose (**8**), followed by debenzylation, deacetylation, and hydrazidation, gave 8-(hydrazinocarbonyl)octyl 4-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**1**). Total yield of **1** from **10** was 25.1%. Tritylation of **10**, followed by acetylation and detritylation, gave a glycosyl acceptor (**18**) with a free hydroxyl group in the C-6 position. Analogous condensation of **18** with **8**, followed by deacetylation and hydrazidation, gave 8-(hydrazinocarbonyl)octyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**2**). Total yield of **2** from **10** was 22.9%. These mannose dimers were coupled to BSA by the acyl azide method. Using the antibodies against the mannose dimers, an enzyme linked immunosorbent assay (ELISA) was established to measure the small amount of

mannose dimers coupled to proteins. These two neoglycoproteins appeared to inhibit the antigen-specific human T cell proliferation over 100 fold more efficiently than free mannose dimers.

INTRODUCTION

Neoglycoproteins, proteins chemically coupled with carbohydrates, have been synthesized to study the role of carbohydrates in various biological reactions, such as binding and uptake by cells through cell-associated lectins.[1,2] Cytokines, proteinous factors, produced by a variety of cell types including lymphocytes, macrophages, granulocytes and endothelial cells, play important roles in immunologic, inflammatory, hematopoietic and homeostatic reactions.[3,4] Most of cytokines are glycosylated. Although the recombinant cyokines generated by cDNA-transfected *E.coli* are devoid of carbohydrates, most of them exhibit the same biological activities *in vitro* as natural glycosylated counterparts. However, *in vivo* it is not well known whether the nonglycosylated cytokines exhibit the same biological activity, tissue distribution and metabolic process as natural cytokines. Mannose, which is known to be a constituent of carbohydrate moiety of glycoprotein, is reported to influence the immunologic reactions.[5-7] D-Mannose inhibits spontaneous monocyte cytotoxicity toward certain erythrocytes and antigen-induced human T cell proliferation.[5] A mannose polymer from yeast mannan is inhibitory to human T cell proliferation,[6] and an immunosuppressive substance purified from human pregnancy urine that inhibits the human T cell proliferation was identified as α-D-Man1-6-D-Man disaccharide.[7] Mannose is also known to be important in glycoprotein recognition by a macrophage-associated lectin[8] and a serum mannose-binding protein.[9] To study the effects of carbohydrates on the cytokine activity *in vitro* and *in vivo* we attempted to develop glycosylated recombinant cytokines by chemical modification with the potential immunoregulatory mannose dimers. However, it is difficult to manipulate cytokines in large quantity because they are quite expensive and difficult to work with. Therefore, here we used bovine serum albumin (BSA) as a model. We chemically synthesized the mannose dimers (Manα1-6Man and Manα1-4Man) with a C9 spacer, and conjugated them to BSA. An ELISA was established to measure the small amount of mannose dimers coupled to proteins. The neoglycoproteins appeared to be immunosuppressive more potently than free mannose dimers.

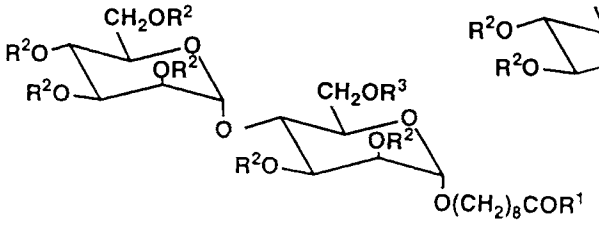
RESULTS AND DISCUSSION

Synthesis of Methyl 9-Hydroxynonanoate (7) - In the literature,[10] compound **7**, with a C9 carbon chain spacer, was prepared from the monomethyl ester of

azelaic acid, via 2-thiazoline-2-thiol ester, by reduction with $NaBH_4$ in aqueous THF. However, we selected 1,9-nonanediol as a starting material for **7**. Partial benzoylation of commercial 1,9-nonanediol in pyridine gave dibenzoate (**3**) and monobenzoate (**4**) in 15.5 and 50.8% yields, respectively. The proton nuclear magnetic resonance (^{1}H NMR) spectrum of **3** showed signals due to aromatic protons at δ 7.28-8.12 (m, 10H), indicating the presence of two benzoyl groups. The infrared (IR) spectrum of **4** showed absorption bands due to hydroxyl and carbonyl functions. The ^{1}H NMR spectrum of **4** showed signals due to aromatic protons from one benzoyl group at δ 7.20-8.12 (m, 5H). Oxidation of **4** by the Jones method (chromic anhydride-dil sulfuric acid-acetone) gave **5** as colourless fine needles in 57.8% yield. Treatment of **5** with methanol in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) gave the methyl ester (**6**) in 93.6% yield. Deacylation by Zemplen's method (sodium methoxide-methanol) gave **7** as a syrup in 66.6% yield. The total yield of **7** from 1,9-nonanediol was 18.3%.

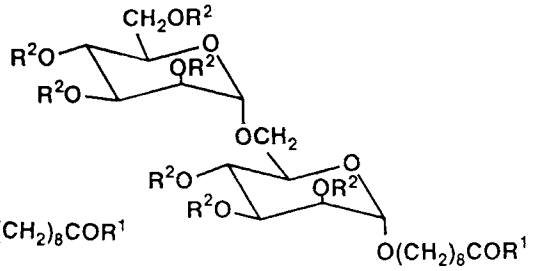
Synthesis of 8-(Hydrazinocarbonyl)octyl 4-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (1) - Reaction of **7** with 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide[11] (**8**) in the presence of mercuric cyanide and Drierite in dry nitromethane was carried out by stirring the mixture at room temperature for 3 days. The ^{1}H NMR spectrum of the product **9**, isolated by column chromatography, showed the signal due to H-1 at δ 4.80 ($J_{1,2}$=1.7 Hz). The coupling constant of H-1, H-2 indicates the stereochemistry of the newly formed glycosidic bond to be α. The carbon-13 nuclear magnetic resonance (^{13}C NMR) spectrum of **9** showed the signal due to C-1 at δ 97.6. The coupling constant of $^1J_{C\text{-}1,H\text{-}1}$ was 171.3 Hz indicating an α-glycoside. Deacetylation of **9**, as described for the preparation of **7** according to Zemplen's method, afforded **10** which was easily crystallized from ethyl acetate as colourless needles. The yield of **10** was 85.9%. The ^{1}H NMR spectrum of **10** showed the signal due to H-1 at δ 4.84 (d, 1H, $J_{1,2}$=1.2 Hz). The chemical shifts of each carbon of **10** were assigned by comparison with literature values for methyl α-D-mannopyranoside.[12] The signal due to C-1 appeared at δ 100.9. The total yield of **10** from acetobromomannose, in two steps, was 49.7%. Compound **10** had been prepared via treatment with mercuric bromide of an 8-(methoxycarbonyl)octyl-orthoester derivative of D-mannose, followed by deacetylation, by Tsui and Gorin[13] with the total yield from acetobromomannose being 59.2% in three steps. Compound **10** had also been prepared by Sugawara, et al.[14] in 71% yield via catalytic hydrogenolysis of 8-(methoxycarbonyl)octyl 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranoside obtained as a minor product (14.9%) by reaction of 2,3,4,6-tetra-*O*-benzyl-α,β-D-mannopyranose, NaH, and 8-(methoxycarbonyl)octyl trifluoromethanesulfonate in anhydrous THF.

Benzylidenation of **10** with benzaldehyde and formic acid, followed by acetylation with acetic anhydride and pyridine, gave the dibenzylidene derivative (**11**) and the mono-



	R^1	R^2	R^3
1	$NHNH_2$	H	H
14	OMe	Ac	Bn
15	OMe	Ac	H
16	OMe	H	H

Bn=benzyl

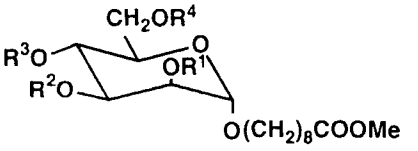


	R^1	R^2
2	$NHNH_2$	H
19	OMe	Ac
20	OMe	H

$R^1O(CH_2)_9OR^2$

	R^1	R^2
3	Bz	Bz
4	Bz	H

Bz=benzoyl



	R^1	R^2	R^3	R^4
9	Ac	Ac	Ac	Ac
10	H	H	H	H
11	benzylidene		benzylidene	
12	Ac	Ac	benzylidene	
13	Ac	Ac	H	Bn
17	Ac	Ac	Ac	Tr
18	Ac	Ac	Ac	H

Tr=trityl

$R^1O(CH_2)_8COOR^2$

	R^1	R^2
5	Bz	H
6	Bz	Me
7	H	Me

benzylidene derivative (**12**) in 17.3 and 56.7% yields, respectively. The ^{1}H NMR spectrum of **11** showed signals due to aromatic protons at δ 7.33-7.60 (m, 10H), indicating the presence of two benzylidene groups. H-1 signals appeared at δ 5.10 and 5.17, those from the methine protons of the 4,6-*O*-benzylidene group at δ 5.64 and 5.52, and those from the 2,3-*O*-benzylidene group at δ 6.29 and 5.96 in an integral ratio of ca. 3:2, respectively. These results indicated that **11** was a mixture of *exo*- and *endo*-2, 3-*O*-benzylidene isomers in ratio of ca. 3:2. According to the literature,[15,16] the methine proton of the benzylidene group on the dioxolane ring resonates at lower field than the protons on the dioxane ring, and the methine proton of the *exo*-benzylidene group resonates at lower field than that of the *endo*-benzylidene group. Therefore, in the *exo*-2,3-*O*-benzylidene acetal derivative, H-1 signals and the methine protons of the 2,3- and 4,6-*O*-benzylidene group were assigned to δ 5.10, 6.29, and 5.64, respectively. In the *endo*-2,3-*O*-benzylidene isomer, H-1 signals and the methine protons of the 2,3- and 4,6-*O*-benzylidene group were assigned to δ 5.17, 5.96, and 5.52, respectively. The ^{13}C NMR specrtum of *exo*-2,3-*O*-benzylidene **11** showed signals due to the methine carbons of 2,3- and 4,6-*O*-benzylidene derivative at δ 103.0 and 102.0, respectively. In the *endo*-isomer, signals due to the methine carbons of 2,3- and 4,6-*O*-benzylidene group appeared at δ 104.1 and 101.7, respectively. These assignments agreed with a report [15] that in a benzylidene acetal of the dioxolane ring an *exo*-methine carbon appeared at higher field than an *endo*-one, and that in a benzylidene acetal of the dioxane ring an *exo*-methine carbon appeared at lower field than an *endo*-one. Signals due to anomeric carbons in *exo*- and *endo*-2,3-*O*-benzylidene isomers appeared at δ 97.8 and 97.6, respectively. The ^{1}H NMR spectrum of **12** showed signals due to two acetyl groups and to aromatic protons at δ 7.10-7.40 (m, 5H).

Cleavage of the benzylidene acetal of **12** with $NaBH_3CN$-HCl according to the method of Garegg, et al.,[17] gave 6-*O*-benzyl ether (**13**) in 81% yield. The ^{13}C NMR spectrum of **13** showed signals due to the methylene carbon of the benzyl group and to C-6 at δ 73.7 and 70.1, respectively, by the "distortionless enhancement by polarization transfer (DEPT)" measurement method. The C-6 signal (δ 70.1) was shifted to lower field as compared with that of C-6 in **18** (δ 61.3) mentioned below. This indicated that the benzyl group is attached at the C-6 position.

Glycosylation of **13** with **8** in the presence of silver triflate and γ-collidine in dichloromethane at -20 ℃ under argon gave a disaccharide derivative (**14**) as a syrup in 72.6% yield after column chromatography. The ^{1}H NMR spectrum of **14** showed signals due to H-1 at δ 4.77 (d, 1H, $J_{1,2}$=1.9 Hz) and to H-1' at δ 5.06 (d, 1H, $J_{1',2'}$=2.5 Hz). The coupling constants of $^1J_{C\text{-}1,H\text{-}1}$ and $^1J_{C\text{-}1',H\text{-}1'}$ were 171.3 and 172.2 Hz, respectively, which indicated the stereochemistry of the newly formed glycosidic bond to be

α. The ^{13}C NMR spectrum of **14** showed signals due to C-1 and C-1' at δ 97.4 and 99.4, respectively. The signal due to C-4 of **14** appeared at δ 74.1, and was deshielded by 6.8 ppm as compared with the chemical shift (δ 67.3) due to C-4 of **13**. However, the chemical shifts (δ 71.6 and 70.8) due to C-3 and C-5 of **14** were shifted upfield by 0.30 and 0.10 ppm as compared with those (δ 71.9 and 70.9) of C-3 and C-5 of **13**, respectively. These results provided unequivocal proof that the newly introduced D-mannopyranosyl linkage in **14** is at C-4. The assignment of spectral data was effected by "homo- and heteronuclear shift correlated spectroscopy (H-H and C-H COSY)" experiments. Removal of the benzyl group in **14** by catalytic hydrogenolysis over Pd catalyst,[18] freshly prepared from $PdCl_2$, gave **15** as a syrup in 90.8% yield. Deacylation of **15** with Zemplen's method as described for the preparation of **7**, followed by lyophylization, gave **16** as a hygroscopic amorphous mass in 85.3% yield. The ^1H NMR spectrum of **16** showed signals due to H-1 at δ 4.82 (s, 1H) and to H-1' at δ 5.27 (s, 1H). The chemical shifts of each carbon of **16** were assigned by comparison with literature values for methyl 4-*O*-α-D-mannopyranosyl-α-D-mannopyranoside.[19] Treatment of **16** with hydrazine hydrate in methanol, followed by dry column chromatography,[20] afforded **1** as a hygroscopic amorphous mass in 97.1% yield. The IR spectrum of **1** showed no absorption band due to a carbonyl group but contained absorption bands due to an amide (1620 and 1525 cm^{-1}) group. The ^1H NMR spectrum of **1** showed two doublets for H-1 at δ 4.86 (1H, $J_{1,2}$=1.8 Hz) and for H-1' at δ 5.25 (1H, $J_{1',2'}$=1.9 Hz), but no resonance due to a methyl ester group.

Synthesis of 8-(Hydrazinocarbonyl)octyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (2) - Tritylation of **10** with trityl chloride in pyridine, followed by acetylation with acetic anhydride, gave **17** as a syrup in 77% yield. The ^1H NMR spectrum of **17** showed signals due to aromatic protons at δ 7.20-7.50 (m, 15H), and to acetyl groups at δ 1.73, 1.96 and 2.16 (3s, 9H). Treatment of **17** with aqueous acetic acid at 70 ℃ for 1.5 h followed by chromatography of the product on a column of silica gel gave **18** as a syrup in 61.9% yield.

Glycosylation of **18** with **8** in the presence of silver triflate and γ-collidine in dichloromethane at -20 ℃ under argon, as described for the preparation of **14**, gave a disaccharide derivative (**19**) as a syrup in 52.9% yield. The ^1H NMR spectrum of **19** showed signals due to H-1 at δ 4.77 (d, 1H, $J_{1,2}$=1.9 Hz) and to H-1' at δ 4.86 (d, 1H, $J_{1',2'}$=1.8 Hz). The coupling constants $^1J_{C\text{-}1,H\text{-}1}$ and $^1J_{C\text{-}1',H\text{-}1'}$ were 171.7 and 172.8 Hz, respectively. These results indicated the stereochemistry of the newly formed glycosidic bond to be α. The ^{13}C NMR spectrum of **19** showed signals due to C-1 and C-1' at δ 97.3 and 97.5, respectively. The signal due to C-6 of **19** appeared at δ 66.6, deshielded by 5.3 ppm as compared with the chemical shift due to C-6 of **18** (δ 61.3).

However, the chemical shift (δ 69.3) due to C-5 of **19** was shifted upfield by 1.3 ppm as compared with that (δ 70.6) of C-5 of **18**. These results provided unequivocal proof of the position (C-6) of the newly introduced D-mannopyranosyl linkage in **19**. Deacylation of **19** with Zemplen's method as described for the preparation of **7**, followed by lyophilization, gave **20** as a hygroscopic amorphous mass in 97.6% yield. The ^{1}H NMR spectrum of **20** showed a singlet for H-1 at δ 4.81 (1H) and for H-1' at δ 4.86 (1H). The chemical shifts of each carbon of **20** were assigned by comparison with literature values for methyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside.[19] Compound **20** had been prepared in 47% yield via catalytic hydrogenolysis of its perbenzyl ether by Sugawara, et al.[14] Treatment of **20** with hydrazine hydrate as described for the preparation of **1**, followed by dry column chromatography,[20] gave **2** as a hygroscopic amorphous mass in 93% yield. The ^{1}H NMR spectrum of **2** showed a singlet for H-1 at δ 4.85 (1H) and a doublet for H-1' at δ 4.90 (1H, $J_{1',2'}$=1.2 Hz).

Coupling of 1 or 2 with bovine serum albumin (BSA) - Compound **1** was transformed to an acyl azide derivative by reaction with hydrogen nitrite. The acyl azide derivative was coupled with BSA in 0.4 M sodium borate buffer (pH 10.0) to afford a glycosylated BSA. The glycosylated BSA was purified by ion exchange column chromatography with DE-52 and deionized with amicon ultrafiltration to give Manα(1-4)Man-BSA as a hygroscopic amorphous powder. Similarly, compound **2** was transformed to an acyl azide derivative, coupled with BSA to give Manα(1-6)Man-BSA as a hygroscopic amorphous powder.

Determination of sugar component combined with BSA - Sugar component contained in Manα(1-4)Man-BSA(1.0 mg) or Manα(1-6)Man-BSA (2.0 mg) was determined by the phenol-sulfuric acid method according to the literature.[21] Manα(1-4)-Man-BSA contained 8.2 residues of α-D-Man-(1-4)-α-D-Man-(1-)-O$(CH_2)_8$CO- per molecule of BSA and Manα(1-6)Man-BSA 9.7 residues of α-D-Man-(1-6)-α-D-Man-(1-)-O-$(CH_2)_8$CO- per molecule of BSA.

Preparation and specificity of the antibody against mannose dimers - Cytokines are quite expensive and difficult to work with. Therefore, it is actually hard to determine the carbohydrate content by a chemical method because it requires large amount of cytokines. To circumvent this, we attempted to establish ELISA. For that purpose we obtained antibodies against the carbohydrates by immunizing rabbits with Manα(1-4)-Man-BSA or Manα(1-6)Man-BSA. As shown in Figure 1, the antibody against Manα(1-6)-Man-BSA reacted with both Manα(1-4)Man and Manα(1-6)Man but not with mannose monomer or BSA. The antibody against Manα(1-4)Man-BSA also reacted with both Manα(1-4)Man and Manα(1-6)Man but not with mannose monomer or BSA (data not shown).

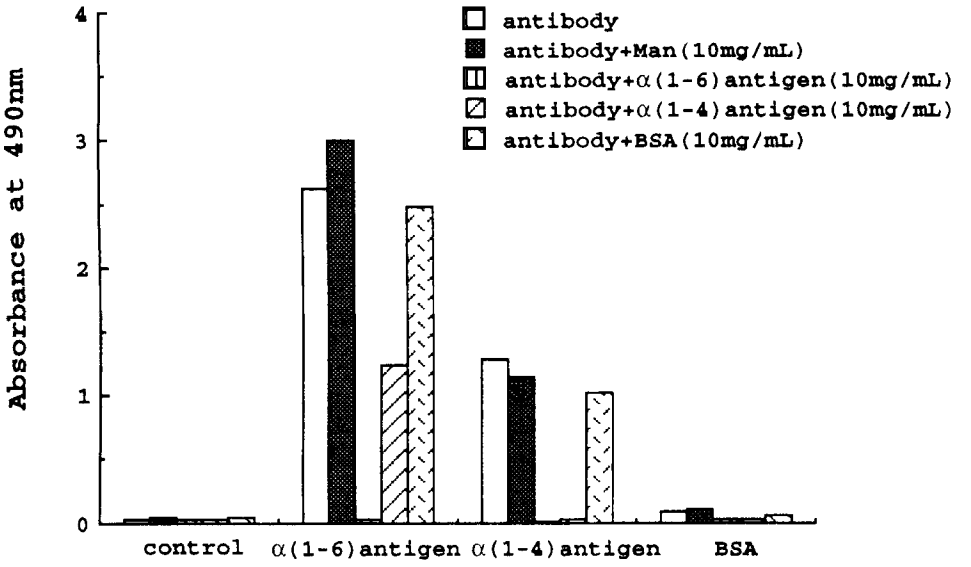


FIG. 1. Specificity of antibody against Manα(1-6)Man-conjugated BSA. Rabbit IgG against Manα(1-6)Man-BSA was pretreated with D-Mannose, Manα(1-6)Man-BSA, Manα(1-4)Man-BSA or BSA, and then the reactivity to Manα(1-6)Man-BSA, Manα(1-4)Man-BSA or BSA was determined.

Development of ELISA - As the antibody against Manα(1-6)Man-BSA strongly reacted with the two mannose dimers but not mannose monomer and BSA, we utilized this antibody for ELISA. As shown in Figure 2, we established ELISA for measuring the small amount of Manα(1-4) and Manα(1-6) coupled to proteins. By this method the carbohydrate content is determinable as small as 2 ng/mL carbohydrates which corresponds to 40 ng/mL of neoglycoprotein. Thus, this ELISA appeared to be applicable for determining the carbohydrate content in a small amount of glycosylated cytokines.

Immunosuppressive effects of mannose dimers and neoglycoproteins - D-Mannose and Manα(1-6)Man are reported to be immunosuppressive.[5-7] Therefore, we determined whether the neoglycoproteins are also immunosuppressive and compared the activity with those of free mannose dimers. As shown in Figure 3, these mannose dimers with or without a C9 spacer inhibited the antigen-specific proliferation of T cells. Mannose dimers with a C9 spacer were more potent than Manα(1-6)Man. These two neoglycoproteins also inhibited the proliferation of T cells in a dose-dependent manner. BSA without carbohydrate did not exhibit any suppressive effect. Manα(1-4)Man-BSA was more potent than Manα(1-6)Man-BSA. Based on the carbohydrate content, these neoglycoproteins were over 100 fold more potent than free carbohydrates. At present

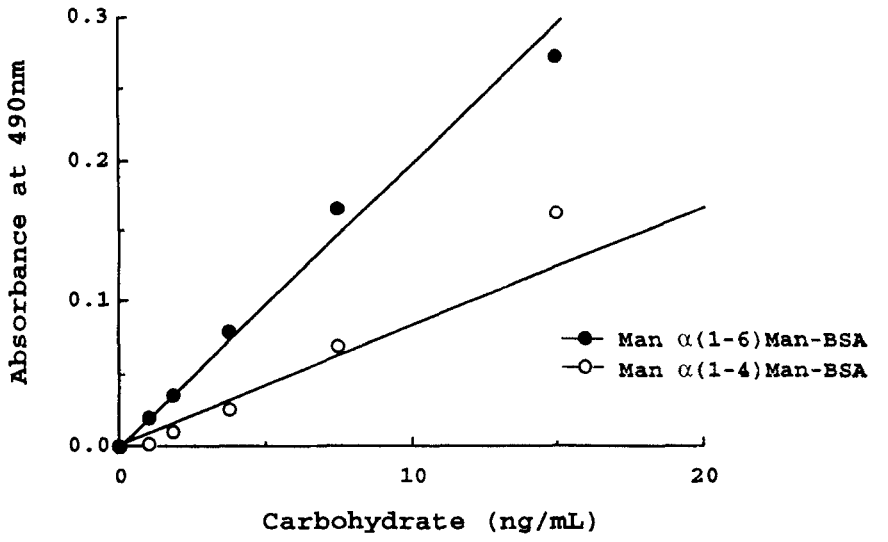


FIG. 2. ELISA with antibody against Manα(1-6)Man-conjugated BSA. An ELISA was performed with rabbit IgG against Manα(1-6)Man-BSA, varying doses of Manα(1-6)Man-BSA and Manα(1-4)Man-BSA. Carbohydrate content in the neoglycoproteins was indicated.

we do not know the reason, but the mannose dimers attached to the surface of BSA may be more accessible to target cell receptors. In order to determine whether these suppressive effects are specific to antigen-stimulated T cell proliferation, the effects of these compounds on the IL-2 dependent cell line proliferation were examined. As shown in Figure 4, at 10^{-2} M all the mannose dimers with or without C9 spacers inhibited the proliferation. Therefore, the inhibitory effects of mannose dimers at the high dose may be nonspecific. In contrast, none of BSA and neoglycoproteins inhibited the proliferation indicating that the immunosuppressive effects of neoglycoproteins were not due to nonspecific action, such as cytotoxicity. Our finding is of interest in considering the application of these neoglycoproteins as immunosuppressive reagents because free carbohydrates are readily metabolized *in vivo*. Therefore, these neoglycoproteins may be a new type of immunosuppressive reagent. *In vivo* trial of these neoglycoproteins for treatment of animal model of autoimmune disease is now under investigation.

EXPERIMENTAL

General Procedures. Melting points were determined with a Yanagimoto MP-S2 micro melting point apparatus and are uncorrected. Solutions were concentrated in a

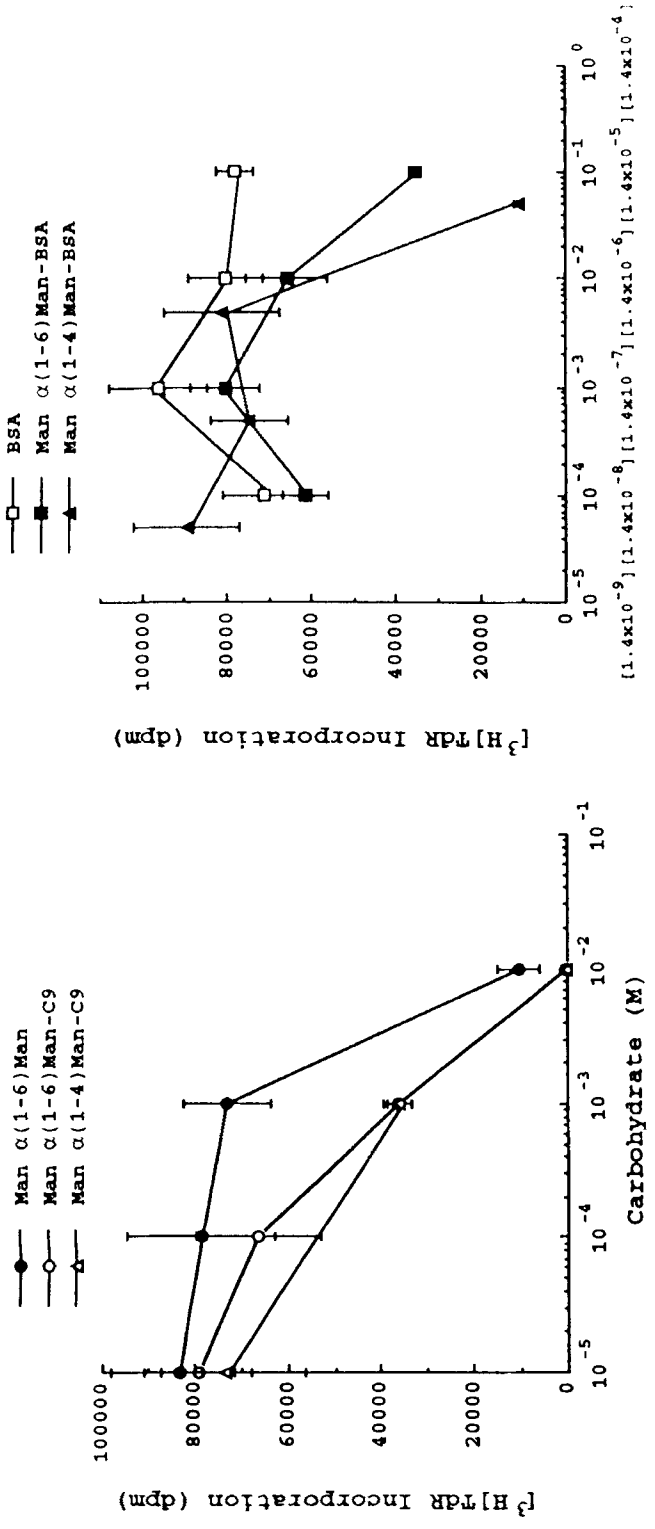


FIG. 3. Effects of mannose dimers and neoglycoproteins on antigen-induced human T cell proliferation. Human peripheral blood mononuclear cells were cultured in the presence of T cell-specific antigen(PPD) with or without varying doses of Manα(1-6)Man, Manα(1-6)Man-C9, Manα(1-4)Man-C9, BSA, Manα(1-6)Man-BSA or Manα(1-4)Man-BSA at 37℃ for 6 days. After culture cell proliferation was determined by [^{3}H]thymidine incorporation. Mean ± S.D. of triplicated cultures is shown. Carbohydrate content in neoglycoproteins is indicated as [Carbohydrate(M)].

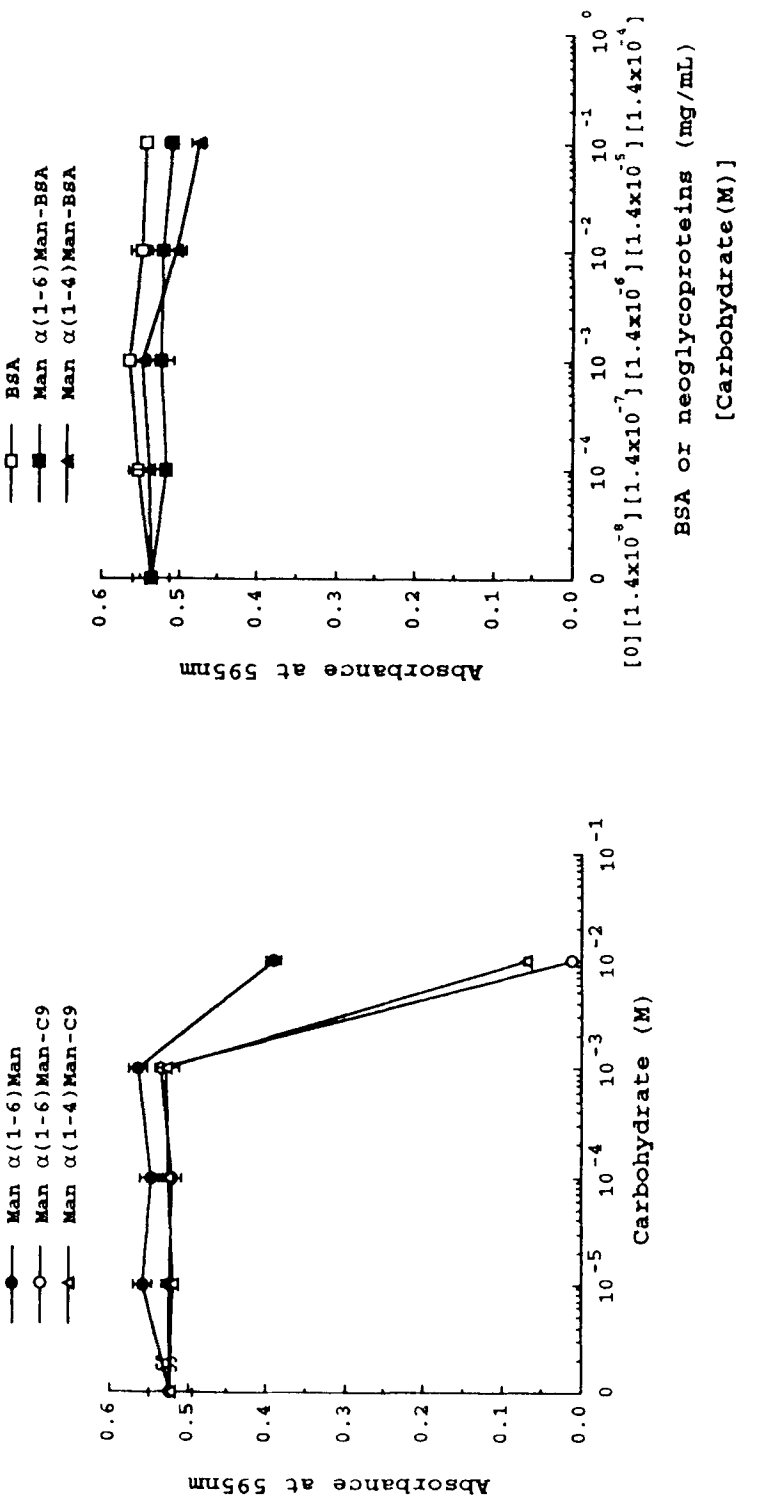


FIG. 4. Effects of mannose dimers and neoglycoproteins on IL-2-dependent cell proliferation. NK-3 cells were cultured in the presence of suboptimal dose of IL-2 with or without varying doses of Manα(1-6)Man, Manα(1-6)Man-C9, Manα(1-4)-Man-C9, BSA, Manα(1-6)Man-BSA or Manα(1-4)Man-BSA at 37°C for 3 days. After culture, cell proliferation was determined by MTT method. Mean ± S.D. of triplicated cultures is shown.

rotary evaporator below 40 ℃ under vacuum. Optical rotations were measured with a Union Giken PM-201 automatic digital polarimeter in a 0.5 dm tube. IR spectra were recorded with a JASCO A-102 spectrometer. ^{1}H NMR spectra were recorded at 100 MHz with a JEOL JNM-FX-100, at 400 MHz with a JEOL GSX-400 or at 500 MHz with a JEOL α-500 spectrometer. ^{13}C NMR spectra were recorded at 25 MHz with a JEOL JNM -FX-100, at 100 MHz with a JEOL GSX-400 or at 125 MHz with a JEOL α-500 spectrometer. Tetramethylsilane was used as an internal standard in $CDCl_3$. 3-(Trimethylsilyl)propionic acid-d_4 sodium salt (TSP) was used as an internal standard in D_2O. Chemical shifts are given on the δ scale and those in D_2O were transformed into TMS standard. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel $60F_{254}$, Merck). Detection was effected with H_2SO_4 or by UV irradiation at 254 nm. Column chromatography was performed on Silica Gel BW-820MH (Fuji-Silysia Chemical Ltd., Nagoya). Solvent combinations for elution of a column chromatography and the developing solvent on TLC are given as v/v.

1,9-Dibenzoylnonanediol (3) and 9-Benzoylnonanediol (4). To a chilled solution of 1,9-nonanediol (15 g, 93.6 mmol) in dry pyridine (100 mL), benzoyl chloride (11 mL, 94.5 mmol) was added dropwise with stirring at 0 ℃, and the mixture allowed to attain room temperature. After stirring overnight, the reaction mixture was treated with ice to decompose the reagent, and concentrated by repeated co-distillation with toluene to afford a syrup which dissolved in dichloromethane. The dichloromethane solution was successively washed with saturated aqueous $NaHCO_3$ and water, dried ($MgSO_4$), and concentrated to afford a syrup which showed two UV positive spots. The syrup was dissolved in a small amount of dichloromethane and chromatographed on a column of silica gel with chloroform-acetone (20:1-6:1) as eluent. Evaporation of the solvent from the first effluent afforded **3** (5.34 g, 15.5%) as a syrup: TLC (20:1 chloroform-acetone), Rf 0.75; IR (neat) 1719 and 1275 (ester), and 711 cm^{-1} (Ph); ^{1}H NMR ($CDCl_3$) δ 1.20-1.92 [m, 14H, $CH_2(CH_2)_7CH_2$], 4.31 (t, 4H, J=6.6 Hz, $2CH_2O$), and 7.28-8.12 (m, 10H, 2Ph); ^{13}C NMR ($CDCl_3$) δ 26.0 (x2), 28.8 (x2), 29.2 (x2), 29.4 [$CH_2(CH_2)_7CH_2$], 65.1 (x2) (CH_2O), 128.3 (x4), 129.5 (x4), 130.6 (x2), 132.8 (x2) (2Ph), and 166.6 (x2) (*C*OPh).

Anal. Calcd for $C_{23}H_{28}O_4$ (368.48): C, 74.97; H, 7.66. Found: C, 74.84; H, 7.83.

Concentration of the second effluent afforded **4** (12.58 g, 50.8%) as a syrup: TLC (20:1 chloroform-acetone), Rf 0.50; IR (neat) 3600-3150 (OH), 1719 and 1315 (ester), and 712 cm^{-1} (Ph); ^{1}H NMR ($CDCl_3$) δ 1.20-1.88 [m, 14H, $CH_2(CH_2)_7CH_2$], 1.98 (broad s, 1H, exchangeable with D_2O, OH), 3.62 (t, 2H, J=6.3 Hz, CH_2OH), 4.31 (t, 2H, J=6.6 Hz, CH_2OBz), and 7.20-8.12 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 25.7, 26.0,

28.7, 29.2, 29.3, 29.5, 32.7 [$CH_2(CH_2)_7CH_2$], 62.9 (CH_2OH), 65.1 (CH_2OBz), 128.3 (x2), 129.5 (x2), 130.5, 132.8 (Ph), and 166.7 (*C*OPh).

Anal. Calcd for $C_{16}H_{24}O_3$ (264.37): C, 72.69; H, 9.15. Found: C, 72.68; H, 9.14.

9-Benzoyloxynonanoic Acid (5). To a chilled solution of **4** (5 g, 18.9 mmol) in acetone (15 mL), a suspension of chromium trioxide (7.53 g, 75.3 mmol) in 3.5 M H_2SO_4 (10 mL) was added dropwise with stirring at 0 ℃. The mixture was allowed to attain room temperature and the stirring was continued for 1.5 h. The mixture was poured into ice-water which was stirred for 30 min and then extracted with dichloromethane. The combined extracts were washed with water, dried ($MgSO_4$), and concentrated to afford a syrup which was dissolved in a small amount of dichloromethane. The dichloromethane solution was chromatographed on a column of silica gel with chloroform-acetone (20:1-6:1) as eluent. Evaporation of the solvent afforded a syrup which was crystallized from diethyl ether-hexane. Recrystallization from the same solvent system gave **5** (3.04 g, 57.8%) as colourless fine needles: mp 44-45 ℃; TLC (10:1 chloroform-acetone) Rf 0.43; IR (KBr) 1718 and 1279 (ester), and 710 cm^{-1} (Ph); ^1H NMR($CDCl_3$) δ 1.08-1.92 [m, 12H,$CH_2(CH_2)_6CH_2$], 2.35 (t, 2H, J=7.3 Hz, CH_2COOH), 4.32 (t, 2H, J=6.6 Hz, CH_2OBz), 7.28 -8.16 (m, 5H, Ph), and 10.27 (broad s, 1H, OH); ^{13}C NMR ($CDCl_3$) δ 24.6, 26.0, 28.7, 29.0, 29.1 (x2) [$CH_2(CH_2)_6CH_2$], 34.0 (CH_2COOH), 65.1 (CH_2OBz), 128.3 (x2), 129.5 (x2), 130.5, 132.8 (Ph), 166.7 (*C*OPh), and 180.0 (COOH).

Anal. Calcd for $C_{16}H_{22}O_4$ (278.35): C, 69.04; H, 7.97. Found: C, 68.93; H, 8.25.

Methyl 9-Benzoyloxynonanoate (6). A solution of **5** (4.04 g, 14.5 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) (3.09 g, 16.1 mmol) in dry methanol (40 mL) was stirred for 2 h. The reaction mixture was concentrated to afford a crystalline residue which was treated with dichloromethane and water. The dichloromethane layer was separated, washed with water, dried ($MgSO_4$), and concentrated to afford a syrup which was chromatographed on a column of silica gel with chloroform-acetone (10:1) as eluent. Evaporation of the solvent gave **6** (3.97 g, 93.6%) as a syrup: TLC (5:1 benzene-diethyl ether) Rf 0.65; IR (neat) 1719 and 1274 (ester), and 712 cm^{-1} (Ph); ^1H NMR ($CDCl_3$) δ 1.20-1.92 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.23 (t, 2H, J=7.6 Hz, CH_2COOMe), 3.66 (s, 3H, MeO), 4.31 (t, 2H, J=6.6 Hz, CH_2OBz), and 7.24-8.16 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 24.9, 26.0, 28.7, 29.0 (x3) [$CH_2(CH_2)_6CH_2$], 34.0 (CH_2COOMe), 51.4 (MeO), 65.0 (CH_2OBz), 128.3 (x2), 129.5 (x2), 130.5, 132.7 (Ph), 166.6 (*C*OPh), and 174.1 (*C*OOMe).

Anal. Calcd for $C_{17}H_{24}O_4$ (292.38): C, 69.84; H, 8.27. Found; C, 69.52; H, 8.49.

Methyl 9-Hydroxynonanoate (7). 0.5 M Methanolic sodium methoxide (3 mL) was added dropwise with stirring to a chilled solution of **6** (4.90 g, 16.8 mmol) in dry methanol (50 mL). After being stirred overnight at room temperature, the mixture was neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated to afford a syrup which was chromatographed on a column of silica gel with chloroform-acetone (30:1-6:1) as eluent. Evaporation of the solvent gave **7** (2.1 g, 66.6%) as a syrup: TLC (5:1 benzene-diethyl ether) Rf 0.33; IR (neat) 3600-3150 (OH), and 1740 and 1199 cm^{-1} (ester); ^1H NMR ($CDCl_3$) δ 1.25-1.70 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.30 (t, 2H, J=7.5 Hz, CH_2COOMe), 2.47 (s, 1H, exchangeable with D_2O, OH), 3.61 (t, 2H, J=6.7 Hz, CH_2OH), and 3.66 (s, 3H, MeO); ^{13}C NMR ($CDCl_3$) δ 25.0, 25.7, 29.1, 29.2 (x2), 32.7 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.5 (MeO), 62.7 (CH_2OH), and 174.4 (*C*OOMe).

Anal. Calcd for $C_{10}H_{20}O_3$ (188.27): C, 63.80; H, 10.71. Found: C, 64.00; H, 10.78.

8-(Methoxycarbonyl)octyl 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranoside (9). A mixture of **7** (0.92 g, 4.89 mmol), 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (**8**) (1.00 g, 2.43 mmol), mercuric cyanide (1.24 g, 4.90 mmol), and Drierite (2 g) in distd nitromethane (20 mL) was stirred for 3 days at room temperature. The mixture was filtered, the residue was washed with dichloromethane, and the combined filtrate and washings were concentrated to a syrup which was dissolved in dichloromethane. The dichloromethane solution was washed with water, 10% KBr solution (x2), dried ($MgSO_4$), and concentrated to afford a syrup which was chromatographed on a column of silica gel with chloroform-acetone (20:1-1:1) as eluent. Evaporation of the solvent gave **9** (0.73 g, 57.9%) as a syrup: $[\alpha]_D^{24}$+40° (*c* 1.02, $CHCl_3$); TLC (20:1 chloroform-acetone) Rf 0.54; IR (neat) 1752 and 1225 cm^{-1} (ester); ^1H NMR ($CDCl_3$) δ 1.23-1.68 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.00, 2.05, 2.10, 2.16 (4s, 12H, 4AcO), 2.31 (t, 2H, J=7.5 Hz, CH_2COOMe), 3.45, 3.68 (m, 2H, CH_2O), 3.67 (s, 3H, MeO), 3.98 (ddd, 1H, $J_{4,5}$=9.7 Hz, $J_{5,6a}$=2.4 Hz, $J_{5,6b}$=5.3 Hz, H-5), 4.11 (dd, 1H, $J_{6a,6b}$=12.0 Hz, H-6a), 4.28 (dd, 1H, H-6b), 4.80 (d, 1H, $J_{1,2}$=1.7 Hz, H-1), 5.23 (dd, 1H, $J_{2,3}$= 3.3 Hz, H-2), 5.27 (dd, 1H, $J_{3,4}$=9.9 Hz, H-4), and 5.35 (dd, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 20.7 (x3), 20.9 (4CO*Me*), 24.9, 26.0 , 29.1 (x2), 29.2 (x2) [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.4 (MeO), 62.6 (C-6), 66.3 (C-4), 68.4 (C-3), 68.5 (CH_2O), 69.2 (C-2), 69.8 (C-5), 97.6 (C-1), 169.8, 169.9, 170.1, 170.7 (4*C*OMe), and 174.3 (*C*OOMe).

Anal. Calcd for $C_{24}H_{38}O_{12}$ (518.57): C, 55.59; H, 7.39. Found: C, 55.38; H, 7.25.

8-(Methoxycarbonyl)octyl α-D-Mannopyranoside (10). A mixture of **9** (167 mg, 0.32 mmol) and 0.5 M sodium methoxide (0.5 mL) in dry methanol (2 mL) was

deacetylated as described for the preparation of **7**. Evaporation of the solvent gave a syrup which was crystallized from ethyl acetate. Recrystallization from the same solvent gave **10** (95.9 mg, 85.9%) as colourless needles: mp 72-74 ℃ [ref.13, mp 70-72 ℃ (ethyl acetate), ref.14, mp 76-77 ℃ (ethyl acetate-diethyl ether)]; $[\alpha]_D^{21}$+43.4° (*c* 1.00, H_2O) [ref.13, $[\alpha]_D^{25}$-47° (*c* 0.75, H_2O), ref.14, $[\alpha]_D^{25}$+52.7° (*c* 0.4, MeOH)]; TLC (10:1 chloroform-methanol) Rf 0.78; IR (KBr) 3600-3200 (OH), and 1734 and 1270 cm^{-1} (ester); ^1H NMR (D_2O) δ 1.20-1.65 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.35 (t, 2H, J=7.4 Hz, CH_2COOMe), 3.49, 3.72 (m, 2H, CH_2O), 3.58 (ddd, 1H, $J_{4,5}$=9.8 Hz, $J_{5,6a}$=5.5 Hz, $J_{5,6b}$=2.4 Hz, H-5), 3.68 (s, 3H, MeO), 3.70 (dd, 1H, $J_{3,4}$=9.1 Hz, H-4), 3.77 (dd, 1H, $J_{2,3}$=3.7 Hz, H-3), 3.79 (dd, 1H, $J_{6a,6b}$=12.2 Hz, H-6a), 3.84 (dd,1H,H-6b), 3.91 (dd, $J_{1,2}$=1.2 Hz, H-2), and 4.84 (d, 1H, H-1); ^{13}C NMR (D_2O) δ 25.6, 26.7, 29.6, 29.7, 29.8, 29.9 [$CH_2(CH_2)_6CH_2$], 34.8 (CH_2COOMe), 52.9 (MeO), 61.8 (C-6), 67.6 (C-4), 68.7 (CH_2O), 71.3 (C-2), 71.9 (C-3), 73.7 (C-5), 100.9 (C-1), and 177.3 (*C*OOMe).

8-(Methoxycarbonyl)octyl *exo*- and *endo*-2,3:4,6-Di-*O*-benzylidene-α-D-mannopyranoside (11) and 8-(Methoxycarbonyl)octyl 2,3-Di-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranoside (12). Compound **10** (2 g, 5.71 mmol) was dissolved in 99% anhydrous formic acid (10 mL), cooled to 0 ℃, and treated with freshly distd benzaldehyde (10 mL). The mixture was stirred for 5 min at 0 ℃ and 2 min at room temperature, and poured, with vigorous stirring, into a cooled mixture of hexane (160 mL) and 30% potassium carbonate solution (60 mL). To the mixture was added dichloromethane, and the organic and the aqueous layers were separated. The organic layer was washed with water and the first aqueous layer was extracted with dichloromethane. The organic layer and extracts were combined, dried (Na_2SO_4), and concentrated to dryness. The residue was acetylated with acetic anhydride (10 mL) and pyridine (10 mL) at room temperature overnight. The mixture was concentrated by repeated co-distillation with toluene to afford a syrup which showed two spots. The syrup was dissolved in dichloromethane, and chromatographed on a column of silica gel with benzene-diethyl ether (20:1-5:1) as eluent. Evaporation of the solvent from the first effluent afforded a crystalline residue which was crystallized from ethanol. Recrystallization from the same solvent gave **11** (0.52 g, 17.3%) as colourless fine needles: mp 97-98 ℃, $[\alpha]_D^{26}$-2.6° (*c* 0.89, $CHCl_3$); TLC (3:1 benzene-diethyl ether) Rf 0.58; IR (KBr) 1734 and 1292 (ester), and 707 and 697 cm^{-1} (Ph); ^1H NMR ($CDCl_3$) δ: *exo*-2,3-*O*-benzylidene isomer; 1.20-1.70 [m, $CH_2(CH_2)_6CH_2$], 2.29 (t, J=7.5 Hz, CH_2COOMe), 3.43, 3.73 (m, CH_2O), 3.65 (s, MeO), 4.15 (d,$J_{2,3}$=5.4 Hz, H-2), 4.65 (dd, $J_{3,4}$ =7.6 Hz, H-3), 5.10 (s, H-1), 5.64 (s, methine proton of 4,6-*O*-benzylidene acetal), 6.29 (s, methine

proton of 2,3-*O*-benzylidene acetal), and 7.33-7.60 (m, 2Ph); *endo*-2,3-*O*-benzylidene isomer; 1.20-1.70 [m, $CH_2(CH_2)_6CH_2$] , 2.31 (t, J=7.5 Hz, CH_2COOMe), 3.43, 3.73 (m, CH_2O), 3.66 (s, MeO), 4.30 (d, $J_{2,3}$=6.2 Hz, H-2), 4.49 (dd, $J_{3,4}$=7.6 Hz, H-3), 5.17 (s, H-1), 5.52 (s, methine proton of 4,6-*O*-benzylidene acetal), 5.96 (s, methine proton of 2,3-*O*-benzylidene acetal), and 7.33-7.60 (m, 2Ph); ^{13}C NMR ($CDCl_3$) δ: *exo*-2,3-*O*-benzylidene isomer; 24.9, 26.1 (x2), 29.1, 29.2, 29.4 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.5 (MeO), 60.4 (C-5), 68.2 (CH_2O), 68.9 (C-6), 75.5, 75.6 (C-3, 4), 77.6 (C-2), 97.8 (C-1), 102.0 (methine carbon of 4,6-*O*-benzylidene acetal), 103.0 (methine carbon of 2,3-*O*-benzylidene acetal), 126.1 (x2), 126.3 (x2), 128.5 (x2), 130.2 (x2), 133.7 (x2), 137.2, 138.7 (2Ph), and 174.3 (*C*OOMe); *endo*-2,3-*O*-benzylidene isomer; 24.9, 26.1 (x2), 29.1, 29.2, 29.4 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.5 (MeO), 60.4 (C-5), 68.1 (CH_2O), 68.9 (C-6), 74.2 (C-4), 78.6 (C-3), 80.7 (C-2), 97.6 (C-1), 101.7 (methine carbon of 4,6-*O*-benzylidene acetal), 104.1 (methine carbon of 2,3-*O*-benzylidene acetal), 126.2 (x2), 126.6 (x2), 128.2 (x2), 128.4 (x2), 133.7 (x2), 137.1, 137.3 (2Ph), and 171.8 (*C*OOMe).

Anal. Calcd for $C_{30}H_{38}O_8$ (526.64): C, 68.42; H, 7.27. Found: C, 68.20; H, 7.44.

Concentration of the second effluent afforded **12** (1.69 g, 56.7%) as a syrup: $[\alpha]_D^{26}$ +27.3° (*c* 0.85, $CHCl_3$); TLC (3:1 benzene-diethyl ether) Rf 0.20; IR (neat) 1720 and 1210 (ester), and 700 cm^{-1} (Ph); ^1H NMR ($CDCl_3$) δ 1.25-1.68 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.02, 2.17 (2s, 6H, 2AcO), 2.30 (t, 2H, J=7.5 Hz, CH_2COOMe), 3.42, 3.70 (m, 2H, CH_2O), 3.66 (s, 3H, MeO), 3.85 (t, 1H, $J_{3,4}$=$J_{4,5}$=10.1 Hz, H-4), 3.96 (ddd, 1H, $J_{5,6a}$=9.2 Hz, $J_{5,6b}$=4.4 Hz, H-5), 4.03 (dd, 1H, $J_{6a,6b}$=10.3 Hz, H-6a), 4.28 (dd, 1H, H-6b), 4.75 (d, 1H, $J_{1,2}$=1.7 Hz, H-1), 5.34 (dd, 1H, $J_{2,3}$=3.5 Hz, H-2), 5.42 (dd, 1H, H-3), 5.58 (s,1H, PhC*H*=), and 7.10-7.40 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 20.8, 20.9 (2CO*Me*), 24.9, 26.0, 29.1 (x2), 29.2, 29.3 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.4 (MeO), 63.8 (C-5), 68.4 (x2) (C-3, CH_2O), 68.8 (C-6), 70.3 (C-2), 76.3 (C-4), 98.6 (C-1), 101.9 (Ph*C*H), 126.2 (x2) , 128.3 (x2), 133.7, 137.2 (Ph), 169.9, 170.0 (2*C*OMe), and 174.4 (*C*OOMe).

Anal. Calcd for $C_{27}H_{38}O_{10}$ (522.60): C, 62.06; H, 7.33. Found: C, 62.33; H, 7.30.

8-(Methoxycarbonyl)octyl 2,3-Di-*O*-acetyl-6-*O*-benzyl-α-D-manno-pyranoside (13). To a suspension of **12** (0.75 g, 1.44 mmol), $NaBH_3CN$ (1.14 g, 18.1 mmol) and molecular sieves 3A (4.5 g) in distd tetrahydrofuran (15 mL) under argon was added hydrogen chloride in diethyl ether until the evolution of gas ceased. The mixture was diluted with dichloromethane and water, filtered, and the organic layer was separated. It was successively washed with water, saturated aqueous $NaHCO_3$, and water,

dried ($MgSO_4$), and concentrated to afford a syrup which was chromatographed with benzene-diethyl ether (10: 1-1:1) as eluent. Evaporation of the solvent gave **13** (0.61 g, 81.0 %) as a syrup: $[\alpha]_D^{26}$+33.5° (*c* 0.95, $CHCl_3$); TLC (1:1 benzene-diethyl ether) Rf 0.50; IR (neat) 3650-3250 (OH), 1747 and 1244 (ester), and 699 cm^{-1} (Ph); ^1H NMR ($CDCl_3$) δ 1.20-1.70 [m, 12 H, $CH_2(CH_2)_6CH_2$], 2.06, 2.11 (2s, 6H, 2AcO), 2.30 (t, 2H, J=7.5 Hz, CH_2COOMe), 3.41, 3.68 (m, 2H, CH_2O), 3.65 (s, 3H, MeO), 3.76 (m, 1H, H-6a), 3.81 (m, 1H, H-5), 3.83 (m, 1H, H-6b), 4.00 (m, 1H, H-4), 4.58, 4.65 (2d, 2H, J= 12.1 Hz, $PhCH_2$), 4.77 (d, 1H, $J_{1,2}$=1.3 Hz, H-1), 5.20 (dd, 1H, $J_{2,3}$=3.5 Hz, $J_{3,4}$=9.9 Hz, H-3), 5.21 (dd, 1H, H-2), and 7.32-7.36 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 20.8, 20.9 (2*COMe*), 24.9, 26.0, 29.0, 29.1 (x2), 29.2 [$CH_2(CH_2)_6CH_2$], 34.0 (CH_2COOMe), 51.4 (MeO), 67.3 (C-4), 68.2 (CH_2O), 69.9 (C-2), 70.1 (C-6), 70.9 (C-5), 71.9 (C-3), 73.7 ($PhCH_2$), 97.5 (C-1), 127.6 (x2), 127.7, 128.4 (x2), 137.8 (Ph), 170.1, 170.8 (2*C*OMe), and 174.3 (*C*OOMe).

Anal. Calcd for $C_{27}H_{40}O_{10}$ (524.62): C, 61.82; H, 7.69. Found: C, 61.74; H, 7.45.

8-(Methoxycarbonyl)octyl 2,3-Di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-*O*-benzyl-α-D-mannopyranoside (14). A solution of **8** (0.94 g, 2.29 mmol) in distd dichloromethane (7 mL) was added dropwise with stirring, with exclusion of light, to a solution of **13** (0.60 g, 1.14 mmol), silver triflate (0.59 g, 2.30 mmol), and γ-collidine (0.33 mL, 2.50 mmol) in distd dichloromethane(3 mL) at -20 ℃ under argon. The mixture was allowed to attain room temperature and continued to stir overnight. After being diluted with dichloromethane, the mixture was filtered and the residue was washed with dichloromethane. The combined filtrate and washings were concentrated to afford a syrup which was chromatographed with chloroform-acetone (50:1-6:1) as eluent. Evaporation of the solvent gave **14** (0.71 g, 72.6%) as a syrup: $[\alpha]_D^{26}$+50.2° (*c* 0.93, $CHCl_3$); TLC (10:1 chloroform-acetone) Rf 0.60; IR (neat) 1748 and 1223 (ester), and 700 cm^{-1} (Ph); ^1H NMR ($CDCl_3$) δ 1.23-1.68 [m, 12H, $CH_2(CH_2)_6CH_2$], 1.98, 2.03, 2.05, 2.06, 2.10, 2.13 (6s, 18H, 6AcO), 2.31 (t, 2H, J=7.7 Hz, CH_2COOMe), 3.43, 3.69 (m, 2H, CH_2O), 3.67 (s, 3H, MeO), 3.76 (dd, 1H, $J_{5,6a}$=1.8 Hz, $J_{6a,6b}$=11.0 Hz, H-6a), 3.86 (dd, 1H, $J_{5,6b}$=4.8 Hz, H-6b), 3.89 (m, 1H, H-5), 3.93 (dd, 1H, $J_{5',6'a}$=2.4 Hz, $J_{6'a,6'b}$= 12.2 Hz, H-6'a), 3.98 (m, 1H, H-5'), 4.12 (dd, 1H, $H_{3,4}$=9.8 Hz, $H_{4,5}$=9.7 Hz, H-4), 4.15 (dd, 1H, $J_{5',6'b}$=4.2 Hz, H-6'b), 4.64 (s, 2H, $PhCH_2$), 4.77 (d, 1H, $J_{1,2}$=1.9 Hz, H-1), 5.06 (d, 1H, $J_{1',2'}$=2.5 Hz, H-1'), 5.14 (dd, 1H, $J_{2',3'}$=2.4 Hz, H-2'), 5.24 (dd, 1H, $J_{3',4}$=9.8 Hz, H-3'), 5.26 (2dd, 2H,$J_{2,3}$=3.7 Hz, $J_{4',5'}$=9.8 Hz, H-2, 4'), 5.31 (dd, 1H, H-3), and 7.30-7.40 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 20.6, 20.7 (x2), 20.8, 20.9 (x2) (6CO*Me*), 24.9, 26.0, 29.1 (x2), 29.2, 29.3 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.4 (MeO), 62.4 (C-6'), 65.9

(C-4'), 68.4 (CH_2O), 68.7 (C-3'), 69.0 (C-6), 69.5 (C-5'), 69.7 (C-2'), 70.0 (C-2), 70.8 (C-5), 71.6 (C-3), 73.5 (PhCH_2), 74.1 (C-4), 97.4 (C-1), 99.4 (C-1'), 127.5 (x2), 127.6, 128.4 (x2), 138.2 (Ph), 169.7 (x2), 169.9, 170.0, 170.1, 170.5 (6*C*OMe), and 174.3 (*C*OOMe).

Anal. Calcd for $C_{41}H_{58}O_{19}$ (854.91): C, 57.60; H, 6.84. Found: C, 57.52; H, 6.91.

8-(Methoxycarbonyl)octyl 2,3-Di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (15). A solution of **14** (300 mg , 0.35 mmol) in dry methanol (10 mL) was hydrogenated for 21 h in the presence of Pd catalyst,[18] freshly prepared from palladium chloride (0.20 g), at room temperature under atmospheric pressure. After the mixture was filtered, the filtrate was concentrated to afford a syrup which was chromatographed on a column of silica gel with chloroform-acetone (6:1) as eluent. Evaporation of the solvent gave **15** (243.7 mg, 90.8%) as a syrup: $[\alpha]_D^{26}$+56.8° (*c* 0.94, $CHCl_3$); TLC (6:1 chloroform-acetone) Rf 0.53; IR (neat) 3650-3200 (OH), and 1720 and 1220 cm^{-1} (ester); ^1H NMR ($CDCl_3$) δ 1.28-1.70 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.00, 2.05 (x2), 2.11, 2.13, 2.14 (5s, 18H, 6AcO), 2.31 (t, 2 H, J=7.6 Hz, CH_2COOMe), 3.41, 3.66 (m, 2H, CH_2O), 3.67 (s, 3H, MeO), 3.78 (m, 1H, H-5), 3.87 (m, 2H, H-6a, 6b), 4.08 (ddd, 1H, $J_{4',5'}$=9.8 Hz, $J_{5',6'a}$=2.5 Hz, $J_{5',6'b}$= 6.1 Hz, H-5'), 4.15 (dd, 1H, $J_{3,4}$=9.8 Hz, $J_{4,5}$= 9.7 Hz, H-4), 4.17 (dd, 1H, $J_{6'a,6'b}$= 12.2 Hz, H-6'a), 4.25 (dd, 1H, H-6'b), 4.74 (d, 1H, $J_{1,2}$=1.8 Hz, H-1), 5.11 (d, 1H, $J_{1',2'}$=2.4 Hz, H-1'), 5.12 (dd, 1H, $J_{2',3'}$=2.5 Hz, H-2'), 5.24 (dd, 1H, $J_{3',4'}$=9.7 Hz, H-4'), 5.26 (dd, 1H, $J_{2,3}$=3.1 Hz, H-2), 5.28 (dd, 1H, H-3'), and 5.31 (dd, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 20.6, 20.7 (x3), 20.8, 20.9 (6CO*Me*), 24.9, 26.0, 29.1 (x2), 29.2, 29.3 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.4 (MeO), 61.5 (C-6), 62.8 (C-6'), 66.3 (C-4'), 68.4 (CH_2O), 68.5 (C-3'), 69.7 (C-2'), 69.8 (C-5'), 70.0 (C-2), 71.1 (C-5), 71.7 (C-3), 73.1 (C-4), 97.6 (C-1), 99.3 (C-1'), 169.7 (x2), 169.8, 170.0 (x2), 170.7 (6*C*OMe), and 174.4 (*C*OOMe).

Anal. Calcd for $C_{34}H_{52}O_{19}$ (764.79): C, 53.40; H, 6.85. Found: C, 53.14; H, 7.06.

8-(Methoxycarbonyl)octyl 4-*O*-α-D-Mannopyranosyl-α-D-mannopyranoside (16). A mixture of **15** (171.5 mg, 0.22 mmol) and 0.5 M sodium methoxide (0.3 mL) in dry methanol (2 mL) was deacetylated as described for the preparation of **7**. Evaporation of the solvent gave a syrup which was dissolved in water, filtered, and lyophilized to give **16** (98.0 mg, 85.3%) as a hygroscopic amorphous mass: $[\alpha]_D^{22}$+86.3° (*c* 0.52, H_2O); TLC (1:1 chloroform-methanol) Rf 0.57; IR (KBr) 3550-3100 (OH), and 1720 and 1200 cm^{-1} (ester); ^1H NMR (D_2O) δ 1.20-1.66 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.33 (t, 2H, J=7.4 Hz, CH_2COOMe), 3.67 (s, 3H, MeO), 4.82 (s, 1H, H-1), and 5.27

(s, 1H, H-1'); ^{13}C NMR (D_2O) δ 25.4, 26.5, 29.5, 29.6, 29.7, 29.8 [$CH_2(CH_2)_6CH_2$], 34.6 (CH_2COOMe), 52.6 (MeO), 61.6, 61.7 (C-6, 6'), 67.3 (C-4'), 68.5 (CH_2O), 71.1, 71.3, 71.6, 72.0, 72.3 (C-2, 2', 3, 3', 5), 74.4, 74.5 (C-4, 5'), 100.6 (C-1), 102.3 (C-1'), and 176.5 (*C*OOMe).

Anal. Calcd for $C_{22}H_{40}O_{13} \cdot 1/2H_2O$ (521.57): C, 50.66; H, 7.92. Found: C, 50.85; H, 7.82.

8-(Hydrazinocarbonyl)octyl 4-*O*-α-D-Mannopyranosyl-α-D-mannopyranoside (1). A mixture of **16** (20.5 mg, 40 μmol) and hydrazine hydrate (0.18 mL, 3.71 mmol) in distd methanol (2 mL) was stirred overnight at room temperature. The solution was concentrated by repeated co-distillation with toluene to afford a syrup which was drychromatographed[20] on a column of silica gel with chloroform-methanol (3:1-2:1) as eluent. Evaporation of solvent afforded a syrup which was dissolved in water, filtered, and lyophilized to give **1** (19.9 mg, 97.1%) as a hygroscopic amorphous mass: $[\alpha]_D^{20}$ +79.7° (*c* 1.40, H_2O); TLC (7:5:3 2-propanol-ethyl acetate-water) Rf 0.50; IR (KBr) 3500-3100 (OH, NH, NH_2), and 1620 and 1525 cm^{-1} (amide); ^1H NMR (D_2O) δ 1.20-1.70 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.21 (t, 2H, J=7.4 Hz, $CH_2CONHNH_2$), 3.54, 3.72 (m, 2H, CH_2O), 4.86 (d, 1H, $J_{1,2}$=1.8 Hz, H-1), and 5.25 (d, 1H, $J_{1',2}$=1.9 Hz, H-1'); ^{13}C NMR (D_2O) δ 26.3, 26.5, 29.3, 29.5 (x2), 29.7 [$CH_2(CH_2)_6CH_2$], 34.9 ($CH_2CONHNH_2$), 62.1, 62.2 (C-6, 6'), 67.8 (C-4'), 69.1 (CH_2O), 71.5 (C-2'), 71.6 (C-3), 71.8 (C-2), 72.4 (x2) (C-5, 3'), 74.9 (C-5'), 75.4 (C-4), 100.8 (C-1), 102.7 (C-1'), and 177.2 ($CONHNH_2$).

Anal. Calcd for $C_{21}H_{40}N_2O_{12} \cdot 3/2H_2O$ (539.58); C, 46.75; H, 8.03; N, 5.19. Found: C, 46.86; H, 7.82; N, 5.00.

8-(Methoxycarbonyl)octyl 2,3,4-Tri-*O*-acetyl-6-*O*-trityl-α-D-mannopyranoside (17). To a solution of **10** (1.29 g, 3.64 mmol) in dry pyridine (80 mL) was added trityl chloride (1.29 g, 4.64 mmol) with exclusion of moisture. The mixture was stirred for 5 h at 95 ℃. After the mixture was cooled at 0 ℃, acetic anhydride (20 mL) was added under stirring and the mixture was left overnight at room temperature. The mixture was concentrated by repeated co-distillation with toluene to afford a syrup which was chromatographed on a column of silica gel with chloroform-acetone (50:1-20:1). Evaporation of the solvent gave **17** (2.04 g, 77.0%) as a syrup: $[\alpha]_D^{25}$+46.3° (*c* 1.02, $CHCl_3$); TLC (20:1 chloroform-acetone) Rf 0.65; IR (neat) 1752 and 1221 cm^{-1} (ester); ^1H NMR ($CDCl_3$) δ 1.28-1.70 [m, 12H, $CH_2(CH_2)_6CH_2$], 1.73, 1.96, 2.16 (3s, 9H, 3AcO), 2.29 (t, 2H, J=7.5 Hz, CH_2COOMe), 3.18 (m, 2H, H-6a, 6b), 3.50, 3.81 (m, 2H, CH_2O), 3.65 (s, 3H, MeO), 3.93 (ddd, 1H, $J_{4,5}$=9.9 Hz, $J_{5,6a}$=4.4 Hz, $J_{5,6b}$= 3.5 Hz, H-5), 4.84 (d, 1H, $J_{1,2}$=1.8 Hz, H-1), 5.23 (dd, 1H, $J_{2,3}$=3.4 Hz, H-2), 5.24 (t, 1H, $J_{3,4}$=9.9 Hz, H-4), 5.32 (dd, 1H, H-3), and 7.20 -7.50 (m, 15H, 3Ph); ^{13}C

NMR ($CDCl_3$) δ 20.6, 20.7, 21.0 (3CO*Me*), 24.9, 26.1, 29.1, 29.2 (x2), 29.3 [CH_2-(CH_2)$_6$$CH_2$], 34.1 (*C*$H_2$COOMe), 51.4 (MeO), 62.6 (C-6), 66.8 (C-4), 68.1 (CH_2O), 69.5 (C-3), 70.0 (C-2), 70.2 (C-5), 86.6 (*C*Ph_3), 97.2 (C-1), 127.0 (x3), 127.8 (x6) , 128.7 (x6), 143.8 (x3) (3Ph), 169.5, 170.0, 170.2 (3*C*OMe), and 174.3 (*C*OOMe).

Anal. Calcd for $C_{41}H_{50}O_{11}$ (718.85): C, 68.51; H, 7.01. Found: C, 68.26 ; H, 7.01.

8-(Methoxycarbonyl)octyl 2,3,4-Tri-*O*-acetyl-α-D-mannopyranoside (18). A solution of **17** (1.58 g, 2.20 mmol) in aqueous 70% acetic acid (60 mL) was heated at 70 ℃ for 1.5 h. The mixture was poured into ice-water, and the resulting triphenylcarbinol removed by filtration. The filtrate was extracted with dichloromethane and the combined extracts were washed with water, dried ($MgSO_4$) and concentrated to dryness to afford a syrup which was chromatographed on a column of silica gel with chloroform-acetone (20:1-6:1) as eluent. Evaporation of the solvent gave **18** (0.65 g, 61.9%) as a syrup: $[\alpha]_D^{20}$+48.5° (*c* 1.75, $CHCl_3$); TLC (10:1 chloroform-acetone) Rf 0.38; IR (neat) 3650-3250 (OH), and 1750 and 1225 cm^{-1} (ester); ^1H NMR ($CDCl_3$) δ 1.28-1.70 [m, 12H, CH_2(*CH*$_2$)$_6$$CH_2$], 2.01, 2.08, 2.15 (3s, 9H, 3AcO), 2.31 (t, 2H, J=7.6 Hz, *CH*$_2$COOMe), 3.43, 3.67 (m, 2H, CH_2O), 3.63, 3.69 (m, 2H, H-6a, 6b), 3.67 (s, 3H, MeO), 3.78 (ddd, 1H, $J_{4,5}$=9.8 Hz, $J_{5,6a}$=1.9 Hz, $J_{5,6b}$=4.3 Hz, H-5), 4.81 (d, 1H, $J_{1,2}$=1.8 Hz, H-1), 5.23 (dd, 1H, $J_{3,4}$=9.8 Hz, H-4), 5.24 (dd, 1H, $J_{2,3}$= 3.6 Hz, H-2), and 5.40 (dd, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 20.7, 20.8, 20.9 (3CO*Me*), 24.9, 26.0, 29.1 (x2), 29.2, 29.3 [CH_2(*CH*$_2$)$_6$$CH_2$], 34.1 (*CH*$_2$COOMe), 51.5 (MeO), 61.3 (C-6), 66.6 (C-4), 68.4 (CH_2O), 69.0 (C-3), 69.8 (C-2), 70.6 (C-5), 97.6 (C-1), 169.9, 170.2, 170.9 (3*C*OMe), and 174.3 (*C*OOMe).

Anal. Calcd for $C_{22}H_{36}O_{11}$ · 1/2H_2O (485.54): C, 54.42; H, 7.68. Found: C, 54.60; H, 7.38.

8-(Methoxycarbonyl)octyl 2,3,4-Tri-*O*-acetyl-6-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (19). To a solution of **18** (0.19 g, 0.40 mmol), silver triflate (0.21 g, 0.82 mmol), and γ-collidine (0.12 mL, 0.91 mmol) in distd dichloromethane (3 mL) was added a solution of **8** (0.33 g, 0.80 mmol) in distd dichloromethane (7 mL) with stirring, with exclusion of light, at -20 ℃ under argon. The reaction mixture was allowed to attain room temperature and continued to stir overnight. It was treated as described for the preparation of **14** to afford a syrup which was chromatographed on a column silica gel with benzene-diethyl ether-methanol (10:10:1) as eluent. Evaporation of the solvent gave **19** (0.17 g, 52.9%) as a syrup: $[\alpha]_D^{27}$+40.2° (*c* 1.45, $CHCl_3$); TLC (10:1 chloroform-acetone) Rf 0.58; IR (neat) 1752 and 1224 cm^{-1} (ester); ^1H NMR ($CDCl_3$) δ 1.30-1.70 [m, 12H, CH_2(*CH*$_2$)$_6$$CH_2$], 1.98, 2.00, 2.05, 2.06, 2.11, 2.15, 2.16 (7s, 21H, 7AcO), 2.31 (t, 2H, J=7.3 Hz, *CH*$_2$COO-

Me), 3.44, 3.70 (m, 2H, CH_2O), 3.57 (dd, 1H, $J_{5,6a}$=2.4 Hz, $J_{6a,6b}$ =11.0 Hz, H-6a), 3.67 (s, 3H, MeO), 3.77 (dd, 1H, $J_{5,6b}$=6.1 Hz, H-6b), 3.94 (ddd, 1H, $J_{4,5}$=9.8 Hz, H-5), 4.08 (ddd, 1H, $J_{4',5'}$=9.8 Hz, $J_{5',6'a}$=2.5 Hz, $J_{5',6'b}$=4.9 Hz, H-5'), 4.13 (dd, 1H, $J_{6'a,6'b}$=12.2 Hz, H-6'a), 4.25 (dd, 1H, H-6'b), 4.77 (d, 1H, $J_{1,2}$=1.9 Hz, H-1), 4.86 (d, 1H, $J_{1',2'}$=1.8 Hz, H-1'), 5.23 (dd, 1H, $J_{2,3}$=3.0 Hz, H-2), 5.25 (dd, 1H, $J_{3,4}$=9.8 Hz, H-4), 5.28 (2dd, 2H, $J_{2',3'}$=3.7 Hz, $J_{3',4'}$=9.8 Hz, H-2', 4'), 5.34 (dd, 1H, H-3'), and 5.35 (dd, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 20.6, 20.7 (x2), 20.8 (x3), 20.9 (7CO-*Me*), 24.9, 26.0, 29.1, 29.2 (x2), 29.3 [$CH_2(CH_2)_6CH_2$], 34.1 (CH_2COOMe), 51.4 (MeO), 62.4 (C-6'), 66.1 (C-4'), 66.6 (C-6), 66.7 (C-4), 68.4 (CH_2O), 68.6 (C-5'), 68.9 (C-3'), 69.2 (C-3), 69.3 (C-5), 69.4 (C-2'), 69.7 (C-2), 97.3 (C-1), 97.5 (C-1'), 169.6, 169.8, 169.9 (x2), 170.0, 170.2, 170.6 (7*C*OMe), and 174.3 (*C*OOMe).

Anal. Calcd for $C_{36}H_{54}O_{20}$ (806.82): C, 53.59; H, 6.75. Found: C, 53.37; H, 6.98.

8-(Methoxycarbonyl)octyl 6-*O*-α-D-Mannopyranosyl-α-D-mannopyranoside (20). A mixture of **19** (60 mg, 0.07 mmol) and 0.5 M sodium methoxide (0.1 mL) in dry methanol (1 mL) was deacylated as described for the preparation of **7**. Evaporation of the solvent gave a syrup which was dissolved in water, filtered, and lyophlized to give **20** (40.2 mg, 97.6%) as a hygroscopic amorphous mass: $[\alpha]_D^{25}$+41.6° (*c* 1.01, H_2O) [ref. 14, $[\alpha]_D^{24}$+55.1° (*c* 0.8, MeOH)]; TLC (1:1 chloroform-methanol) Rf 0.58; IR (KBr) 3550-3050 (OH), and 1720 and 1205 cm^{-1} (ester); ^1H NMR (D_2O) δ 1.20-1.66 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.35 (t, 2H, J=7.4 Hz, CH_2COOMe), 3.65 (s, 3H, MeO), 4.81 (s, 1H, H-1), and 4.86 (s, 1H, H-1'); ^{13}C NMR (D_2O) δ 25.4, 26.5, 29.6 (x2), 29.8 (x2) [$CH_2(CH_2)_6CH_2$], 34.5 (CH_2COOMe), 52.4 (MeO), 61.7 (C-6'), 66.2 (C-6), 67.2 (C-4), 67.4 (C4'), 68.5 (CH_2O), 70.8, 71.1 (C-2, 2'), 71.5, 71.8, 71.9 (C-3, 3', 5), 73.4 (C-5'), 100.4 (C-1'), 100.8 (C-1), and 176.4 (*C*OOMe).

8-(Hydrazinocarbonyl)octyl 6-*O*-α-D-Mannopyranosyl-α-D-mannopyranoside (2). A mixture of **20** (98.4 mg, 0.19 mmol) and hydrazine hydrate (0.90 mL, 18.5 mmol) in distd methanol (10 mL) was stirred overnight at room temperature. It was treated as described for the preparation of **1**. After lyophilization, **2** (91.5 mg, 93.0 %) was obtained as a hygroscopic amorphous mass: $[\alpha]_D^{23}$+96.1° (*c* 0.17, H_2O); TLC (7:5:2 2-propanol-ethyl acetate-water) Rf 0.50; IR (KBr) 3500-3100 (OH, NH, NH_2), and 1625 and 1530 cm^{-1} (amide); ^1H NMR (D_2O) δ 1.20-1.70 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.21 (t, 2H, J=7.3 Hz, $CH_2CONHNH_2$), 3.56, 3.72 (m, 2H, CH_2O), 4.85 (s, 1H, H-1), and 4.90 (d, 1H, $J_{1',2'}$=1.2 Hz, H-1'); ^{13}C NMR (D_2O) δ 26.3, 26.5, 29.3, 29.4, 29.5, 29.7 [$CH_2(CH_2)_6CH_2$], 34.9 ($CH_2CONHNH_2$), 62.1 (C-6'), 66.9 (C-6), 67.8 (C-4), 68.0 (C-4'), 69.2 (CH_2O), 71.2 (C-2'), 71.3 (C-2), 71.8 (C-3'), 72.1 (x2) (C-3, 5), 73.9 (C-5'), 100.7 (C-1'), 101.1 (C-1), and 177.2 ($CONHNH_2$).

Anal. Calcd for $C_{21}H_{40}N_2O_{11} \cdot 3/2H_2O$ (539.58): C, 46.75; H, 8.03; N, 5.19. Found: C, 47.07; H, 7.94; N, 5.14.

Coupling of 1 or 2 with BSA - Compound **1** (19.9 mg, 38.8 μmol) was dissolved in water (0.5 mL) and the solution chilled on ice. To the chilled solution, cold 4 M HCl (70 μL) and sodium nitrite (8.7 mg) were added. After the solution was kept at room temperature for 15 min, solid ammonium sulfamate (14.4 mg) was added and the mixture was kept at room temperature for 15 min in order to destroy excess HNO_2. This mixture (containing the acyl azide) was added to ice-cooled 0.4 M sodium borate buffer (pH 10.0) containing 50 mg of BSA (1 mL). The pH was quickly adjusted to 9.0-9.5 with 4 N NaOH solution with stirring. After the reaction mixture was stirred for 60 min at room temperature, it was neutralized with 0.1 M acetic acid. The mixture was dialyzed against distd water for 4 days and lyophilized to provide an amorphous powder which was purified by ion exchange column chromatography with DE-52 (adsorption buffer: 0.02 M sodium phosphate buffer, pH 6.0. elution buffer: 1 M sodium chloride/ 0.02 M sodium phosphate buffer, pH 6.0). The fractions were monitored with the phenol-sulfuric acid method[21] and absorbance at 280 nm. The positive fractions were collected, deionized with amicon ultrafiltration, and lyophilized to give Manα(1-4)Man-BSA (41.6 mg) as a hygroscopic amorphous powder.

Similarly, compound **2** (102.9 mg, 201 μmol) in water (2 mL) was transformed to the acyl azide derivative with 4 M HCl (0.28 mL), sodium nitrite (34.8 mg), and ammonium sulfamate (57.7 mg), coupled with 0.4 M sodium borate buffer (pH 10.0) containing 70 mg of BSA (1 mL), to give Manα(1-6)Man-BSA (70.4 mg) as a hygroscopic amorphous powder after column chromatography with DE-52.

Preparation of rabbit IgG against glycosylated BSA - Each glycosylated BSA (1 mg/mL in saline) was mixed with equal volume of Freund Complete Adjuvant and 400 μL of the solution were subcutaneously injected into female Japanese white rabbits (body weight 1.5-2.5 kg, Japan Biosupply, Tokyo, Japan). The rabbits were booster injected with the same antigen two times every 3 weeks. One week after the final injection antiserum was obtained, and IgG was purified by the use of Protein A-Sepharose column (Amersham, Buckinghamshire, U.K.).

ELISA - Antigens, glycosylated BSA, were dissolved in 0.05 M Na_2CO_3-$NaHCO_3$ buffer (pH 9.6). 100 μL of the antigen solutions were added to each well of 96-well ELISA plate (Sumitomo Bakelite, Tokyo, Japan), and then incubated overnight at 4 ℃. After washing the plate three times with phosphate buffered saline pH 7.4 (PBS), 200 μL of 1% human serum albumin (HSA) in PBS were added, and further incubated for 1 h at room temperature. After washing the plate three times with 0.05% Tween 20 in PBS, rabbit IgG against glycosylated BSA, 1000 fold diluted with 0.1% BSA in Tween 20/PBS, were added and incubated for 1 h at room temperature. The plate was washed

three times with Tween 20/PBS, and then peroxidase-conjugated goat IgG against rabbit IgG (Bio-Rad Laboratories, Richmond, CA) were added and incubated for 1 h at room temperature. After finally washing the plate three times with Tween 20/PBS, 100 μL of substrate solution (1.1 mg/mL *o*-phenylenediamine and 0.01% H_2O_2 in 0.025 M citric acid, 0.05 M Na_2HPO_4 buffer pH 5.0) were added. After incubation for 10-30 min at room temperature, 10 μL of stop solution, 8 N H_2SO_4, were added and the plate was read at 490 nm with an automatic plate reader (Bio-Rad Laboratories, Richmond, CA).

Antigen-specific proliferation assays - Heparinized peripheral blood was centrifuged for 5 min at 200 g and the buffy coat was collected. Mononuclear cells were purified from the buffy coat by standard procedures using lymphocyte separation medium. Mononuclear cells were washed twice with Hanks balanced solution (Nissui Pharmaceutical Co., Tokyo, Japan) and then $2x10^5$ cells in 100 μL RPMI1640 (Sigma Chemical Co.,St.Louis, MO) containing 100 U/mL of Penicillin G, 100 μg/mL of Streptomycin, 15 mM HEPES and 10% autologous plasma were added to flatbottomed, 96-well tissue culture plates (Falcon, Lincoln, NJ). 50 μL of medium containing mannose dimer or BSA were added to the well to give indicated final concentration. Finally 50 μL of PPD solution (50 μg/mL final concentration) or tissue culture medium were added and the plates were allowed to incubate in humidified 5% CO_2 incubator at 37 ℃ for 6 days. These cells were pulsed with 0.5 μCi [^{3}H]thymidine (2 Ci/mM, New England Nuclear, Boston, MA), allowed to incubate for a further 4.5 h, and then harvested on filter paper disks and counted for [^{3}H]thymidine incorporation.

IL-2-dependent cell line proliferation - IL-2-dependent murine NK cell line NK3 was provided by Dr. Kumagai of Tohoku University (Sendai, Japan). The cells were maintained in RPMI1640 containing antibiotics, HEPES, 5% heat-inactivated FBS (Bocknek, Toronto, Canada) and human recombinant IL-2 (100 U/mL, Shionogi Co., Osaka, Japan). For the assay for proliferation, 50 μL of NK3 cell suspension ($2x10^5$ cells/mL) in culture medium containing IL-2 (200 U/mL) and equal volume of medium containing test reagents were added to each flatbottomed well of 96-well microtiter plate, and cultured at 37 ℃ in 5% CO_2 in air for 3 days. After culture, cell proliferation was assessed by MTT method.[22] After solubilizing the formazan with 20% SDS, 50% dimethylformamide in water, the absorbance at 595 nm was measured by an ELISA autoreader.

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