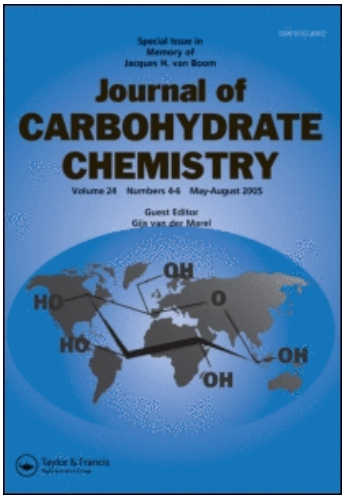

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Mannose-BSA Conjugates: Comparison Between Commercially Available Linkers in Reactivity and Bioactivity

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Mannose-BSA Conjugates: Comparison Between Commercially Available Linkers in Reactivity and Bioactivity

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ABSTRACT

Mannosyl ethanolamine and BSA were conjugated together by their amino groups with various homobifunctional cross-linker reagents: disuccinimidyl carbonate (**DSC**), disuccinimidyl glutarate (**DSG**), disuccinimidyl suberate (**DSS**), ethylene glycolbis-(succinimidylsuccinate) (**EGS**), 1,5-difluoro-2,4-dinitrobenzene (**DFDNB**), diethyl squarate (**DES**), and thiophosgene (**TP**). The resulting mannose-BSA conjugates were subjected to an enzyme-linked lectin assay (ELLA) to investigate their affinity to concanvalin A (ConA). With these results, the seven linkers were evaluated on the basis of five criteria, i.e., cost, reactivity, sugar loading, homogeneity, and affinity to ConA. Thus, **DSS**, **DFDNB**, and **DES** seemed to have advantages over the other cross-linking reagents.

Key Words: Neoglycoprotein; Linker; Mannose-BSA conjugate; MALDI-TOF MS; Concanavalin A.

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INTRODUCTION

Bioactive forms of carbohydrates are provided sometimes as neoglycoproteins, the proteins carrying carbohydrate pendants.[1–3] Neoglycoproteins are needed to elicit antibodies against the carbohydrate haptens, and thus several anticancer vaccines have been synthesized by linking tumor associated carbohydrate antigens to a carrier protein, e.g., keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), or tetanus toxoid.[4–6] Neoglycoproteins are also used to exert a cluster effect on carbohydrate-receptor recognition events, as illustrated by a strong binding affinity of a galactose-BSA conjugate toward a hepatic lectin.[1]

In an effort to design new neoglycoproteins, it is always frustrating to select the best cross-linking reagent. The efficiency of the cross-linking, the homogeneity in the number of the linked carbohydrates per protein, and the influence of the linker on the bioactivity, in addition to the cost of the cross-linker reagents and the steps and tasks required for the cross-linking, would be the major concerns for most researchers. However, there is little comprehensive information about these properties of the reagents and hence some researchers might be caught up in the indecision on selecting one, as was the case for us in the course of our own synthetic studies of thiosugar-linked neoglycoproteins. We thus decided to address this issue by examining several linkers including those that had not been used for neoglycoproteins.

There are almost infinite combinations of carbohydrates, linkers, and proteins available for preparing neoglycoproteins. In addition, we need to select the functional groups by which a protein and a carbohydrate are combined together through a linker. We focused on the amino functional groups of the 59 lysine residues in BSA,[7] the richest functional groups among available functional groups in this least expensive protein. Homobifunctional cross-linkers, including the ones that had not been used for the neoglycoprotein syntheses, were selected, because of their simple linking methods, requiring minimal synthetic steps and operational tasks, and their high commercial availability.[8] Thus, disuccinimidyl carbonate (**DSC**), disuccinimidyl glutarate (**DSG**), disuccinimidyl suberate (**DSS**), ethylene glycol bis(succinimidylsuccinate) (**EGS**), 1,5-difluoro-2,4-dinitrobenzene (**DFDNB**), diethyl squarate (**DES**), thiophosgene (**TP**) were of choice as linking reagents (Figure 1). Among these reagents, only **DES** has been frequently used for neoglycoprotein syntheses,[9–16] while a few examples have been reported for **TP**[17,18] and **DSS**.[19] The amino function was accordingly added to mannose as 2-aminoethyl glycoside, in order to link it to BSA. Mannose was selected as the sugar moiety, since the cluster effect can be assessed by use of concanavalin A (ConA),[20,21] the least expensive and most thoroughly studied carbohydrate-binding lectin. With these kinds of materials in hand, we compared the efficiency of carbohydrate loading on BSA by MALDI-TOF mass spectroscopy, and studied the affinity of these mannose-BSA conjugates (MBC) toward ConA by enzyme-linked lectin assay (ELLA).

RESULTS AND DISCUSSION

The Preparation of Mannosyl-linker Intermediates

A drawback in the use of homobifunctional linkers for the sugar-protein conjugation is a possibility of linking between the same species to provide sugar-

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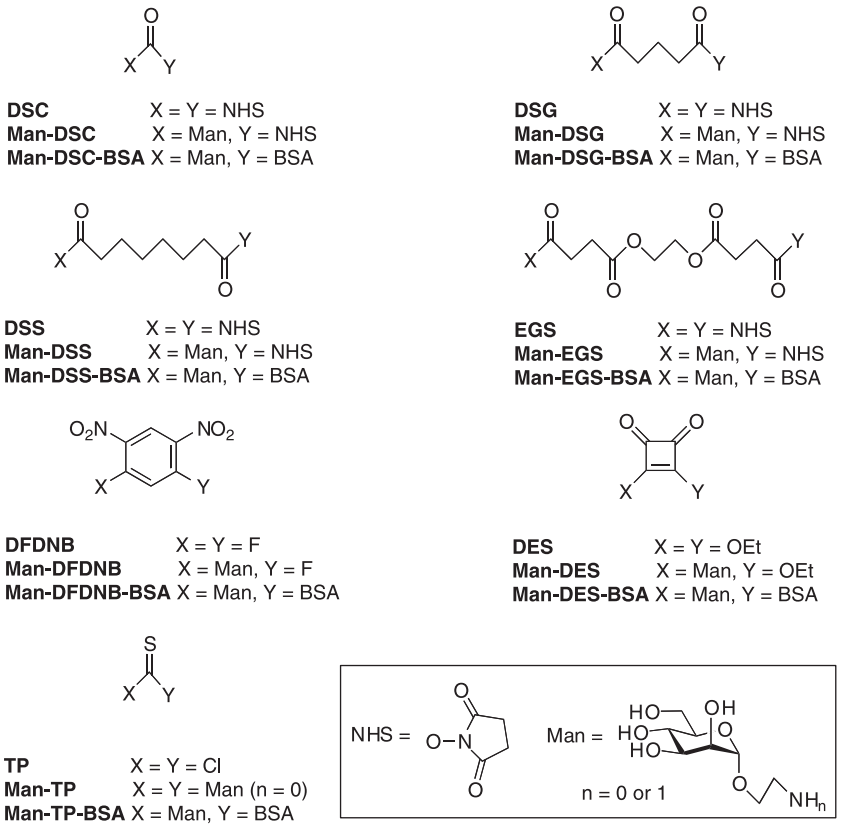

Figure 1. The structures of the seven linkers used in this study and their derivatives.

sugar and/or protein-protein conjugates. However, it has been well established that two-step conjugation methods would enable an effective heteroconjugation even with the homobifunctional linkers to afford sufficient amounts of sugar-protein conjugates.[8] We thus isolated the mannosyl-linker intermediates prepared by coupling between the homobifunctional linker reagents and the 2-aminoethyl α-D-mannopyranoside (**Man**) as shown in Table 1, prior to coupling with BSA. Moderate yields of **Man-DSG**, **-DSS**, and **-EGS** were due to the hydrolysis of NHS ester. Excess amount of **DSG**, **DSS**, and **EGS** were used because reactions with less than 5 equivalents gave bis-mannose conjugates as side products. Although these linkers were expensive, using excess reagents was not a problem since unreacted reagents could be precipitated by adding water to the reaction and recovered easily by filtration. Since the isothiocyanate intermediate produced with the linker reagent **TP** was too labile to isolate, the one-pot procedure was employed for the **TP**-linker coupling. Namely, to a stirred buffer solution of **Man** was added a chloroform solution of **TP**, the chloroform layer was separated after completion of the reaction, and BSA was added to the aqueous layer.

Table 1. The synthesis of the mannosyl-linker intermediates.

Reagent	Amount (equiv)	Additive and solvent	Product	Yield (%)
DSC	1.0	Et_3N/DMF	**Man-DSC**	83
DSG	5.0	DMSO	**Man-DSG**	69
DSS	5.0	DMSO	**Man-DSS**	69
EGS	10.0	DMSO	**Man-EGS**	63
DFDNB	2.0	DMSO/$KHCO_3$-$Na_2B_4O_7$ buffer (pH 9.0)	**Man-DFDNB**	91
DES	1.3	EtOH/0.1 M phosphate buffer (pH 7.0)	**Man-DES**	67

The Synthesis of Mannose-BSA Conjugates (MBC)

The purified mannosyl-linker intermediates were coupled to BSA and the molecular weights of the coupled products were determined by MALDI-TOF mass spectroscopy. The typical mass spectra for BSA and a mannose-BSA conjuagate were shown in Figure 2. The broadened peak for the conjugate compared to that of a bare BSA suggests the heterogeneous distribution of the sugar loadings and the half width would indicate the extent of distribution. The sugar loadings were calculated from the mass increment based on the bare BSA.

Although the reactions with most of the *N*-succinimide intermediates completed within 5 h and with **Man-DSC** in 32 h, those with **Man-DSC**, **-DFDNB**, **-DES**, and **-TP** required longer periods to level off as shown in Figure 2. Up to ca 60 sugars were loaded per BSA molecule with more than 240 equivalents each of **Man-DSS**, **-DSG**, or **-DFDNB** (Figure 3). While the maximum loadings of 60 sugars appears to be consistent with the 59 lysine residues and one *N*-termini of BSA, the broadened peaks derived from heterogeneity in the sugar loadings suggest the presence of neoglycoproteins with more than 60 sugars, and as such the reacting species other than the amino group. These may include only one thiol group from Cys, the 19 phenolate groups from Tyr, and the 17 imidazole rings from His. The varied half values over different linkers can be explained by different reactivities of the cross-linker reagents toward these versatile functional groups. The yield with 60 equivalents of **Man-DSS**, **-DSG**, or **-DFDNB** was satisfactory in that half of them were loaded on BSA. With **DFDNB** linker, the homogeneity was lowest among the tested linkers, as indicated by the relatively large half values in MALDI spectra. The yields with **DES** and **EGS** linkers were acceptable, but the saturated loadings of 60 sugars were not achieved within the examined conditions.[a] The short linkers **TP** and **DSC** were impractical, allowing for only a small amount of sugars on BSA, probably due to a steric clash between the protein and the mannose residue.

[a]Maximum loading, however, is not always important for inducing immune response by neoglycoproteins.[22]

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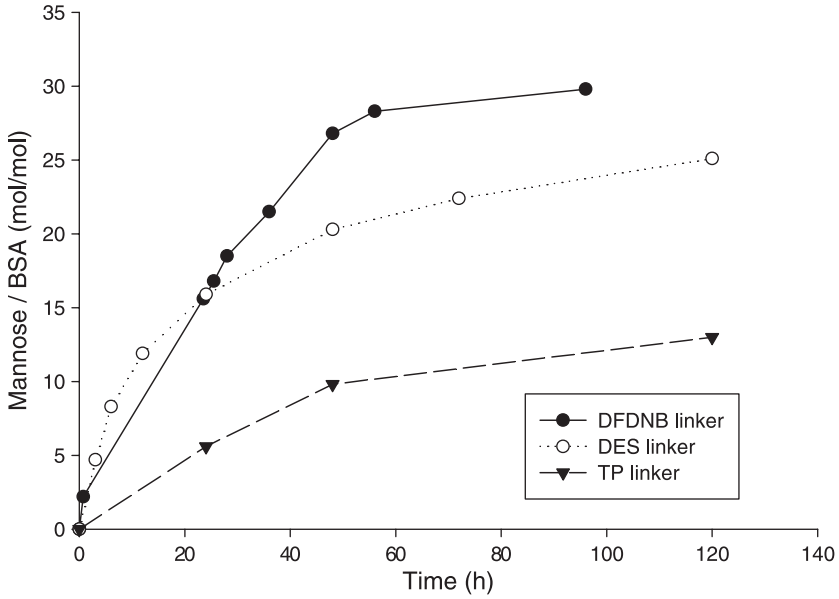

Figure 2. Time course of the coupling between mannose and BSA via three types of linkers. All the reactions were run in 0.1 M $KHCO_3/Na_2B_4O_7$ buffer (pH 9.0) with 60 equivalents of the linker reagents. For other linkers examined, DSS, DSG, and EGS linkers were consumed in 5 h, and DSC linker was consumed in 32 h.

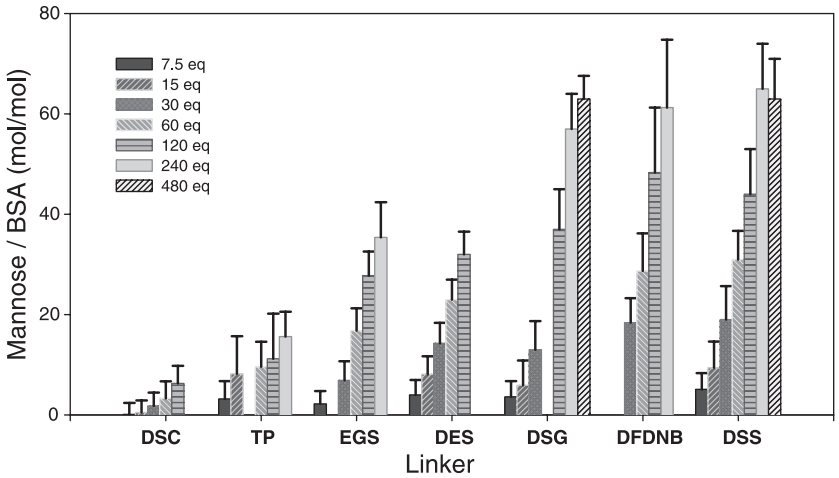

Figure 3. Relationship between the amounts of the seven linker reagents and of the mannose loaded on BSA. All the reactions were run in 0.1 M $KHCO_3/Na_2B_4O_7$ buffer (pH 9.0) for 5 h (EGS, DSG, DSS), 32 h (DSC), 48 h (TP), 120 h (DES), and 96 h (DFDNB). The error bars stand for the half values of the observed peaks for mannose-BSA conjugates in MALDI-TOF mass spectra.

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The effects of pH (from 7 to 11) and buffer components on the sugar loading were examined for **DSS** and **DES** (Figure 5).[b] To examine the effect of pH, all reactions were run in 0.1M $KHCO_3$-$Na_2B_4O_7$ for 16 h (**DSS**) and 67 h (**DES**) with 60 equivalents of the linker reagents. **DSS** afforded almost constant sugar loadings between 25 and 30 sugars per BSA (mol/mol) over the pH range between 8 and 11 within the buffer systems examined, though there was a slight increase from 25 to 30 (mol/mol) at pH 7 to 8. On the other hand, **DES** showed variable results. In the neutral conditions, **DES** scarcely gave the linked products as has been usually observed.[9] However, as pH was raised, the sugar loadings gradually increased to 6.4 (mol/mol) at pH 9, 7.5 at pH 10, and 12 at pH 11. This increase of the sugar loadings with pH is consistent with what has been observed for the coupling of Le^b-tetrasaccharide to human serum albumin (sugar loadings of 6 at pH 9 and 8 at pH 10).[15] Moreover, the buffer systems significantly influenced the number of the **Man-DES** coupled to BSA (Figure 4). We examined four buffer systems each comprising two components from carbonates, phosphates, and a borate, and the sugar loadings changed dramatically from 7 (mol/mol) with the phosphate buffer to 23 with the carbonate-borate buffer. On the other hand, **Man-DSS** was unaffected by the used buffer systems, constantly giving ca 27 sugar loadings.

The Binding Ability of MBC to ConA

An MBC linked by a guanylmethylthio linker [Man-S-CH_2C(=NH)-NH-BSA] has been tested by Lee and coworkers[23,24] for binding ability to mammalian mannose-binding proteins by measuring IC_{50} against a reference radio-labeled MBC. As a result, it has been demonstrated that the affinity increased, down to a nanomolar scale in IC_{50}, with an increasing number of the sugar loading and reached plateau at 20 (mol/mol). However, the effect of the linker structure on the affinity has not been examined. This kind of information is very important for future developments of sugar-based vaccines and sugar clusters. Thus we selected four linkers, **DSS**, **DSG**, **DES**, and **DFDNB**, to be tested for the affinity of MBC to ConA. The assay was performed by ELLA, the enzyme-linked lectin assay, in which the inhibition by MBC of the binding between the horseradish peroxidase (HRP)-labeled ConA and the yeast mannan coated on a microtiter plate was evaluated by monitoring the absorption at 415 nm derived from the oxidation of 2,2′-azino diethylbenzothiazolinesulfonic acid (ABTS). As shown in Figure 5, MBC with **DSS** linker showed the greatest affinity to ConA over the whole range of the tested sugar-loadings, those with **DSG** and **DFDNB** the middle, and that with **DES** the lowest. These results suggest that the longer and less constrained linkers are appropriate for constructing MBC with a stronger affinity for ConA.

Usefulness of the Linkers

Recently, **DES** has become widely used for the syntheses of neoglycoproteins, and its superiority to other cross-linker reagents has been pointed out, such as the mild

[b]The effect of the concentration of sugar-linker on the sugar loading using DES has been reported.[13]

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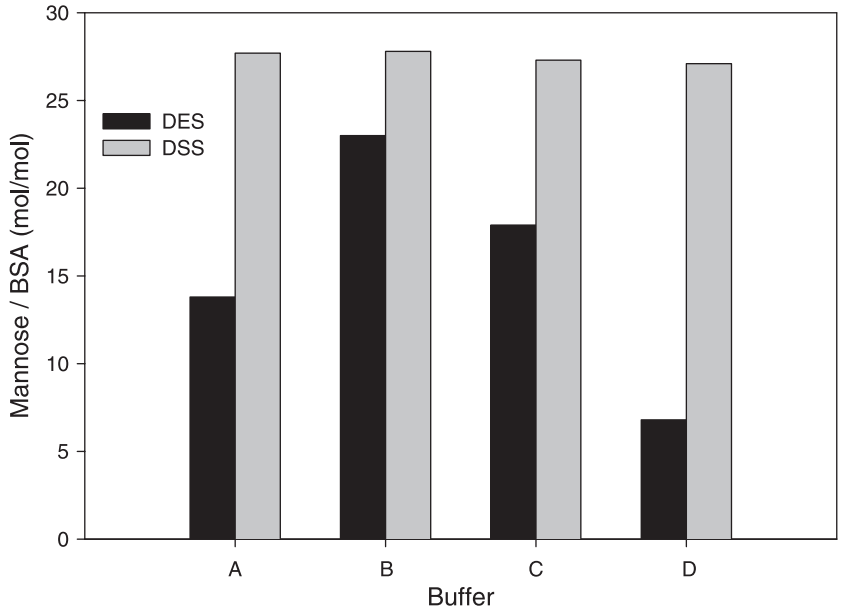

Figure 4. The effect of the buffer components on the sugar-loadings in the coupling reaction between mannose and BSA. All the reactions were run in the specified buffer (pH 9.0) for 60 h (DES) and 16 h (DSS) with 60 equivalents of the linker reagents. Buffers: A, $NaHCO_3$-Na_2CO_3; B, $KHCO_3$-$Na_2B_4O_7$; C, KH_2PO_4-$Na_2B_4O_7$; D, KH_2PO_4-Na_3PO_4.

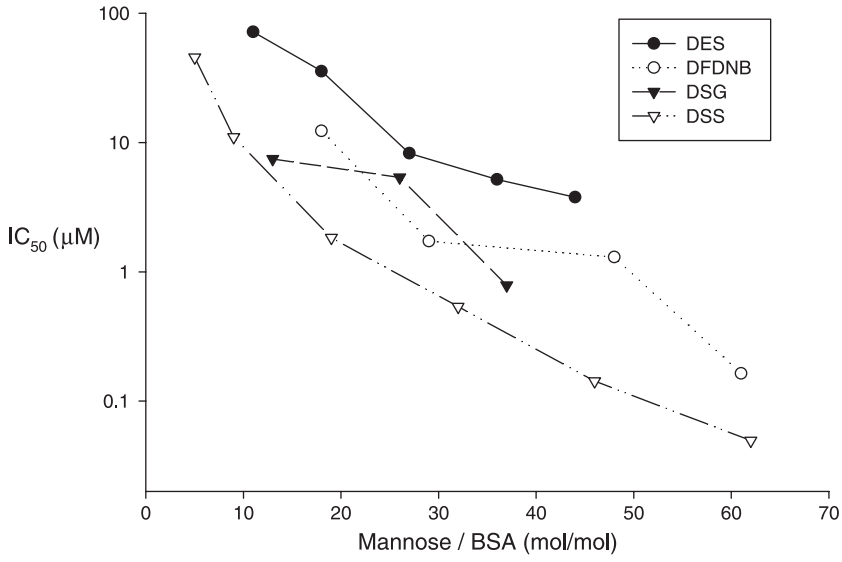

Figure 5. The relationship between the sugar loading of the mannose-BSA conjugates with four types of linkers (DES, DFDNB, DSG, and DSS) and their inhibition against the mannan-binding to ConA in IC_{50} (μM).

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reaction conditions and the recovery of the reagent with ease.[16] However, as we demonstrated in this study, some other cross-linker reagents, including those never examined for neoglycoprotein syntheses, are comparable to **DES**. Here we summarize our evaluation of the seven mannosyl-BSA linking reagents as follows: 1) **TP** and **DSC** are impractical, permitting only insignificant amounts of sugar loadings, though they may be used in case a small loading is needed. 2) Disuccinimidyl reagents (**DSS**, **DSG**, **EGS**) are useful since they usually give high sugar loadings in relatively short periods and are probably easy to handle as illustrated by the insensitivity of **DSS** to the reaction conditions, only their high prices are a problem (ca \$140/g for **DSS** and **EGS**; ca \$1000!/g for **DSG** at this point). 3) **DES** and **DFDNB** are inexpensive compared to the disuccinimidyl reagents and characteristic in their highest and lowest homogeneities, respectively, among the tested reagents, as shown in their half values of the sugar loadings. 4) The cross-linker having long spacer arms such as **DSS** could have beneficial effects for the cluster-binding of neoglycoproteins to carbohydrate-recognizing proteins.

The information gained in this study should be helpful in considering the type of linkers that may be used to prepare new vaccines and sugar clusters, whatever the sugars and proteins are. Among the tested linkers, **DFDNB** was used to link a sugar and a protein for the first time, to the best of our knowledge, and gave excellent results. Although **DFDNB** was inferior to **DSS** in regard to its reactivity, homogeneity, and affinity to ConA, it can be a good alternative because of the lower price of the reagent. It should also be mentioned for **DFDNB** that, compared to **DES**, the effect of this linker to immune response is unknown, an important factor in preparing vaccines. The differences might be trivial as compared with the superiority in the price of the reagent.

EXPERIMENTAL

Materials. Bovine serum albumin (fraction V) was purchased from Sigma and was used without purification. DSS, DSG, and EGS were purchased from Pierce, DSC and TP were from Aldrich, and DFDNB was from Acros Organics. DES was prepared from 3,4-dihydroxy-3-cyclobutene-1,2-dion (Tokyo Kasei Kogyo) accroding to Ohno's method.[25] 2-Aminoethyl α-D-mannopyranoside was prepared according to Lindhorst's method.[26] Concanavalin A labeled with horse radish peroxidase (L-6397) was purchased from Sigma.

General methods. Reactions were monitored by TLC was performed on Kieselgel 60 F_{254} (Merck) with detection by UV light, then charring with either 10% H_2SO_4 in MeOH or 1% cerium sulfate and 1.5% ammonium molybdate in 10% H_2SO_4. For flash chromatography, Merck silica gel 60 or Wako C300 was used. For the reverse phase separation of compounds, Sep-Pak C18 cartridge (Waters) and/or octadecyl silica gel (ODS; Tokyo Kasei Kogyo) was used. Each fraction was checked by normal phase TLC with detection methods mentioned above. For neoglycoprotein purifications, Econo-Pac 10DG columns (Bio-Rad) were used. The concentration determination for the neoglycoproteins was performed with BCA protein assay kit (Pierce), which was checked by absorbance at 280 nm using a cell length of 1 cm with a UV mini-1240

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spectrometer (Shimadzu), when it was appropriate. Optical rotations were measured on a JASCO DIP-4 polarimeter (0.5 dm cell length). Mass spectra were measured on a Mariner ESI-TOF (PerSeptive) or a Voyager-DE MALDI-TOF (PerSeptive). For the MALDI measurements, 3,5-dimethoxy-4-hydroxycinnamic acid was used as a matrix. NMR spectra were measured on a JEOL JNM-EX270 (270 MHz for ^{1}H and 67.8 MHz for ^{13}C NMR). Chemical shifts are in ppm, relative to internal $SiMe_4$ (0 ppm) in $CDCl_3$ or internal CH_3OD (3.35 for ^{1}H and 49.3 for ^{13}C NMR) in D_2O. *J*-Values are in Hz. The enzyme linked lectin assay was carried out using the method of Pagé and coworkers.[20] Optical absorbance at 405 nm in ELLA was measured on a Model 550 Microplate Reader (Bio-Rad).

2′-*N*-[6-(Succinimidooxycarbonyl)heptan-1-oyl]-aminoethyl α-D-mannopyranoside (Man-DSS). To a stirred solution of disuccinimidyl suberate (**DSS**; 165 mg, 433 μmol) in DMSO (1.1 mL), was added dropwise a solution of 2-aminoethyl α-D-mannopyranoside (**Man**; 9.4 mg, 42.2 μmol) in DMSO (0.2 mL) at room temperature. After 20 min, TLC analysis showed that all the **Man** was consumed. To the solution was added ice-cold water (10 mL), and the resulting white precipitates (**DSS**) were removed by suction filtration. The filtrate was chromatographed on a column of ODS with MeOH/H_2O (0, 10, 20, 30, 40, 50%; each 10 mL) as a stepwise gradient. The fraction of 30% MeOH was concentrated to give **Man-DSS** (13.9 mg, 69%) as a syrup. For analytical purpose, **Man-DSS** was further purified by flash chromatography ($CHCl_3$-MeOH = 4:1). R_f = 0.63 ($CHCl_3$-MeOH-H_2O = 6:4:1); $[\alpha]_D^{23}$ + 29 (*c* 0.69, MeOH); ^{1}H NMR (CD_3OD) δ 4.75 (d, 1H, *J* = 1.7 Hz, H-1″), 3.85–3.33 (m, 10H), 2.82 (s, 4H), 2.62 (t, 2H, *J* = 7.4 Hz), 2.20 (t, 2H, *J* = 7.4 Hz), 1.78–1.57 (m, 4H), 1.48–1.33 (m, 4H); ^{13}C NMR (CD_3OD) δ 176.1, 171.7, 170.1, 101.6, 74.7, 72.5, 72.0, 68.5, 67.2, 62.9, 40.2, 36.9, 31.6, 29.7, 29.4, 26.7, 26.5 (2C), 25.5; HR-ESI calcd for $C_{20}H_{33}N_2O_{11}$ $[M + H]^+$ 477.2086. Found 477.2108.

2′-*N*-[4-(Succinimidooxycarbonyl)butan-1-oyl]-aminoethyl α-D-mannopyranoside (Man-DSG). To a stirred solution of disuccinimidyl glutarate (**DSG**; 50.0 mg, 153 μmol) in DMSO (0.5 mL) was added dropwise a solution of **Man** (7.7 mg, 34.5 μmol) in DMSO (0.2 mL) at room temperature. After 20 min, TLC analysis showed that all the **Man** was consumed. To the solution was added ice-cold water (10 mL), and the resulting white precipitates (**DSG**) were removed by suction filtration. The filtrate was chromatographed on a column of ODS with MeOH/H_2O (0, 10, 20, 30, 40%; each 10 mL) as a stepwise gradient. The fraction of 20% MeOH was concentrated to give **Man-DSG** (10.3 mg, 69%) as a syrup. For analytical purpose, **Man-DSG** was further purified by flash chromatography ($CHCl_3$-MeOH = 4:1). R_f = 0.78 ($CHCl_3$-MeOH-H_2O = 6:4:1); $[\alpha]_D^{23}$ + 32 (*c* 1.1, MeOH); ^{1}H NMR (CD_3OD) δ 4.75 (d, 1H, *J* = 1.6 Hz, H-1″), 3.84–3.33 (m, 10H), 2.83 (s, 2H), 2.66 (s, 2H), 2.56–2.48 (m, 2H), 2.36–2.24 (m, 2H), 2.04–1.93 (m, 2H); ^{13}C NMR (CD_3OD) δ 174.7, 171.7, 169.7, 101.6, 74.7, 72.5, 72.0, 68.6, 67.2, 62.9, 40.3, 35.4, 30.9, 26.5, 22.0; HR-ESI calcd for $C_{17}H_{27}N_2O_{11}$ $[M + H]^+$ 435.1615. Found 435.1599.

2′-*N*-[5,8-Dioxa-4,9-dioxo-6-(succinimidooxycarbonyl)undecan-1-oyl]-aminoethyl α-D-mannopyranoside (Man-EGS). To a stirred solution of ethylene glycol bis(succinimidylsuccinate) (**EGS**; 63.3 mg, 139 μmol) in DMSO (0.5 mL) was added

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dropwise a solution of **Man** (3.6 mg, 16.1 μmol) in DMSO (0.2 mL) at room temperature. After 30 min, TLC analysis showed that all the **Man** was consumed. To the solution was added ice-cold water (10 mL), and the resulting white precipitates (**EGS**) were removed by suction filtration. The filtrate was passed through SepPak C18, the cartridge washed with H_2O (10 mL), and the absorbed substance eluted with MeOH (10 mL). This operation was repeated. The eluate was chromatographed on a column of ODS with MeOH/H_2O (0, 10, 20, 30, 40%; each 5 mL) as a stepwise gradient. The fraction of 20% MeOH was concentrated to give **Man-EGS** (5.7 mg, 63%) as a syrup. For analytical purpose, **Man-EGS** was further purified by flash chromatography ($CHCl_3$-MeOH = 4:1). R_f = 0.60 ($CHCl_3$-MeOH-H_2O = 6:4:1); $[\alpha]_D^{23}$ + 23 (*c* 1.4, MeOH); ^{1}H NMR (CD_3OD) δ 4.75 (d, 1H, *J* = 1.6 Hz, H-1″), 4.33–4.28 (m, 4H), 3.85–3.36 (m, 10H), 2.98–2.93 (m, 2H), 2.83 (s, 4H), 2.77–2.72 (m, 2H), 2.65–2.59 (m, 2H), 2.52–2.47 (m, 2H); ^{13}C NMR (CD_3OD) δ 174.2, 174.0, 172.6, 171.5, 169.4, 101.6, 74.7, 72.5, 72.0, 68.6, 67.2, 63.8, 63.5, 62.9, 40.4, 31.3, 30.3, 29.5, 27.1, 26.5; HR-ESI calcd for $C_{22}H_{33}N_2O_{15}$ $[M + H]^+$ 565.1881. Found 565.1922.

2-*N*-(Succinimidooxycarbonyl)-aminoethyl α-D-mannopyranoside (Man-DSC). To a stirred solution of *N,N′*-disuccinimidyl carbonate (**DSC**; 15.0 mg, 58.6 μmol) in DMF (0.8 mL) was added dropwise a solution of **Man** (13.0 mg, 58.2 μmol) in DMF (0.5 mL) and triethylamine (8.2 μL, 112 μmol) at room temperature. After 30 min, TLC analysis showed that all the **Man** was consumed. The reaction mixture was concentrated and chromatographed on a column of silica gel with $CHCl_3$-MeOH (3:1) to give **Man-DSC** (17.7 mg, 83%) as a syrup. R_f = 0.27 ($CHCl_3$-MeOH = 3:1); $[\alpha]_D^{23}$ + 34 (*c* 2.1, MeOH); ^{1}H NMR (CD_3OD) δ 4.78 (d, 1H, *J* = 1.7 Hz, H-1″;), 3.87–3.29 (m, 10H), 2.79 (s, 4H); ^{13}C NMR (CD_3OD) δ 174.2, 153.8, 100.2, 73.4, 71.1, 70.6, 67.3, 66.3, 61.5, 41.6, 25.7; HR-ESI calcd for $C_{13}H_{20}N_2NaO_{10}$ $[M + Na]^+$ 387.1016. Found 387.1010.

2-Isothiocyanatoethyl α-D-mannopyranoside (Man-TP). The mixture of **Man** (15.3 mg, 68.5 μmol) in 0.02 M $KHCO_3/Na_2B_4O_7$ buffer (pH 9.0; 3 mL) and thiophosgene (**TP**; 7.7 μL, 101 μL) in $CHCl_3$ (1.5 mL) was vigorously stirred for 1 hour at room temperarature. The water layer was washed three times with $CHCl_3$ and bubbled with argon gas for 10 min and the solution was used for the coupling to BSA without further purification. R_f = 0.61 ($CHCl_3$-MeOH-H_2O = 6:4:1).

2′-*N*-(2-Ethoxy-3,4-dioxocyclobut-1-en-1-yl)-aminoethyl α-D-mannopyranoside (Man-DES). To a stirred solution of **Man** (99.1 mg, 448 μmol) in 0.75 M phosphate buffer (pH 7.0, 10 mL) was added dropwise a solution of diethyl squarate (**DES**; 100 mg, 590 μmol) in EtOH (10 mL). After 2.5 h, TLC analysis showed that all the **Man** was consumed. The reaction mixture was concentrated and chromatographed on a column of silica gel with $CHCl_3$-MeOH (4:1) and then on Sephadex G-15 (H_2O) to give **Man-DES** (105 mg, 67%) as white amorphous solids. R_f = 0.27 ($CHCl_3$-MeOH-H_2O = 60:20:3); $[\alpha]_D^{25}$ + 31.4 (*c* 1.46, MeOH); ^{1}H NMR (D_2O) δ 4.72-4.59 (m, 5H), 3.76–3.40 (m, 8H), 1.33–1.26 (m, 3H); ^{13}C NMR (D_2O) δ 189.2, 183.7, 99.7, 73.5, 71.3, 70.6, 67.2, 66.8, 61.5, 44.6, 44.5, 15.8.

Anal. Calcd for $C_{14}H_{21}NO_9$: C, 48.41; H, 6.09; N, 4.03. Found: C, 48.09; H, 5.76; N, 3.87.

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2′-*N*-(3-fluoro-4,6-dinitrophenyl)-aminoethyl α-D-mannopyranoside (Man-DFDNB). To a stirred solution of 1,5-difluoro-2,4-dinitrobenzene (**DFDNB**; 104 mg, 508 μmol) in DMSO (1 mL) was added dropwise a solution of **Man** (50.5 mg, 226 μmol) in 0.1 M $KHCO_3/Na_2B_4O_7$ buffer (pH 9.0; 0.5 mL) at room temperature. After 10 min, TLC analysis showed that all the **Man** was consumed. To the solution was added ice-cold water (10 mL), and the solution was chromatographed on a column of ODS with MeOH/H_2O (0, 10, 20, 30, 40%; each 10 mL) as a stepwise gradient. The fractions of 30 and 40% MeOH were concentrated and further chromatographed on a column of silica gel ($CHCl_3$-MeOH = 8:1) to give **Man-DFDNB** (83.5 mg, 91%) as yellow solids. R_f = 0.24 ($CHCl_3$-MeOH = 5:1); mp = 92–94; $[\alpha]_D^{23}$ + 26.0 (*c* 0.68, DMSO); ^{1}H NMR (CD_3OD) δ 9.03 (d, 1H, $J_{H,F}$ = 8.1 Hz, H-5), 7.04 (d, 1H, $J_{H,F}$ = 14.3 Hz, H-2), 4.81–4.79 (brs, 1H, H-1″), 4.02 (m, 1H, H-2a′), 3.81–3.62 (m, 7H, H-1a′, 1b′, 2b′, 2″, 3″, 6a″, 6b″), 3.58 (t, 1H, *J* = 9.3 Hz, H-5″); ^{13}C NMR (CD_3OD) δ 159.8, 128.5 (C-5), 103.1 (d, J = 28.2 Hz, C-2), 101.9 (C-1″), 75.1 (C-4″), 72.6 (C-3″), 72.0 (C-2″), 68.4 (C-5″), 66.8 (C-1′), 62.9 (C-6″), 44.1 (C-2′).

Anal. Calcd for $C_{14}H_{18}FN_3O_{10}$: C, 41.28; H, 4.45; F, 4.66; N, 10.32. Found: C, 40.93; H, 4.40; F, 4.71; N, 10.08; HR-ESI calcd for $C_{14}H_{18}FN_3O_{10}Na$ $[M + Na]^+$ 430.0874. Found 430.0880.

Synthesis of neoglycoproteins (Man-linker-BSA). A solution of BSA (2.5 mg, 38 nmol) in a buffer (100 μL) was placed in a microtube (1 mL) and rotated for 30 min (ca. 20 rpm) by an autorotation apparatus. To the solution was added an aqueous solution (400 μL) of the **Man**-linker reagents synthesized above (from 282 nmol to 18.0 μmol) and the reaction mixture was rotated for the period specified in the main text. Purification was performed using an Econo-Pak 10DG column and the molecular weight was measured by MALDI-TOF mass spectroscopy.

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