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Inhibition of Adhesion of Type 1 Fimbriated *Escherichia coli* to Highly Mannosylated Ligands

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The inhibitory potencies of a number of mannosides, di- and trivalent mannosides, a set of mannose-terminating dendrimers, and five types of mannose-bearing neoglycoproteins were determined by using a binding assay that measures the binding of ^{125}I-labeled, highly mannosylated neoglycoprotein to a type 1 fimbriated Escherichia coli *(K12) strain in suspension. The IC_{50} values (the concentration of inhibitor that causes 50 % reduction in the bound ^{125}I-ligand to* E. coli*) obtained by this method were much lower than the equivalent values obtained by hemagglutination or in assays that involve microplate immobilization. Two important factors that strongly influence the affinity to* E. coli *adhesin are: 1) the presence of an α-oriented aglycon that has a long aliphatic chain or an aromatic group immediately next to the glycosyl oxygen, and 2) the presence of multiple mannosyl residues that can span a distance of 20 nm or longer on a relatively inflexible structure. The two best inhibitors, which are a highly mannosylated neoglycoprotein with the longest linking arm between a mannose and protein amino group and the largest mannosylated dendrimer (fourth generation), exhibited sub-nM IC_{50} values.*

KEYWORDS:

dendrimers · FimH adhesin · glycoproteins · inhibitors · mannose-specific adhesin

Introduction

Many bacteria including pathogenic ones express carbohydrate-specific adhesin on their fimbriae. These fimbrial adhesins are often implicated in the initial recognition/binding of bacteria to host cells or persisting colonization of bacteria on certain host cell surfaces. For example, mannose-specific adhesin (known as the FimH protein) of type 1 fimbriated *E. coli*[1] is known to cause common urinary tract infection.[2] The presence of type 1 fimbria also appears to be important for the verotoxin-producing *E. coli* strain O157:H7 that causes hemorrhagic colitis to colonize and persist on the human epithelial cells and brush border membranes.[3] Another example is the P-fimbriated *E. coli* that expresses galabiose (Galα(1,4)Gal) specific adhesin on its fimbrial tip, through which it colonizes the human kidney and causes pyelonephritis.

High-affinity ligands for these adhesins may be useful as therapeutics for preventing or mitigating pathological symptoms. As described in this paper, we investigated the factors that are important in determining the inhibitory potency of a ligand for *E. coli* type 1 adhesin (the FimH protein). The two important factors are the presence of an α-oriented hydrophobic group located immediately next to mannose and the presence of a large number of such mannosides on one molecule. The best inhibitors we produced are neoglycoproteins and dendrimers that are substituted with large numbers of α-mannopyranosides with either a long aliphatic chain or an aromatic ring immediately next to the sugar, and that have a long Man-to-Man span (possibly reaching around 20 nm). Such multivalent inhibitors expressed IC_{50} values (the concentration of inhibitor that causes 50 % reduction in the binding of the labeled reference ligand) in the sub-nM level, which represents an affinity enhancement of up to 1600-fold over the monomeric counterpart.

Results

Neoglycoproteins and other multivalent mannosides

In this study, we used a few different designs of multivalent mannosides as inhibitors to investigate how multivalency enhances avidity towards the mannose-specific adhesin on *E. coli* K12 cells. We used five types of bovine serum albumin (BSA) or human serum albumin (HSA) based mannoside-containing neoglycoproteins, and their linking arm structures (all linked through amino groups of protein) are shown in Table 1. The AI ($-CH_2C(=NH)-$) and AD ($-CH_2CONH(CH_2)_2$) type

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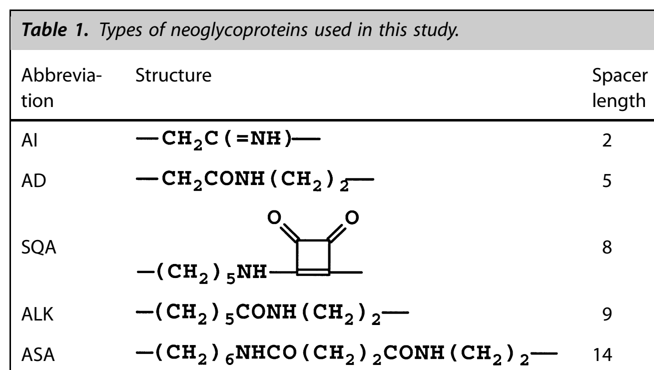


Table 1. Types of neoglycoproteins used in this study.

Abbreviation	Structure	Spacer length
AI	$-CH_2C(=NH)-$	2
AD	$-CH_2CONH(CH_2)_2-$	5
SQA	$-(CH_2)_5NH-$ (O, O)	8
ALK	$-(CH_2)_5CONH(CH_2)_2-$	9
ASA	$-(CH_2)_6NHCO(CH_2)_2CONH(CH_2)_2-$	14

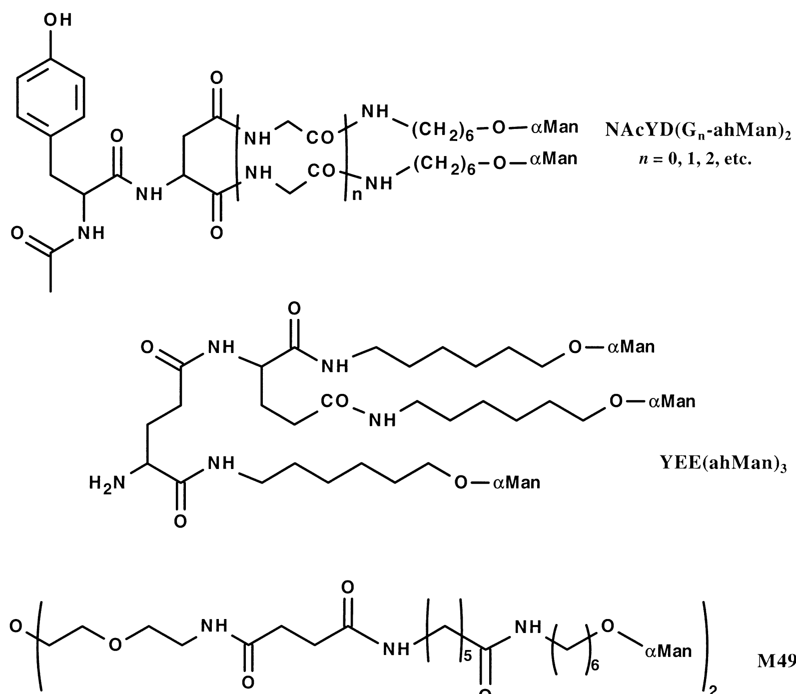
neoglycoproteins contain thioglycosides and have been used extensively in the study of hepatic and other lectins.[4, 5] Three new types of neoglycoprotein used in this study all contain *O*-mannosides, and their linking arms are ethylaminocarboxypentyl (ALK-type), *N*-(3,4-dioxo-1-cyclobuten)aminopentyl (SQA-type), and 6-*N*-(ethylaminocarboxyethylcarboxy)aminohexyl (ASA-type). HSA was used to make ALK- and SQA-type neoglycoproteins, and BSA was used for ASA-type neoglycoproteins.

As to small, paucivalent mannosides, all the divalent mannosides, with the sole exception of compound M-49, are derivatives of L-aspartic acid containing terminal mannosyl residues as shown in Scheme 1. The linking arm was elongated by the addition of zero to four glycyl residues. Similarly, the trivalent structure is based on γ-L-glutamyl-L-glutamic acid to provide three arms.[6] M-49 is a divalent mannoside in which two mannosyl residues are separated by 49 atoms (C, N, and O atoms). All these compounds have also been used in many lectin studies.[6, 7]

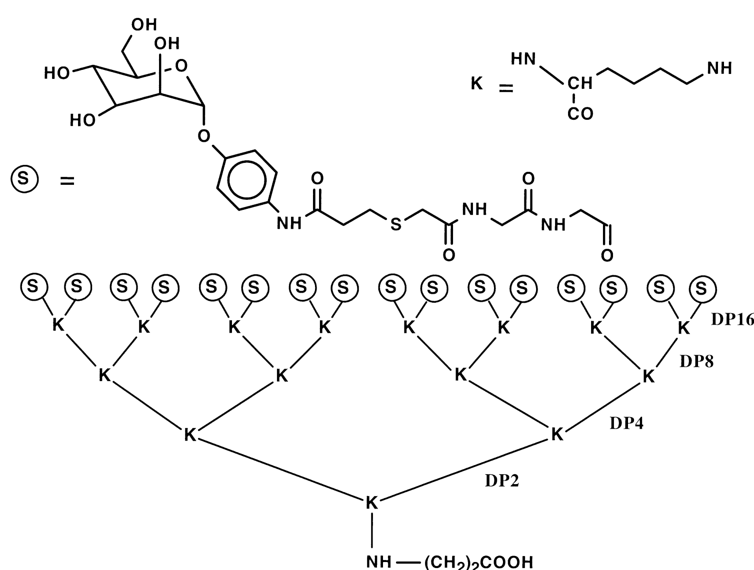
L-Lysine-based mannose dendrimers have been used in the study of legume lectins,[8] and their structural designs are given in Scheme 2. DP-2, DP-4, DP-8, and DP-16 dendrimers contain backbones consisting of 1, 3, 7, and 15 interconnected lysyl residues as scaffolds that will provide 2, 4, 8, and 16 terminal amino groups, respectively, through which the mannosides are linked.

Hemagglutination inhibition assay

The hemagglutination inhibition assay is a simple and useful method for screening the carbohydrate specificity of bacterial adhesions.[9, 10] For the type 1 fimbriated *E. coli*, the sensitivity of hemagglutination with erythrocytes varies a great deal depending on the species, the strongest being guinea pig, horse, and fowl, followed by human, sheep, goat, and ox, in that order. Therefore, we used horse red blood cells to test the hemagglutination strength of *E. coli* cultures during five passages of cultivation, and also to determine the inhibitory potency of various proteins and neoglycoproteins. Inhibition results are presented in Table 2. As found by others,[9, 11] mannose-containing compounds all have inhibitory potency to varying degrees, while the corresponding compounds containing galactose were totally ineffective. Among Man-containing neoglycoproteins, the two types that have short linking arms, that is, Man_{32}-AI-BSA and Man_{35}-AD-BSA (see Table 1 for structures), were very poor inhibitors compared to Man_{32}-SQA-HSA, which has similar degrees of Man substitution but in which the sugars are linked by a longer arm. Understandably, the level of Man substitution is also important, since within the same neoglycoprotein type the higher substitution resulted in the better inhibitor. Mannose-containing natural glycoproteins were also effective inhibitors. Invertase,



Scheme 1. Structures of di- and trivalent cluster ligands.



Scheme 2. Structure of the dendrimeric ligand.

Table 2. *Inhibition of* E. coli *mediated hemagglutination of horse red blood cells.*

Inhibitor	IC_{min}[a] [nM]
Man_{32}-SQA-HSA	313
Man_{19}-SQA-HSA	625
Man_{21}-ALK-HSA	625
Man_{11}-ALK-HSA	1250
Man_{32}-AI-BSA	25000
Man_{35}-AD-BSA	12500
invertase	1850
soybean agglutinin	18000
Me α-Man	500000
Me α-Gal	NI[b] (500 mM)
Gal_{34}-AI-BSA	NI (25 µM)
Gal_{33}-AD-BSA	NI (25 µM)
HSA	NI (29 µM)
BSA	NI (50 µM)

[a] Minimum concentration required to inhibit the agglutination. [b] NI: no inhibition at the highest concentration tested, which is shown in parenthesis.

which has a massive amount of mannose,[12] is a better inhibitor than soybean agglutinin, which has only four high-mannose-type *N*-glycans (12 terminal mannoses) per molecule.

Binding of ^{125}I-Man_{21}-ALK-HSA by *E. coli*

A series of tubes containing a fixed concentration of *E. coli* (6×10^9 cells mL^{-1}) and varying concentrations of ^{125}I-Man_{21}-ALK-HSA (with unlabeled Man_{21}-ALK-HSA as the carrier) were incubated for one and two hours in the cold. Similarly iodinated ^{125}I-HSA was used as a control. The amount of protein bound (*y* axis) was plotted against the dose of radiolabeled protein (*x* axis) as shown in Figure 1. Since the one- and two-hour data overlapped almost

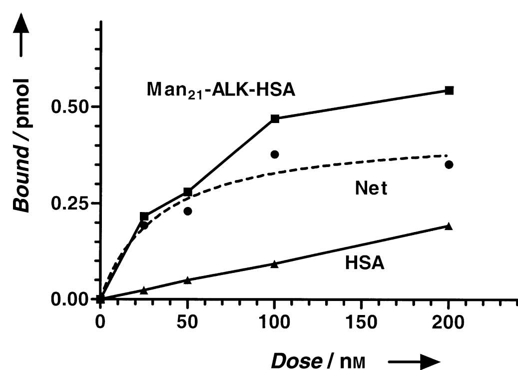


Figure 1. *Isothermal binding curves of ^{125}I-Man_{21}-ALK-HSA and ^{125}I-HSA binding to* E. coli *cells.* ■, *^{125}I-Man_{21}-ALK-HSA;* ▲, *^{125}I-HSA;* ●, *difference in the bound amounts between the two.*

completely, only the former data were shown. The results showed that: 1) there is no difference in the amounts of bound ligand between one- and two-hour incubations, 2) the amount of bound HSA increased linearly with its concentration, whereas the binding of Man_{21}-ALK-HSA was much higher and increased in a hyperbolic manner, and 3) subtraction of the bound HSA as nonspecific binding from the amount of bound Man_{21}-ALK-HSA yielded a curve which indicated that saturation was reached at around 100 nM of Man_{21}-ALK-HSA.

Inhibition assays with ^{125}I-Man_{21}-ALK-HSA as a tagged reference ligand

It is known from the existing reports that mannose appears to be the only monosaccharide bound tightly by the *E. coli* adhesin.[9] We first tested methyl α-D-mannopyranoside (Me α-Man) as an inhibitor, since this glycoside has been frequently used by others as the reference inhibitor.[13–15] The assays carried out at three different concentrations of *E. coli* (1×10^9, 3.1×10^9, and 8×10^9 cells mL^{-1}) yielded IC_{50} values of 5.7, 8.8, and 11.5 µM, respectively, showing only a twofold increase in IC_{50} value with an eightfold increase in *E. coli* concentration. In contrast, Old reported that the minimum inhibition concentration (MIC) of hemagglutination was highly dependent on the *E. coli* concentration.[9] For instance, an 8-fold increase in bacterial concentration caused a 16-fold increase in the MIC value for mannose.

A total number of *E. coli* per incubation tube of approximately 1.5×10^9 cells (or 6×10^8 cells for pelleting) was used in all the inhibition assays, since assays carried out under such conditions gave adequate radioactive signals of the bound ligand with the minimum number of *E. coli* cells. Inhibitors used in this study include simple glycosides, synthetic multivalent mannosides, and various Man-containing neoglycoproteins. Some examples of inhibition curves are shown in Figure 2. The IC_{50} values

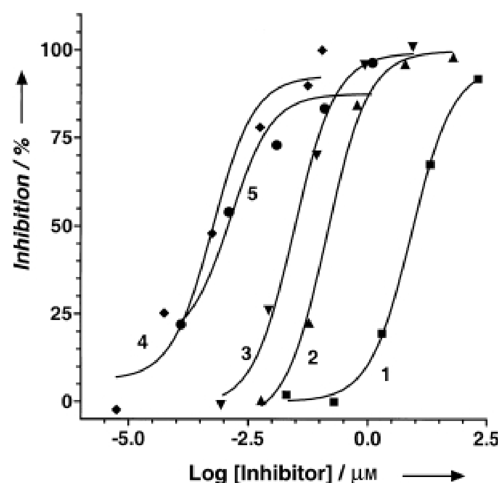


Figure 2. *Some examples of inhibition curves. Percent inhibition (y axis) was plotted against the logarithm of inhibitor concentration in µM (x axis). Curve 1 (■), Me α-Man; curve 2 (▲), PNP α-Man; curve 3 ▼, $Man_{7.3}$-ALK-HSA; curve 4 ◆, Man_{25}-ASA-HSA; curve 5 (●) DP-16 Man-dendrimer.*

obtained by nonlinear regression of the data are presented respectively in Tables 3, 4, and 5. As seen in Table 3, β-D-mannopyranosides were considerably inferior inhibitors than the corresponding α-D-mannopyranosides; IC_{50} values of the former were at least tenfold higher than those of the latter. Old reported that Me β-Man was a very poor inhibitor, but he was unable to

Table 3. *Inhibition potencies of glycosides towards binding of* ^{125}*I-Man*$_{21}$*-ALK-HSA to* E. coli.

Inhibitor	IC_{50} [μM]	
	α	β
methyl Man	10 ± 3	104
ethyl Man	2.6	23 ± 7
PNP Man	0.074 ± 0.019	21.5
TFA-ah Man	0.074 ± 0.009	–
TFA-ah Gal	6400	10800
ZG-ah Man	0.09 ± 0.025	–
Manα(1,6)[Manα(1,3)]Manα-OMe	0.49 ± 0.03	–
Manα(1,2)Manα(1,6)Manα-OMe	3.9 ± 0.1	–

obtain an inhibition value (MIC) by his hemagglutination assay.[9] The main reason that we could obtain IC_{50} values of β-mannosides probably stems from the fact that the IC_{50} values obtained in our assay were lower across the board than the values reported by others. For instance, the MIC value of Me α-Man obtained by Old at mid-level *E. coli* concentrations was around 0.15 mM,[9] as compared to the IC_{50} value of 0.01 mM obtained by us. This discrepancy in inhibition potency will be discussed in detail in the Discussion section. For the same reason of having lower overall IC_{50} values, we were also able to estimate the IC_{50} value of Me α-Glc to be around 200 – 500 mM, which means that it is at least a 10^4-fold weaker inhibitor than Me α-Man. As reported by others,[13, 16] an aromatic aglycon (for example, *p*-nitrophenyl (PNP)) increased the inhibitory potency tremendously. The enhanced inhibitory activity of PNP α-Man over Me α-Man was 135-fold in our assay as compared to 30-fold reported by Firon et al.[13] This enhancement effect appears to be rather nonspecific, since we demonstrated that an aliphatic aglycon, 6-trifluoroacetamidohexyl (TFA-ah), had a similar affinity enhancement to a PNP group (Table 3). Since the TFA-ah group gave a very strong enhancement of affinity, we tested the hydrophobic aglycon portion alone as inhibitors, as well as the TFA-ah α/β-glycosides of a nonbinding sugar, Gal. 6-*N*-(Trifluoroacetamido)hexanol began to inhibit at around 5 mM, but 50% inhibition was never attained. A rough estimate of the IC_{50} value is approximately 200 mM. As shown in Table 3, the presence of a galactosyl residue decreases the IC_{50} value to 6.4 mM and 10.8 mM for the α- and β-D-galactosides, respectively. Thus, even a nonbinding sugar, Gal, has a positive effect on the binding. Since TFA-ah β-Gal is a slightly weaker inhibitor than the α counterpart, the preference, albeit very small, for α- over β-glycosides holds even for galactoside. Comparison of TFA-ah α-Man and TFA-ah α-Gal shows that Man is preferred over Gal by about 10^5-fold. Of the two isomeric Man trisaccharides tested, the trimannosyl core structure, (Manα(1,3)[Manα(1,6)]Manα-OMe), showed 20-fold higher affinity than Me α-Man, whereas the linear Man trisaccharide (see Table 3) was only slightly more inhibitory than Me α-Man. Firon et al. reported the affinity increase of a tri-Man core structure over Man of 10.5-fold,[13] which is comparable to our result of 20-fold. Thus, the increase in affinity caused by the presence of the second and third Man residues is considerably smaller than that generated by a hydrophobic aglycon. Moreover, our linear trisaccharide and the results of Firon et al.,[13] who tested a large number of Man oligosaccharides, suggest that the extent of enhancement appears to be highly dependent on the linkage as well as the overall structure of the Man oligosaccharides. In general, oligosaccharides that contain the tri-Man core structure are better inhibitors than most others.

Preparation of a number of multivalent mannosides of different structural designs and their inhibitory potencies have been reported.[14, 15, 17] The enhancement in inhibition potency of these cluster mannosides with two to eight Man residues linked by the ordinary *O*-glycosidic linkage ranged from 10- to 250-fold over Me α-Man. However, since the nature of aglycon greatly influences the binding affinity of type 1 *E. coli* adhesin, the true affinity enhancement by these cluster mannosides is difficult to assess. Table 4 gives our results on the inhibition potency of

Table 4. *Inhibition potencies of multivalent mannosides towards binding of* ^{125}*I-Man*$_{21}$*-ALK-HSA by* E. coli.

Inhibitor	IC_{50} [nM]	Number of atoms between sugars	IC_{50} per Man [nM]
monovalent			
Me α-D-Man	10000		
Et α-D-Man	2600		
PNP α-D-Man	74		
divalent			
NAcYD(ah-Man)$_2$	45	18	90
NAcYD(GG-ah-Man)$_2$	12 ± 4	30	24
NAcYD(GGGG-ah-Man)$_2$	14 ± 1	42	28
M-49	22	49	44
trivalent			
YEE(ah-Man)$_3$	11 ± 3	19	33
dendrimer			
DP-2	22	41	44
DP-4	5	55	20
DP-8	2.8	68	22.4
DP-16	0.9	83	14.4

multivalent glycosides. The number of atoms (C, N, and O atoms) in the chain and the calculated IC_{50} values per mannosidic residue are also given in the table. If we use Me α-Man as the reference inhibitor as others have done, our cluster glycosides all show affinity increases much larger than those reported by others, the highest one being 12500-fold for the dendrimer of DP-16. However, all of our compounds have either a $-(CH_2)_6NH-$ moiety or phenyl group immediately next to the mannosyl residue, so that the expected IC_{50} value of each mannoside in the cluster glycosides is likely to be around 80 nM. When we use this number as the reference IC_{50} value, the column of IC_{50} value per Man residue in Table 4 indicates that there is no affinity enhancement for the shortest divalent structure, NAcYD(ah-Man)$_2$, and others with somewhat longer arms only exhibited affinity enhancements of up to fourfold. The most significant affinity enhancement was observed with the Man-based dendrimers, whose affinity increased with increasing valency and molecular size; the largest enhancement was 100-fold for the largest dendrimer of DP-16. This dependence of affinity increase on the number of Man residues per dendrimer may be as a result

of these Man residues being engaged in binding at many fimbrial binding sites, or due to the increased probability of Man residues fitting in two combining sites. This aspect will be discussed further in the Discussion section.

Table 5 gives IC_{50} values of various neoglycoproteins on the protein molar basis and on the basis of Man concentration of neoglycoproteins. Several conclusions can be made from the results. It is quite obvious that multivalency helps to increase the inhibitory potency, since all the Man-containing neoglycoproteins of SQA, ALK, and ASA types have lower IC_{50} values than TFA-ah α-Man. The examination of the ALK series of neoglycoproteins shows that affinity increases with increasing number of Man residues; around a sixfold increase in affinity is observed when the number of Man moieties is increased from 7 to 21 per molecule of HSA. The second point is that the affinity enhancement is dependent on the arm length; the longer the arm length, the larger the enhancement is. Comparison of ALK- and ASA-type neoglycoproteins, which have similar structures (either aminopentyl or aminohexyl) immediately next to the sugar residue, shows that at a comparable level of sugar substitution (20 – 25 mol mol^{-1}), an ASA neoglycoprotein was a four to five times better inhibitor than the ALK-type neoglycoprotein. The third point is that the two shortest neoglycoprotein types, AI and AD, had IC_{50} values much higher than other neoglycoprotein types, in fact even higher than that of TFA-ah α-Man, even at very high sugar substitution levels. This fact suggests that the chemical nature of the linking arm must be a very important factor in addition to the arm length. The AI-neoglycoproteins have a positively charged amidino group ($-SCH_2C(=NH)NH-$) adjacent to mannose, and the AD series has a peptido linkage ($-SCH_2CONH(CH_2)_2NH-$) close to the sugar residue; therefore the results suggest that the presence of a hexyl (or pentyl) moiety or a phenyl group in the other types of neoglycoproteins is a dominant factor in generating an effective neoglycoprotein inhibitor. Neither the presence of the thioglycosidic linkage nor the carrier (BSA in the AI- and AD-type) can be a significant factor in their low inhibitory potency, because ethyl α-thio-D-mannopyranoside was actually a better inhibitor than Me α-Man (Table 3), and the best type of neoglycoprotein, the ASA-type, had BSA as the carrier. The column of IC_{50} value per Man residue has considerable variation among neoglycoproteins. Interestingly, aside from the AI- and AD-type neoglycoproteins that have unfavorable linking arms, the three neoglycoproteins that manifested the lowest IC_{50} value per Man residue had the highest levels of Man substitution (>25). This suggests that for the purpose of increasing affinity it is not wasteful to have very large numbers of Man residues. Although perhaps only a few Man residues in a neoglycoprotein are actually bound, the presence of a large number of Man residues seems to improve the probability of binding greatly. It is quite obvious from these observations that a long hydrophobic arm and very high levels of Man substitution are two important factors for the high inhibitory potency of neoglycoproteins.

***Table 5.** Inhibitory potencies of neoglycoproteins towards binding of ^{125}I-Man$_{21}$-ALK-HSA to* E. coli.

Inhibitors	IC_{50} [nM]	IC_{50} per monosaccharide [nM]
TFA-ah-α-Man	74	74
Man$_{29}$-AI-BSA	175	5075
Man$_{35}$-AD-BSA	87	3045
Man$_{19}$-SQA-HSA	5.7	108
Man$_{32}$SQA-HSA	1.0	32
Man$_{7.3}$-ALK-HSA	29	212
Man$_{11}$-ALK-HSA	21 ± 0.7	231
Man$_{18}$-ALK-HSA	12	216
Man$_{21}$-ALK-HSA	5.0 ± 1.5	105
Man$_{25}$-ASA-BSA	0.88 ± 0.2	22
Man$_{34}$-ASA-BSA	0.2 ± 0.01	6.8

Discussion

Mannose-specific binding of type 1 fimbriated bacteria of *enterobacteriaceae*, such as *E. coli* and those in *Salmonella* and *Klebsiella* genera, is perhaps the most common and the best studied of all the carbohydrate-mediated bacterial adherence. The relationship between the binding affinity and the ligand structure has been studied by a number of groups, and their results suggest that an effective binding by this adhesin requires an α-Man configuration,[9] that the presence of aromatic aglycon enhances the affinity tremendously,[13, 16] and that certain oligomannosyl structures increase the affinity over Me α-Man.[13] The relative inhibitory potencies of various compounds obtained in this study agree largely with these conclusions. However, one major difference between our results and those of others is the absolute values of IC_{50} obtained in our system. The IC_{50} values generated in our assay are much lower than those reported by others. For example, the IC_{50} value of Me α-Man in our study was 10 μM, while that reported by Firon et al.[18] using a yeast agglutination assay was around 0.3 mM, and that determined by an ELISA with yeast mannan coated plates was 2.5 – 4 mM.[15, 17] As mentioned in the Results section, because of generally much lower IC_{50} values obtained in our assay, we were also able to determine or estimate the IC_{50} values of some very poor inhibitors. For instance, Me α-Glc, the 2-epimer of Me α-Man, had 10^4-fold lower affinity than Me α-Man, suggesting that the axial 2-OH group of Man is a very important element in the binding by the adhesin. Inversion of the second OH group at the 4-position (that is, Gal) caused further decrease, but only by an additional 8.6-fold.

This difference in inhibition data most likely results from the assay methods used. Assays used most often by others are based on *E. coli* mediated yeast or erythrocyte agglutination[16, 18] or ELISAs with microtiter wells coated with yeast mannan.[14, 15] In both of these methods, the binding of bacteria to ligand involves a highly clustered network of potential ligand residues, so that the binding of bacteria is perhaps not freely reversible, and an inhibitor would not have a fair chance of competing. In our assay, the tagged ligand was a Man-containing neoglycoprotein, which contained a large number of Man-bearing chains, each chain terminating in a single residue of α-mannose. Also, the incubation mixture containing bacteria, tagged ligand, and inhibitor was constantly mixed. Under such conditions, the

binding of each Man residue by the fimbrial adhesin will perhaps be freely reversible, which allows more efficient competition by inhibitors and results in lower IC_{50} values.

The IC_{50} value of 10 μM for Me α-Man is surprisingly low for a simple mannoside. For comparison, IC_{50} values of the same glycoside for the legume lectins, Con A, pea lectin, and lentil lectin, are 0.13 mM, 0.6 mM, and 1.4 mM, respectively.[19] It appears that the FimH binding pocket is well designed to engage in tight interactions with mannose. Indications thereto are that the solvent-accessible surface area buried by the interaction of FimH with mannose is 368 $Å^2$ as compared to 312 $Å^2$ for Me α-Man bound to Con A (J. M. J. Bouckaert, personal communication). There are also more hydrogen bonds involved at the FimH binding site than at the Con A site. The large affinity enhancement (approximately 135-fold) due to the presence of an α-oriented hydrophobic aglycon suggests that it lies close to a hydrophobic surface of the adhesin. Indeed, the recently elucidated X-ray structure of the FimH adhesin binding area has an extensive hydrophobic patch surrounding the Man-binding crevice.[20] It is interesting to note that PNP β-Man is a 290-fold poorer inhibitor than PNP α-Man, while a smaller aglycon (Me and Et) has only a tenfold difference between the α and β anomers. This suggests that a β-oriented aglycon does not make good contact with the hydrophobic surface, so that one cannot reap the benefit of a large hydrophobic binding energy by the presence of a β-oriented hydrophobic aglycon.

Many interactions between lectins and multivalent carbohydrate ligands have varied degrees of affinity enhancement, which is known as the glycoside cluster effect,[21] ranging from affinity being slightly better than to almost a geometrical increase over the one-site binding. The best example of strong affinity enhancement is that of mammalian hepatic asialoglycoprotein receptor (ASGP-R).[21] This receptor on the isolated rat hepatocyte surface binds simple galactoside with an IC_{50} value in the mM range, while certain Gal-terminated, biantennary and triantennary glycans were bound with IC_{50} values in the μM and nM range, respectively. The reason for this extraordinary affinity enhancement stems from a near-perfect complementarity between the preferred conformations of complex-type, Gal-terminated glycans and tightly organized ASGP-R subunits on the hepatocyte surface, thus reducing the entropical energy expenditure during the binding process. For the *E. coli* FimH adhesin system, the enhancement by divalent mannosides was rather small, as shown in Table 4; it amounted only to about threefold of the monomeric parent structure excluding the concentration effect. However, highly substituted Man-containing neoglycoproteins (Table 5) with the longest connecting arm (ASA-type) and the largest dendrimer (DP-16, Table 4) exhibited much higher affinity with IC_{50} values at the sub-nM level (approximately a 400-fold enhancement), which suggests that at least two fimbrial adhesin binding sites were occupied. The fact that affinity enhancement produced by neoglycoproteins is dependent on the length of the Man-bearing chain, and that the longest one has the highest affinity, suggests that these sites are quite far apart and the optimal length of the Man-to-Man span may not have been reached. This is also true for the Man-containing dendrimers. The reason for the dependence of affinity increase on the Man density within the same neoglycoprotein type is probably due to an improved chance of a mannose residue being favorably spatially oriented for binding.

FimH adhesin of *E. coli* is located at the tip of fimbria.[22] There is also some compelling evidence to suggest that adhesin also resides along the length of fimbrial stalk.[23] However, such laterally oriented FimH subunits may not have binding capability.[24] It is most likely, therefore, that Man residues on the high-affinity neoglycoproteins and dendrimers bind two fimbrial tips. BSA is an oblong-shaped molecule whose longer span is around 14 nm, and with the added length of Man-terminated chains, the span of two Man residues may reach 20 nm for the ASA-type neoglycoproteins. Electron micrograph images of fimbriated *E. coli* and immunostained fimbriae suggest that this distance of 20 nm is probably far shorter than the average fimbrial tip separation or average adhesin separation within a single fimbria.[1, 23, 25] However, it is probably quite possible for two fimbrial tips to approach the distance of 20 nm or less, at least temporarily. Because of this large distance of separation and the built-in flexibility between the binding sites, the affinity enhancement due to binding at two or more sites for the *E. coli* fimbrial systems is expected to be rather small. However, the *E. coli* fimbrial adhesin system can probably still generate biologically relevant affinity by binding at two or three fimbriae, since binding affinity at each site is considerably greater (100-fold or more), even without a hydrophobic aglycon, than that of ASGP-R binding a simple galactoside.

For the design of a potential inhibitor of *E. coli* adhesion that is medically applicable, it is obviously prudent to incorporate a long aliphatic chain or an aromatic residue immediately next to mannose. For affinity enhancement by multivalency, the *E. coli* fimbrial adhesin appears to require a very large distance ($>$20 nm) between two mannose residues. A dendrimer containing mannosides with hydrophobic aglycon may be convenient and effective for this purpose, since the synthesis of dendrimers involves repetition of simple chemical reactions and the products obtained seem to possess a higher degree of rigidity than linear polymers due to general crowding of both scaffolds and sugar residues. Although we have not studied inhibitory potency of linear multivalent inhibitors, relatively rigid or restrictive architecture should improve the inhibitory potency by lowering the entropical energy expenditure. In reality, very few mannose residues may actually be engaged in the binding process, but a large number of mannoses oriented on the periphery of molecule should improve the efficacy of binding tremendously. Neoglycoproteins, although excellent as scaffolding for mannose presentation, are perhaps not as suitable due to their strong immunogenicity.

Experimental Section

Materials: Methyl α- and β- and *p*-nitrophenyl (PNP) α- and β-D-mannopyranosides, bovine serum albumin (BSA), invertase, and horse red blood cells were from Sigma Chem. Co. (St. Louis, MO). Boron trifluoride diethyl etherate, pyridine-borane, 3,4-diethoxy-3-cyclobutene-1,2-dione (diethyl squarate), ε-caprolactone, 1,8-diaza-

bicyclo[5.4.0]undec-7-ene (DBU), and 5-aminopentanol were from Aldrich Chem. Co. (Milwaukee, WI). *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) was from Research Organics (Cleveland, OH). Fluorenylmethoxycarbonyl (Fmoc) chloride was from Advanced ChemTech (Louisville, KY). Human serum albumin was kindly provided by the American Red Cross. Trifluoroacetic acid was from Fluka (Milwaukee, WI), and bicinchoninic acid (BCA) reagent was from Pierce (Rockford, IL). Soybean agglutinin was prepared as described elsewhere.[26] Active carbon (Carbograph) cartridges were from Alltech (Deerfield, IL).

Preparations of the following compounds have been described elsewhere: 6-(Trifluoroacetamido)hexyl (TFA-ah) α-D-mannopyranoside,[27] 6-(*N*-benzyloxyglycyl)aminohexyl (ZG-ah) α-D-mannopyranoside,[26] Manα(1,3)[Manα(1,6)]Manα-OMe (tri-Man core) and Man-α(1,2)Manα(1,6)Manα-OMe.[28] Ethyl α- and β-thio-D-mannopyranosides were prepared by the standard procedure. Di- and trivalent mannose-containing cluster glycosides based on Asp and γ-glutamylglutamic acid were prepared basically by the method as described.[6, 7] M-49 is a divalent mannoside, as shown in Scheme 1, that has 49 C, N, or O atoms between the two mannosyl residues. Preparation of M-49 and related compounds will be described elsewhere. Mannose-containing dendrimers based on oligolysyl structures were prepared as described elsewhere.[8] Structures of these compounds are shown in Schemes 1 and 2.

Preparations of AI- and AD-type BSA derivatives (see Table 1 for structures) have been reported elsewhere.[4, 29] Preparation of ASA-type BSA derivatives will be described elsewhere.

General: Sugar content in the neoglycoproteins was determined by the phenol–sulfuric acid method.[30] Protein concentration was determined either with the microBCA[31] or Bradford assay.[32] ^{1}H NMR spectra were recorded with a Bruker AMX-300 NMR spectrometer in $CDCl_3$, CD_3OD, or D_2O. Synthetic reactions were monitored with TLC on a silica gel F_{254} layer precoated on aluminum sheet (E. Merck, Darmstadt, Germany). After chromatography, TLC plates were dried and sprayed with H_2SO_4 (15%) in 50% ethanol and heated for detection of carbohydrates, with $KMnO_4$ (5%) for double bonds, with ninhydrin (5%) in 95% ethanol and heated for amino groups, and with 2,4-dinitrophenylhydrazine (DNPH) reagent (0.2% DNPH, 1 M HCl in 48% ethanol) for aldehydo groups. *E. coli* strain K12 was inoculated in Luria–Bertani (LB) medium (5 mL; triptone (10 g), yeast extract (5 g), and NaCl (5 g) in water (1 L), and autoclaved) in a test tube and incubated at 37 °C for 48 h under static conditions. A portion of white pellicle on the surface was transferred to a fresh LB medium, and the cultivation under static conditions was repeated until strong hemagglutination activity was observed (serial passage method). After five passages, bacteria were grown in LB medium (500 mL) at 37 °C for 48 h under static conditions. The cells were harvested by centrifugation at 5000 rpm for 15 min and washed with phosphate-buffered saline (PBS, 25 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl). Cells were suspended in PBS to give $1.6-1.7\times10^{10}$ cells mL^{-1}. Cell concentration was determined by measuring absorbance at 600 nm, where $A_{600}=1.0$ corresponds to 10^9 cells mL^{-1}. The cells were kept frozen at -80 °C until use.

Proteins were iodinated with carrier-free Na^{125}I as described elsewhere.[33] Briefly, chloramine T (40 µg in 10 µL) was added to a mixture containing Na^{125}I (0.5 mCi; 5 µL) and 0.25 M sodium phosphate buffer (pH 7.5; 25 µL) that contained protein (10 µg). After 40 s, iodine was quenched with $NaHSO_3$ and KI, and the mixture was fractionated on a 5-mL Sephadex G-25 column in 25 mM HEPES buffer (pH 7.2) containing 0.15 M NaCl. Fractions containing protein were combined and stored in the cold with BSA (0.2 mg).

Hemagglutination: *E. coli* cells (1.6×10^8 cells in 10 µL) were mixed at room temperature in the U-shaped wells of microtiter plates in PBS (20 µL) with or without a potential inhibitor. After 15 min at room temperature, horse red blood cells (10 µL; Sigma, 10% hematocrit) were added, and after approximately 30 min, the area of the dark circle was noted. A small dark circle indicates the absence of hemagglutination. In the inhibition assay, the lowest concentration of inhibitor that causes noticeable reduction in the agglutinated area is observed.

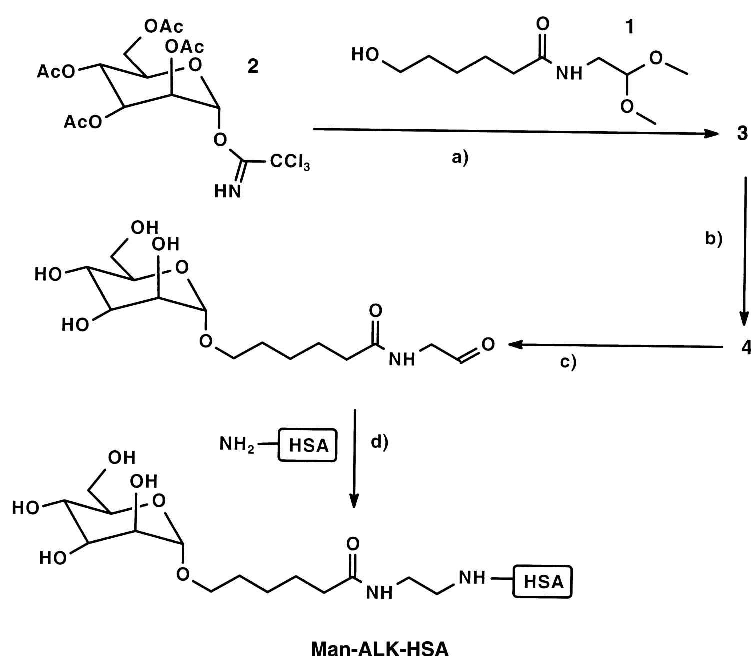
***E. coli* ligand-binding assay**: The incubation mixture contained ^{125}I-Man$_{21}$-ALK-HSA and *E. coli* ($5\times10^8-4\times10^9$ cells) in a total volume of 0.5 mL of 25 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.1% BSA in a 1.5-mL microcentrifuge tube. Tubes were placed in an ice-water bath and tumbled end-over-end at approximately 10 rpm for up to 2 h. Two 200-µL aliquots taken from each incubation mixture were placed in chilled, narrow microcentrifuge tubes (0.4-mL) that had been filled with an oil mixture (silicon oil/light mineral oil, 3.8:1) to about half the length of tubes. Tubes were centrifuged in a Fisher microcentrifuge (Model 235B) for about 1 min. The tips of the tubes containing cell pellet were clipped off, and placed in a 13×100 mm glass tube, and the radioactivity was measured by using a gamma counter (Packard MINAXIγ). To assess the inhibitory potency of various compounds, *E. coli* cells were incubated with ^{125}I-Man$_{21}$-ALK-HSA (around 133-fold dilution of the stock) in the presence of a test compound at five different concentrations (10-fold serially diluted solutions). The range of concentration was chosen so that 0% and 100% inhibitions were achieved at the lowest and the highest inhibitor concentrations, respectively. The highest concentration that can be tested for any inhibitor is around 0.1 M, since incubation mixtures with larger amounts of inhibitor have a propensity to cause inversion of oil and aqueous layers when centrifuged. Radioactive counts obtained without inhibitor were set as 0%, and the counts obtained in the presence of Me α-Man at 50 mM were set as 100% inhibition. The IC_{50} value is defined as the concentration of inhibitor that causes 50% inhibition, and was obtained from the inhibition curves generated by plotting percent inhibition versus inhibitor concentration in logarithmic scale. Examples of inhibition curves are shown in Figure 2. Most inhibitors were tested by at least two independent assays.

Man-ALK-HSA [Man-O$(CH_2)_5$CONH$(CH_2)_2$NH-HSA]:

This synthesis is summarized in Scheme 3.

N-(2,2-Dimethoxyethyl)-6-hydroxyhexanamide (**1**): The method of Zhang and Kovac[34] was used for preparation of **1**. ε-Caprolactone (50 g, 0.44 mol) and 2-aminoacetaldehyde dimethylacetal (5.2 mL, 47.6 mmol) were mixed and kept at room temperature for one week, during which time the formation of product was monitored by TLC (ethyl acetate/acetone, 1:1) by using dinitrophenylhydrazine (DNPH) spray to develop the plates. The reaction mixture was fractionated on a column of Sepadex LH-20 (5×190 cm) in three batches with 95% ethanol as eluant. Fractions were analyzed by TLC, and those containing product **1** were combined and evaporated. The yield of syrupy **1** was 98% based on 2-aminoacetaldehyde dimethylacetal. The ^{1}H NMR spectrum in $CDCl_3$ had the correct ratio of OCH_3 to methylene signals.

N-(2,2-Dimethoxyethyl)aminocarboxypentyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**3**): *O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)trichloroacetimidate (**2**) was prepared from 2,3,4,6-tetra-*O*-acetylmannopyranose and trichloroacetonitrile with DBU as the catalyst.[35] $BF_3\cdot OEt_2$ (0.32 mL, 2.54 mmol) was added at 0 °C to a mixture of **1** (8.7 g, 39.9 mmol), **2** (6.06 g, 12.7 mmol), and 4 Å molecular sieves in dry dichloromethane under nitrogen atmosphere. After one night at room temperature, triethylamine (2.1 mL) was added and the



Scheme 3. Preparation of Man-ALK-HSA. Conditions: a) $BF_3 \cdot OEt_2$, CH_2Cl_2, 0 °C → RT, overnight; b) TEA, MeOH, RT, 22 h; c) 50 mM aq TFA, heat, 20 min; d) pyridine – borane (pH 7.0), RT, 48 h. TEA = triethylamine, TFA = trifluoroacetic acid.

precipitate was filtered off. The filtrate was washed with saturated $NaHCO_3$, dried with anhydrous Na_2SO_4, filtered, and evaporated. The product was purified by gel filtration through the Sephadex LH-20 as described above for preparation of **1**. The correct fractions (as monitored by TLC) were pooled and evaporated to yield **3** (2.84 g, 5.49 mmol). NMR analysis revealed that the product was not homogeneous, even though it appeared as a single spot on TLC (R_f = 0.25; toluene/acetone (1:4)). However, the two products could be separated after *O*-deacetylation as described below.

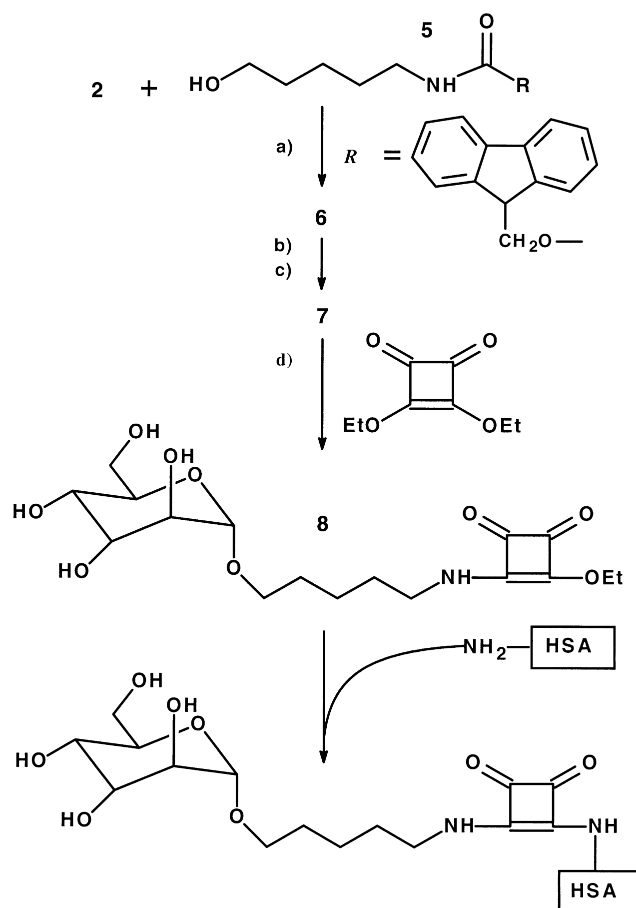
N-(2,2-Dimethoxyethyl)aminocarboxypentyl α-D-mannopyranoside (**4**): The product obtained above (210 mg, 0.41 mmol) was deacetylated in dry methanol (3 mL) containing 10% triethylamine. After 22 h at room temperature, the reaction mixture contained essentially two products of equal charring intensity by TLC (R_f = 0.45 and 0.3; chloroform/methanol, (5:1 v/v)). The mixture was evaporated and separated by silica gel chromatography with chloroform/methanol (5:1) as the eluant. NMR spectra showed that the low R_f material was the desired glycoside **4**, and its yield was estimated to be approximately 48%. ^{1}H NMR (300 MHz, D_2O): δ = 5.11 (d, 1 H, J(1,2) = 2.42 Hz; H-1), 4.13 (dd, 1 H, J(2,3) = 3.82 Hz; H-2), 4.08 (t, 1 H; CH_2CH), 3.43 – 3.28 (m, 4 H; H-3, H-4, H-6ab), 3.22 (t, 2 H; OCH_2), 3.09 (s, 6 H; OCH_3), 3.00 (d, 2 H; $CONHCH_2$), 2.94 (m, 1 H; H-5), 1.88 (t, 2 H; CH_2CONH), 1.34 (s, 3 H; CH_3), 1.30 – 1.05 (m, 6 H, CH_2) ppm.

Conjugation of **4** to HSA: ω-Acetal glycoside **4** (105 mg, 0.3 mmol) was converted into the corresponding aldehyde by heating it in 50 mM trifluoroacetic acid (1 mL) in water at 100 °C for 20 min. The mixture was coevaporated with dry ethanol, and the residue was used for conjugation to the protein. A two-fold excess of pyridine – borane (relative to the aldehydo compound) was added to the mixtures containing HSA (100 mg, 1.45 μmol) and either 148 or 74 μmol of the aldehyde in 0.2 M sodium phosphate buffer (pH 7.0; 0.7 mL). After 48 h at room temperature, the mixtures were dialyzed against water in the cold after adding 0.5 M NaCl into the dialysis tubing, and lyophilized. The mannose incorporations for the above two preparations determined by the phenol – sulfuric acid assay and Bradford assay were 21 and 11 mol per mol of HSA, respectively. Man_{73}-ALK-HSA and Man_{18}-ALK-HSA were prepared similarly, but with a 10-fold lower amount of HSA and 10-fold lower overall concentration of reactants with 300- and 1000-fold molar excess of the aldehyde over HSA.

Man-SQA-HSA [Man-O(CH_2)$_5$NH(C_4O_2)NH-HSA]:

This synthesis is summarized in Scheme 4.

5-*N*-(9-Fluorenylmethoxycarbonyl)aminopentyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**6**): 5-*N*-(9-Fluorenylmethoxycarbonyl)pentanol (**5**) was prepared by reacting 5-aminopentanol with 1.1 equiv of Fmoc chloride in dioxane/water (1:1) containing 2 mol equiv of sodium carbonate at 0 °C. After stirring at room temperature overnight, the mixture was partitioned between chloroform and water, and the organic layer was washed with cold H_2SO_4 (0.5 M) and NaCl (1.5 M), dried (Na_2SO_4), filtered, and evaporated. The residue was crystallized from chloroform/hexanes to afford **5** in 92% yield. The ^{1}H NMR spectrum in $CDCl_3$ indicated a correct ratio of aromatic to methylene proton signals. A mixture containing glycosyl donor, **2** (3.06 g, 6.4 mmol), glycosyl acceptor **5** (2.0 g, 6.15 mmol), and 4 Å molecular sieves in dichloromethane (10 mL) was cooled to 0 °C, and $BF_3 \cdot OEt_2$ (0.16 mL,



Scheme 4. Preparation of Man-SQA-HSA. Conditions: a) $BF_3 \cdot OEt_2$, CH_2Cl_2, 0 °C → RT, 3.5 h; b) TEA, MeOH, RT, 48 h; c) piperidine, MeOH, RT, 48 h; d) triethylamine carbonate buffer (pH 7.3), RT, overnight; e) triethylamine carbonate buffer (pH 9.0), RT, 18 h.

1.28 mmol) was added. The mixture was stirred for 3.5 h at room temperature, after which time triethylamine (1.1 mL) was added and the precipitate was filtered off through Celite. The filtrate was washed with saturated $NaHCO_3$ and 1.5 M NaCl, and dried (Na_2SO_4). After filtration and evaporation, the resulting syrup was purified by silica gel chromatography, with successive elution with 4:1 and 2:1 (v/v) toluene/ethyl acetate to yield pure **6** (1.92 g, 48%). $R_f = 0.7$ (toluene/ethyl acetate (1:1)); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.79-7.14$ (m, 8H; aromatic H), 5.37 (dd, 1H, $J(3,4) = 9.9$ Hz; H-3), 5.29 (t, 1H; H-4), 5.25 (dd, 1H, $J(2,3) = 3.3$ Hz; H-2), 4.82 (d, 1H, $J(1,2) = 1.6$ Hz; H-1), 4.40 (d, 2H; CH_2-Fmoc), 4.30 (dd, 1H, $J(5,6a) = 5.3$, $J(6a,6b) = 12.2$ Hz; H-6a), 4.23 (t, 1H; CH in Fmoc), 4.12 (dd, 1H; H-6b), 4.00 (m, 1H, $J(5,6b) = 2.5$ Hz; H-5), 3.75–3.67 and 3.54–3.43 (m, 2H; OCH_2), 3.25–3.19 (m, 2H; CH_2NH), 2.17, 2.11, 2.05, and 2.00 (4s, 12H; 4 $OCOCH_3$), 1.74–1.40 (m, 6H, CH_2) ppm.

5-Aminopentyl α-D-mannopyranoside (**7**): Compound **6** (1.04 g, 1.59 mmol) was first *O*-deacetylated by treating it in a mixture of dry methanol (4 mL) and triethylamine (1 mL) for 48 h at room temperature. The mixture was evaporated, and the residue was stirred for 48 h at room temperature overnight in a mixture of dry methanol (7 mL) and piperidine (1 mL) to remove the Fmoc group. The white solid formed was filtered off, and the filtrate was treated with activated charcoal, filtered, and evaporated to give a yellow syrup. The syrup was dissolved in water (5 mL) and purified by passing it through a 150-mg Carbograph solid-phase extraction cartridge (Alltech, Deerfield, IL), washing it with water, and then eluting with water/methanol (95:5) to give pure **7** in 98% yield. $R_f = 0.5$ (isopropanol/acetic acid/water (2:1:1)); ^{1}H NMR (300 MHz, D_2O): $\delta = 4.83$ (s, 1H; H-1), 3.89–3.49 (m, 7H; H-2, H-3, H-4, H-5, H-6ab, and 1H of OCH_2), 3.22 (m, 1H; OCH_2), 2.92 (m, 2H; CH_2NH), 1.65–1.32 (m, 6H; CH_2) ppm.

Conjugation of **7** to HSA through a squaric acid derivative: 3,4-Diethoxy-3-cyclobuten-1,2-dione (diethyl squarate, 8.6 µL, 58 µmol) was added to a solution of **7** (14 mg, 52.8 µmol) in 0.1 M triethylamine carbonate buffer (pH 7.3; 0.5 mL), and the mixture was left to stand at room temperature overnight. TLC in *n*-butanol/ethanol/water (2:1:1) showed two char-positive spots ($R_f = 0.78$ and 0.44), of which the upper spot (≈ 40%) was determined to be the amino-reactive species (results not shown). The mixture was extracted with diethyl ether (3 mL), the aqueous solution was evaporated, and the resulting syrup containing *N*-(2-ethoxy-3,4-dioxo-1-cyclobuten)aminopentyl α-D-mannopyranoside (**8**) was immediately dissolved in 0.1 M triethylamine (1 mL). The solution was adjusted to pH 9.0 by addition of dry ice and then added to HSA (50 mg, 0.625 µmol). The conjugation reaction was carried out in the dark for 18 h at room temperature. The mixture was dialyzed against water with 0.5 M NaCl added to the dialysis tubing. Lyophilization yielded Man-SQA-HSA (41 mg). The incorporation of mannose was 19 mol per mol of HSA. Man_{32}-SQA-HSA (53 mg) was prepared from 50 mg (0.63 µmol) of HSA and 1 mmol of **7** using the same reaction scheme.

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