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Inhibition of the type 1 fimbriae-mediated adhesion of *Escherichia coli* to erythrocytes by multiantennary α-mannosyl clusters: The effect of multivalency

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α-Mannosyl glycoclusters and glycodendrimers were tested as multivalent inhibitors of the type 1 (mannose-specific) fimbriae of a recombinant *E. coli* HB101 strain. Inhibition of haemagglutination of guinea pig erythrocytes was determined on microtiter plates. The effect of multivalency is pronounced for up to three mannosyl residues in the molecule, whereas larger derivatives do not have an appreciable effect on binding to the fimbrial carbohydrate binding domain. The best glycoclusters tested reach the binding potency of the known potent inhibitor pNPMan (3). The results support the idea of a monovalent recognition site at the adhesive protein FimH, which might best accommodate molecules with the size of a trisaccharide or those which expose up to three α-mannosyl residues, such as the glycocluster 8. The results obtained with the thiourea-bridged α-mannosyl clusters, possessing defined sugar valencies, facilitate the development of high affinity inhibitors of the fimbrial lectin on type 1 fimbriae.

Keywords: **antiadhesives, glycoclusters and glycodendrimers, mannose sensitive adhesion, type 1 fimbriae, *Escherichia coli***

Introduction

Carbohydrate-protein interactions are of enormous importance for cell–cell communication in a large number of physiological processes [1, 2]. However, they also play a crucial role in pathogenic events such as microbial infection [3–5]. In the majority of cases, adherence of the microbe to the host cell is a crucial step prior to successful infection of the cell. Bacteria carry specific lectins at organelles on their surfaces called fimbriae or pili. Bacterial adhesion is accomplished by the interaction of fimbrial lectins with surface oligosaccharide structures of the host cell. The structural characterization of carbohydrate binding sites of bacterial lectins may lead to custom-designed carbohydrate derivatives which can effectively inhibit a specific lectin-sugar interaction. This may eventually help to prevent bacterial infection.

Fimbriae are classified according to their carbohydrate specificities. Type 1 fimbriae recognize mannose-containing ligands. They were termed mannose-sensitive (MS) as they can be inhibited by mannose derivatives, typically shown with mM concentrations of methyl α-D-mannoside. Type 1 fimbriae are widely distributed among enterobacteria of which one of the most important is *Escherichia coli*. *E. coli* cells express between 100 and 400 type 1 fimbriae on their surfaces. These are composed of a repeating major subunit, FimA (approx. 18 kD), accounting for more than 98% of the fimbrial protein [6, 7]. The FimH protein (approx. 32 kD) is the mannose-specific adhesin [8], where the carbohydrate-binding site is located. FimH is localized at the lateral ends of the fimbriae (rather than at the very tips) and is also distributed along the shaft of the fimbriae [9, 10]. To date it remains uncertain if all FimH sites of the fimbriae possess the same affinity to mannose structures [11].

E. coli is an abundant inhabitant of the intestinal tract of mammals and has been recognized as an important pathogen involved in a variety of intestinal and extra-intestinal diseases. These include urinary tract infections, sepsis, bacteria-related traveler's diarrhea and newborn meningitis [12]. Fimbrial adhesins play a role in pathogenicity and virulence, and also enable the bacteria to attach to eukaryotic cells. Adherence of the bacteria to their potential host cells protects them from being swept away by the normal cleansing mechanisms operating on mucosal surfaces such as urinary flow, thereby increasing their ability

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to colonize the epithelia, multiply and invade the host. Different types of fimbriae are considered to be important in urinary tract infections, including P fimbriae and type 1 fimbriae; the latter also facilitate the adhesion of other fimbriated strains and thus contribute to virulence [13].

Firm microbial adhesion is a result of multiple ligand-receptor interactions. Multivalency is of general importance for effective carbohydrate-protein interactions and this is based on oligo-antennary saccharide structures on cell surface glycoconjugates on one hand and on clustered lectins on the other [14]. Multivalent glycomimetics have consequently served as excellent inhibitors of carbohydrate-protein interactions in many cases. This has impressively been demonstrated for mammalian hepatic lectins and cluster galactosides by Y.C. Lee and coworkers [15–17]. Since then a variety of further multivalent glycomimetics have been designed as inhibitors of carbohydrate-protein interactions. Among those, so-called glycodendrimers have received special attention because they combine high sugar valencies with an exactly defined structure in every case [18, 19]. We have contributed to the synthesis of multi-antennary glycomimetics by introducing thiourea-bridged glycoclusters [20]. Here we used this chemistry to investigate the structural requirements of type 1 fimbriae on *E. coli* with synthetic multi-antennary carbohydrate derivatives which carry a precise number of α-mannosyl residues (depicted in Figure 1b and 1c) *in vitro*.

Experimental procedures

General methods

Flash chromatography was performed on silica gel 60 (0.040–0.063 mm, Merck, Germany). For size exclusion chromatography, Sephadex G-15 was used with a 20 mM aqueous NH_4HCO_3-buffer as eluent. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AMX 400 (400 MHz for ^1H and 100.62 MHz for ^{13}C NMR) or a DRX 500 (500 MHz for ^1H and 125.76 MHz for ^{13}C NMR if not otherwise stated). Chemical shifts are given relative to $CDCl_3$ (7.24 ppm for ^1H and 77.00 for ^{13}C NMR), D_4-methanol (3.33 ppm for ^1H and 49.00 for ^{13}C NMR), (D_6-dimethyl sulfoxide (DMSO)) (2.49 ppm for ^1H and 39.7 for ^{13}C NMR), and in case of D_2O relative to added D_6-acetone (2.04 ppm for ^1H and 29.8 for ^{13}C NMR). Where necessary two-dimensional NMR experiments were performed for full assignment of the peaks. Optical rotation values were measured on a Perkin-Elmer polarimeter 243 or 341. Fast atom bombardment (FAB)-mass spectra were recorded on a VG Analytical 70-250S and electrospray-MS was performed on a Finnigan MAT 95.

Carbohydrate derivatives and controls

D-Mannose and methyl α-D-mannoside were purchased from Merck (Germany), p-nitrophenyl α-D-mannoside (pNPMan) was purchased from SENN Chemicals (Switzerland), p-nitrophenyl α-D-glucopyranoside (pNPGlc) was from Fluka (Germany), the trivalent core amine tris(2-aminoethyl)amine from Aldrich (Germany). Thiourea-bridged glycoclusters **6–9**, **11–13**, **15** and **16** were synthesized according to a published general procedure [20]. The core polyamines such as **14**, which were used for α-mannosyl clustering, were obtained following established protocols for the synthesis of Starburst® dendrimers [21]. All tested compounds are depicted in Figure 1.

Synthesis of the monovalent thiourea derivative **4** and the divalent α-D-mannopyranosyl ligands **6** and **7**

The synthesis of α-mannosyl clusters from α-mannosyl isothiocyanate and branched polyamines has been described in the literature for trivalent, hexavalent and octavalent examples [20]. The same procedure was carried out with α-mannosyl isothiocyanate and methylamine (8 M solution in ethanol), ethylenediamine or diaminohexane, to yield **4**, **6** and **7**, respectively, after deacetylation with sodium methanolate in methanol (Figure 2). The deprotected compounds were purified by gel permeation chromatography on Sephadex G-15.

Spectroscopic and analytical data for **4** and the divalent clusters **6** and **7**

Methylthiourylene α-D-mannopyranoside (**4**)

$[\alpha]_D^{20}$ + 107.3° (c = 1.00, H_2O); ^1H NMR (400 MHz, D_2O) δ 5.10 (br s, 1H, H-1), 3.71 (br s, 1H, H-2), 3.51 (m, 2H, H-3, H-6), 3.41 (dd, 1H, H-6′), 3.33 (dd ≈ t, 1H, H-4), 3.21 (ddd, 1H, H-5), 2.71 (s, 3H, NCH_3) ppm; $J_{3,4}$ = 9.2, $J_{4,5}$ = 9.2, $J_{5,6}$ = 5.6, $J_{6,6'}$ = 12.2 Hz; ^{13}C NMR (100.62 MHz, D_2O) δ 182.10 (C = S), 82.68 (C-1), 73.80 (C-5), 70.79 (C-2), 69.77 (C-3), 67.24 (C-4), 61.14 (C-6), 32.00 (NCH_3) ppm; FAB-MS 253.2 $[M + H]^+$ (252.07 calculated for $C_8H_{16}N_2O_5S$).

1,2-Bis-(α-D-mannopyranosylthiourylene)ethane (**6**)

$[\alpha]_D^{20}$ + 41.7° (c = 0.50, H_2O); ^1H NMR (400 MHz, D_2O) δ 5.26 (br s, 2H, 2 H-1), 3.82 (dd ≈ s, 2H, 2 H-2), 3.70-3.47 (m, 10H, 2 H-3, 2 H-6, 2 H-6′, 2 CH_2), 3.42 (dd ≈ t, 2H, 2 H-4), 3.34 (m, 2H, 2 H-5) ppm; $J_{3,4}$ = 9.7, $J_{4,5}$ = 9.7, $J_{5,6}$ = 2.5 $J_{5,6'}$ = 6.1, $J_{6,6'}$ = 12.2 Hz; ^{13}C NMR (62.89 MHz, D_2O) δ 184.65 (C = S), 85.00 (2 C-1), 76.12, 72.99, 71.99, 69.45 (2 C-2, 2 C-3, 2 C-4, 2 C-5), 63.46 (2 C-6), 46.66 (2 CH_2N) ppm; FAB-MS 503.3 $[M + H]^+$ (502.14 calculated for $C_{16}H_{30}N_4O_{10}S_2$).

1,2-Bis-(α-D-mannopyranosylthiourylene)hexane (**7**)

$[\alpha]_D^{20}$ + 35.3° (c = 0.50, MeOH); ^1H NMR (400 MHz, D_2O) δ 5.35 (br s, 2H, 2 H-1), 3.94 (dd ≈ s, 2H, 2 H-2), 3.70-3.47 (m, 6H, 2 H-3, 2 H-6, 2 H-6′), 3.42 (dd ≈ t, 2H, 2 H-4), 3.34 (m, 6H, 2 H-5, 2 NCH_2), 1.51 (m, 4H, 2 NCH_2CH_2), 1.27 (m, 4H, 2 $NCH_2CH_2CH_2$) ppm; $J_{3,4}$ = 9.7, $J_{4,5}$ = 9.7, $J_{5,6}$ = 2.1 $J_{5,6'}$ = 5.6, $J_{6,6'}$ = 11.7 Hz; ^{13}C NMR (62.89 MHz, D_2O) δ 183.95 (C = S), 84.06 (2 C-1), 76.22, 72.98, 71.96, 69.52

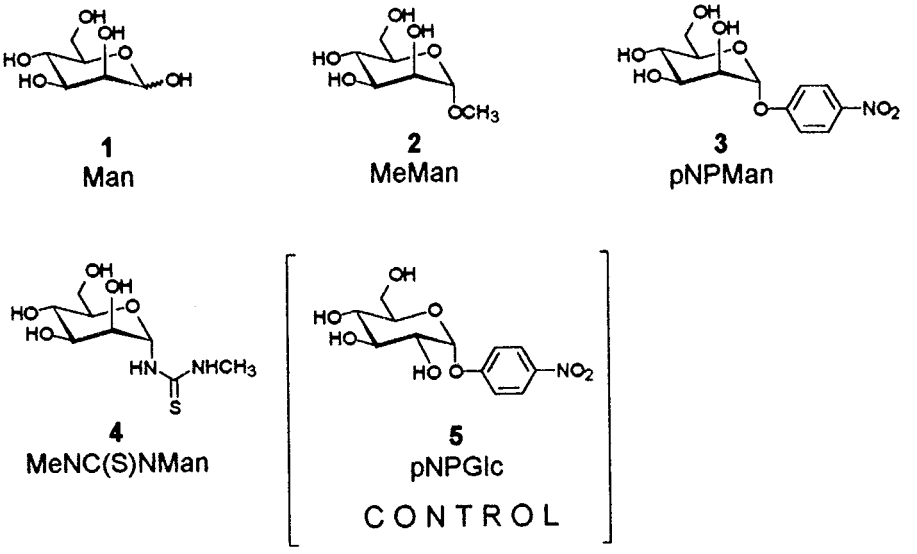

Figure 1a. Monovalent reference compounds.

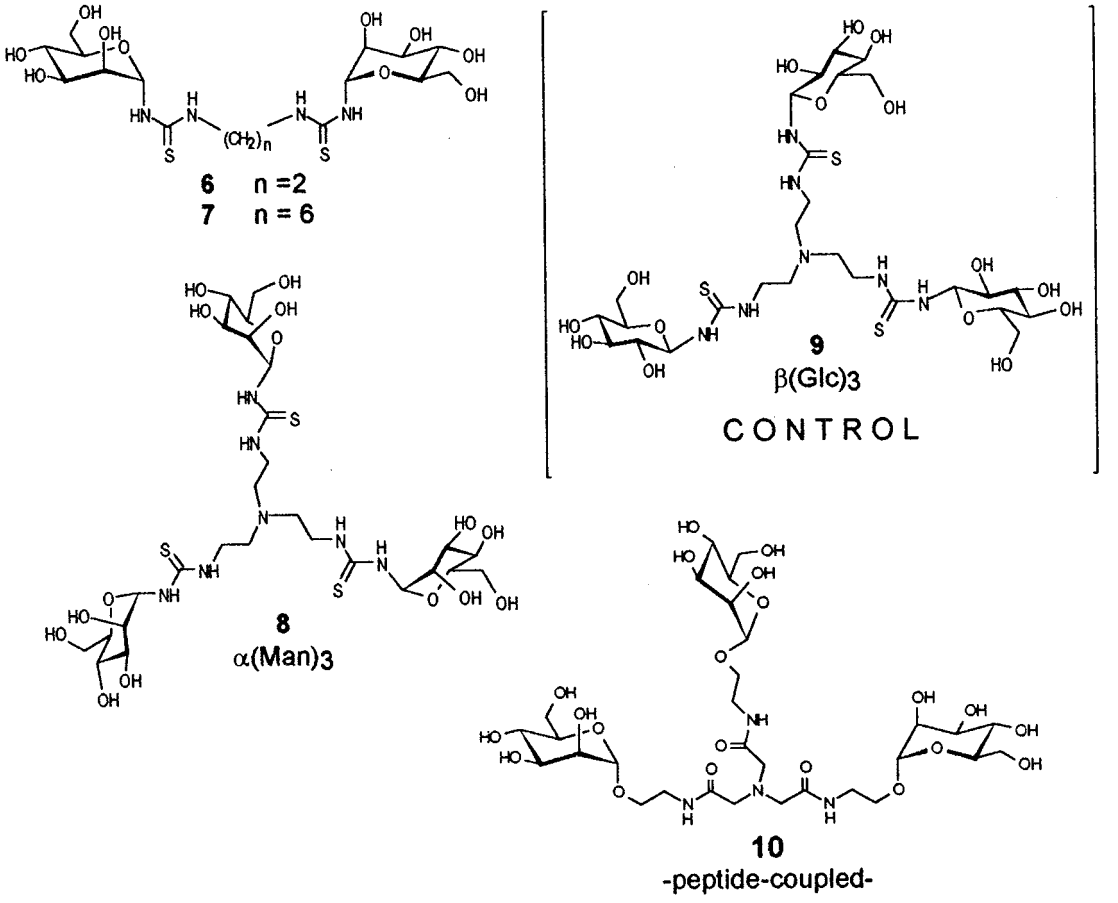

Figure 1b. Divalent and trivalent reference compounds.

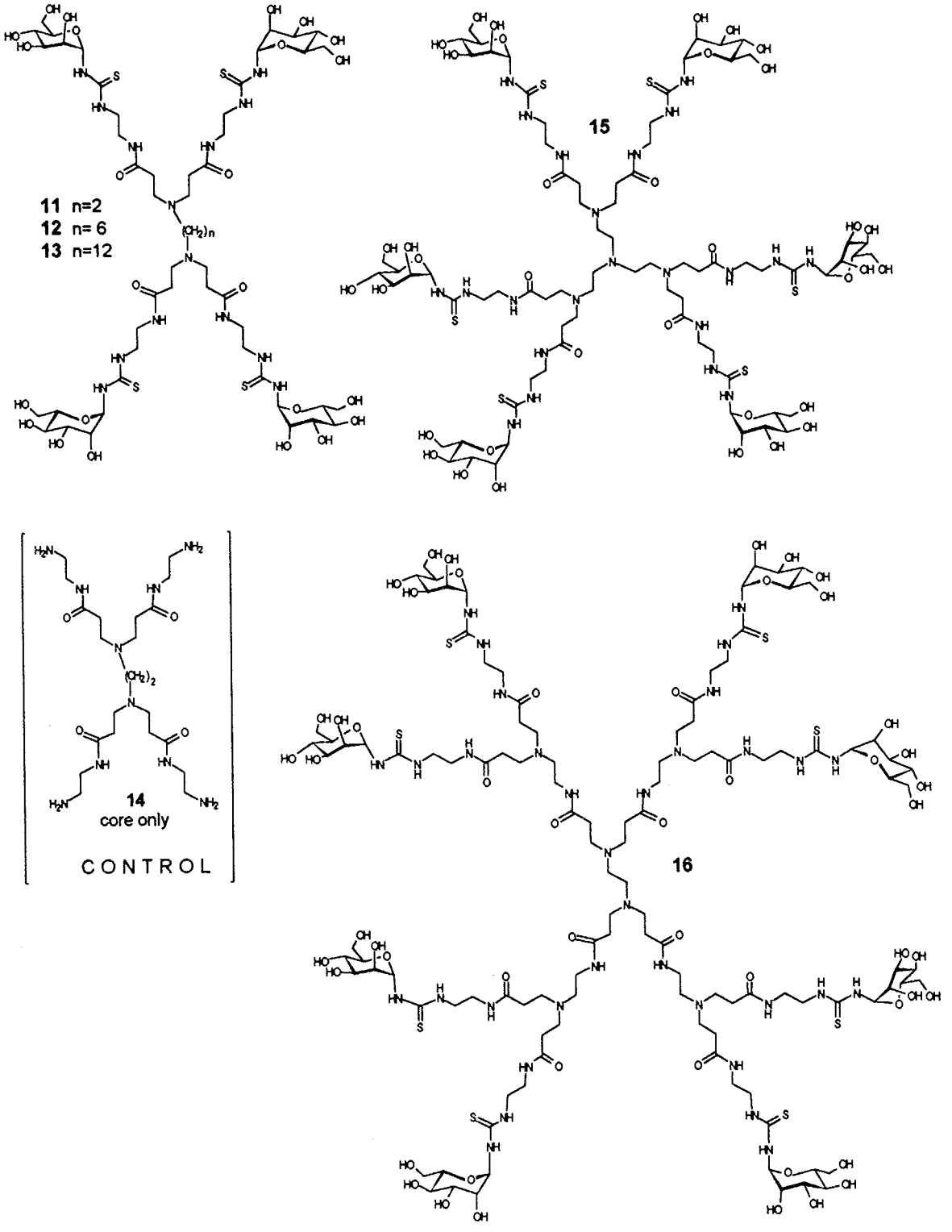

Figure 1c. Tetra-, hexa- and octavalent glycoclusters and glycodendrimers.
Figure 1. Structures of all tested compounds.

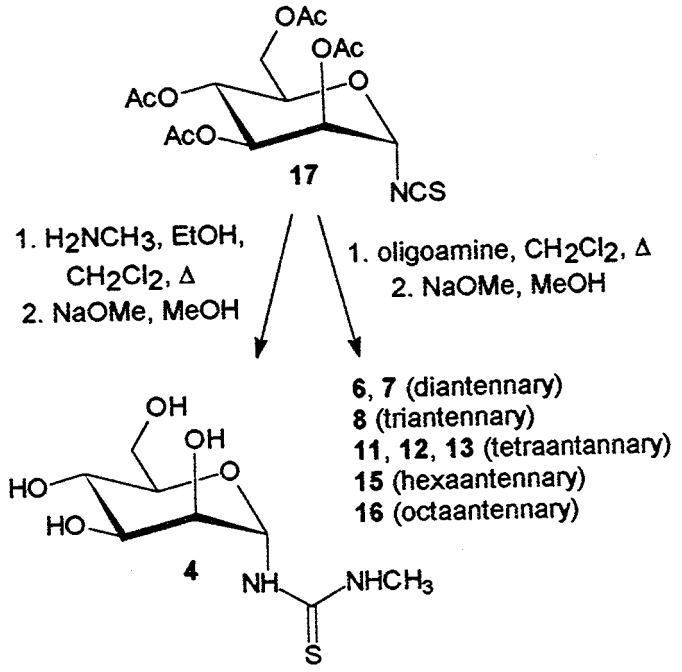

Figure 2. Synthesis of the monovalent reference compound **4** and the thiourea-bridged *a*-mannosyl clusters.

(2 C-2, 2 C-3, 2 C-4, 2 C-5), 62.96 (2 C-6), 45.36 (2 CH_2N), 29.64 (2 $NCH_2CH_2CH_2$), 28.00 (2 $NCH_2CH_2CH_2$) ppm; ESI-MS (+ VE, + HMR): 581.3 $[M + Na]^+$ (581.19 calculated for $C_{20}H_{38}N_4O_{10}S_2Na$).

2-Amino-ethyl 2,3,4,6-tetra-O-benzoyl-α-D-mannopyranoside (**19**)

2-Azido-ethyl α-D-mannopyranoside [22] (**18**, 1.00 g, 4.00 mmol), benzoyl chloride (2.8 g, 2.3 ml, 5 equiv.) and a catalytic amount of (DMAP) 4-dimethylamino-pyridine were dissolved in pyridine (50 ml) and the mixture was stirred at room temperature for 3 h. The solvent was evaporated by co-distillation with toluene and the obtained protected azide, 2-azido-ethyl 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranoside, was purified by flash chromatography (ethylacetate/toluene, 15:1). Subsequent hydrogenation with Pd-C (10%) in a 1:1-ethyl acetate-ethanol mixture afforded the benzoylated amine **19** (1.85 g, 2.89 mmol, 72% overall yield). $[\alpha]_D^{20}$ −21.5° (c = 0.50, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12-7.22 (m, 20 H, aryl-H), 6.12 (dd ≈ t, 1H, H-4), 5.93 (dd, 1H, H-3), 5.73 (dd, 1H, H-2), 5.16 (dd, 1H, H-1), 4.70 (dd, 1H, H-6), 4.51-4.43 (m, 2H, H-5, H-6′), 3.91 (ddd, 1H, OCH_aH_b), 3.80 (ddd, 1H, OCH_aH_b), 3.10-2.92 (m, 2H, CH_2N), 1.62 (br s, NH_2) ppm; $J_{1,2} = 1.5$, $J_{2,3} = 3.1$, $J_{3,4} = 10.2$, $J_{4,5} = 10.2$, $J_{5,6} = 2.6$, $J_{6,6'} = 12.2$, $J_{Ha,Hb} = 10.2$, $J_{Ha,CH2} = 5.08$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 166.16, 165.60, 165.48, 165.41 (4 C = O), 133.03, 132.99, 132.75, 132.62 (4 p-aryl-CH), 129.41, 129.37, 129.34, 129.29 (8 aryl-H), 128.87, 128.54, 128.21 (4 aryl-C), 97.47 (C-1), 70.45 (OCH_2), 70.02, 69.67, 68.53, 66.51 (C-2, C-3, C-4, C-5), 62.45 (C-6), 41.51 (CH_2NH_2) ppm; FAB-MS: 640.6 $[M + H]^+$ (639.65 calculated for $C_{36}H_{33}NO_{10}$).

Nitrilotriacetic acid tris[2-(2,3,4,6-tetra-O-benzoyl-α-D-mannopyranosyl)ethyl] amide (**20**)

Titriplex I (76.5 mg, 0.4 mmol) and the amino-functionalized mannoside **19** (850 mg, 1.3 mmol) were dissolved in DMF (20 ml), and DCC (246 mg, 1.2 mmol) and 1-HOBT (184 mg, 1.2 mmol) were added. The mixture was stirred for 6 days at room temperature, evaporated to dryness and the residue was dissolved in CH_2Cl_2. Subsequently the dicyclohexyl urea by-product was removed by filtration and the remaining solution was evaporated *in vacuo*. Flash chromatography on silica gel (ethyl acetate/toluene, 5:2) afforded the protected trisamide **20** (450 mg, 0.22 mmol, in 55%). $[\alpha]_D^{20}$ −37.8° (c = 1.00, CH_2Cl_2); ^{1}H NMR (500 MHz, DMSO) δ 8.10-7.22 (m, 60H, aryl-H), 7.19-7.13 (m, 3H, 3 NH), 6.00 (dd, 3H, 3 H-4), 5.80 (dd, 3H, 3 H-3), 5.68 (dd, 3H, 3 H-2), 5.21 (d, 3H, 3 H-1), 4.68-4.60 (m, 3H, 3 H-6), 4.56-4.49 (m, 6H, 3 H-5, 3 H-6′), 3.85 (ddd, 3H, 3 OCH_aH_b), 3.69 (ddd, 3H, 3 OCH_aH_b), 3.55-3.35 (m, 12H, 3 $NHCH_2$, 3 NCH_2) ppm; $J_{1,2} = 1.9$, $J_{2,3} = 3.2$, $J_{3,4} = 10.1$, $J_{4,5} = 10.1$ Hz; ^{13}C NMR (125.76 MHz, DMSO) δ 171.66 (3 CONH), 166.18, 165.86, 165.61, 165.54 (4 × 3 CO), 143.89 (3 aryl-C), 134.60-123.89 (15 aryl-C), 98.09 (3 C-1), 71.28 (3 C-3), 70.95 (3 C-2), 69.26 (3 C-5), 67.66 (3 OCH_2), 67.41 (3 C-4), 63.26 (3 C-6), 59.03 (3 $NHCH_2$), 39.25 (3 NCH_2) ppm; FAB-MS: 2057 $[M + H]^+$, 2079 $[M + Na]^+$, 2095 $[M + K]^+$ (2056.07 calculated for $C_{114}H_{102}N_4O_{33}$).

Nitrilotriacetic acid tris[2-(α-D-mannopyranosyl)ethyl] amide (**10**)

The acylated trisamide (**20**, 120 mg, 0.06 mmol) was deprotected in dry methanol and sodium methanolate according to the standard Zemplén procedure. After neutralization and evaporation of the solvent, the product was purified by gel permeation chromatography on Sephadex G-15. Lyophilization gave **10** (40 mg, 0.05 mmol, 85%) as a syrup. $[\alpha]_D^{20}$ + 9.8° (c = 0.50, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.81 (d, 3H, 3 H-1), 3.86 (dd, 3H, 3 H-2), 3.81 (dd, 3H, 3 H-6), 3.78-3.69 (m, 6H, 3 OCH_aH_b, 3 H-3), 3.67 (dd, 3H, 3 H-6′), 3.63 (dd ≈ t, 3H, 3 H-4), 3.54 (m, 6H, 3 H-5, 3 OCH_aH_b), 3.49-3.39 (m, 6H, 3 $NHCH_2$), 3.38 (s, 6H, 3 NCH_2) ppm; $J_{1,2} = 1.5$, $J_{2,3} = 3.1$, $J_{3,4} = 10.1$, $J_{4,5} = 10.1$, $J_{5,6} = 2.0$, $J_{6,6'} = 12.2$ Hz; ^{13}C NMR (100.61 MHz, D_2O) δ 175.28 (3 C = O), 102.12 (3 C-1), 75.34 (3 C-5), 73.03 (3 C-3), 72.51 (3 C-2), 69.21 (3 C-4), 68.18 (3 OCH_2), 63.41 (3 C-6), 60.42 (3 NCH_2), 41.33 (3 $NHCH_2$) ppm; FAB-MS: 807.6 $[M + H]^+$, 829.5 $[M + Na]^+$ (806.33 calculated for $C_{30}H_{54}N_4O_{21}$).

Cultivation of bacteria

A recombinant type 1 fimbriated *E. coli* strain, *E. coli* HB101 (pPKl4) [23], was used for the tests. *E. coli* HB

101 (pPKL4) was cultivated on LB (5 g NaCl, 10 g tryptone and 5 g yeast extract were dissolved in distilled deionized water up to 1000 ml total volume; then 12 g agar was added and the mixture was autoclaved for 15 min at 121 °C) [16] + ampicillin (100μg ml^{-1}) plates for 1 day at 37 °C, harvested, washed twice with PBS (phosphate buffered saline) (Saline: 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \times 2H_2O$ and 0.2 g KH_2PO_4 were dissolved in distilled deionized water up to 1000 ml total volume (pH 7.0)) [17] and suspended to a concentration of 10 mg ml^{-1} wet weight, corresponding to about 3×10^9 (between 1 and 4×10^9) cfu ml^{-1} (cfu = colony forming units).

Blood

Guinea pig blood was freshly isolated and stabilized by 3.3% sodium citrate. After centrifugation (10 min, 2500 upm, on a Herens Christ Labofuge I), the sediment was carefully suspended in PBS and this procedure of centrifugation and washing of the sediment was repeated twice. The thoroughly washed sediment of erythrocytes is referred to as packed erythrocytes. 5 ml of packed erythrocytes were suspended in 95 ml PBS and stored at 4 °C.

Haemagglutination of guinea pig erythrocytes

The carbohydrate derivatives (see Figure 1) were suspended in water and were serially diluted twofold in distilled deionized water or PBS. As the solubility of compounds **3**, **5** and **16** was limited in water, up to 20% methanol was used for the preparation of the stock solutions. This amount of methanol is not detrimental to erythrocytes *in vitro*.

Haemagglutination (= erythrocyte agglutination) in V-microtiter plates

5 μl of the sugar solutions (serially diluted) were thoroughly mixed with 5 μl bacteria in wells of V-shaped microtiter plates. Then 5 μl guinea pig erythrocytes were added and the haemagglutination was read after approximately 15 min at room temperature on a microtiter plate mirror (Cooke Mikrotiter System, Dyntech, England). Storing the plates at least 30 min at 4 °C, putting them in an upright position at room temperature and reading the inhibition of haemagglutination again after 5 minutes ensured reproducible results in all cases. The '*haemagglutination inhibition titre*' was the lowest sugar concentration that inhibited haemagglutination.

Results

From prior work on the specificity of the carbohydrate binding site on type 1 fimbriae [24, 25], it is known that the mannosyl moieties must have α-configuration at the anomeric centre for good binding to the fimbrial lectin. The synthesized glycoclusters were designed with regard to this structural requirement.

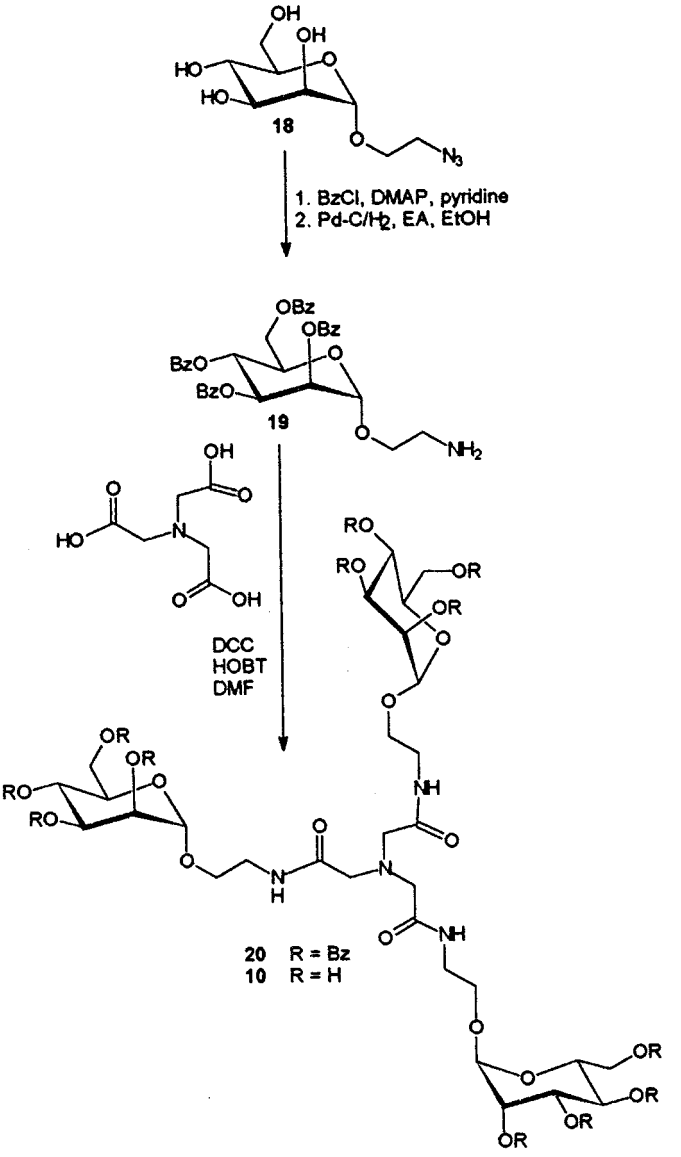

Figure 3. Synthesis of the peptide-linked glycocluster **10**.

Synthesis

The reaction between amines and isothiocyanates leads to thiourea derivatives in often close to quantitative yields. It can be successfully applied to the synthesis of multivalent glycomimetics using branched oligoamines and isothiocyanato-functionalized carbohydrate derivatives such as glycosyl isothiocyanates, giving rise to the thiourea-bridged glycoclusters and glycodendrimers [20] depicted in Figure 1b and 1c. The synthesis of the divalent, tri-, tetra-, hexa- and octa-antennary α-mannosyl derivatives **6–9, 11–13, 15** and **16** followed a published procedure which includes the coupling step and subsequent deprotection (Figure 2). This method also led to the monovalent thiourea-bridged mannosyl analog **4** which was prepared for comparison with the standard natural mannoside **2**.

To compare the structural effect of the thiourea-bridges in the synthesized sugar clusters with other types of linkages, the peptide-linked glycocluster **10** was prepared on the basis of a titriplex core (Figure 3). 2-Azido-ethyl α-D-mannoside **18**, which is easily available from D-mannose, was benzoylated and hydrogenated to give the amino-functionalized mannoside **19**. With benzoyl esters as protecting groups acyl migration during the reduction step was avoided. The amino-functionalized mannoside **19** was coupled to titriplex I following a classical peptide coupling procedure using dicyclohexyl carbodiimide (DCC) and 1-hydroxy-1H-benzotriazole (HOBT) leading to **20** in fair yield. Deprotection using Zemplén conditions gave the unprotected triantennary peptide cluster mannoside **10**. An alternative route to **10**, using the unblocked aminoethyl mannoside and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) as the coupling reagent failed to provide sufficient amounts of the product.

Inhibition experiments

The applied inhibition agglutination test is a classical test in immunology. Initially, the haemagglutination with guinea pig erythrocytes and *E. coli* was examined along with the agglutination of yeast cells [26]. The relative inhibition titres (RITs) revealed analogous results in both tests. However, the haemagglutination test proved to be more sensitive and accurate to perform, so the yeast assay was omitted. All tests were independently performed three times with a fresh erythrocyte preparation in every case. The bacteria used were always grown from fimbriae-positive colonies. The results have to be considered as half-quantitative, as the minimum concentration for 100% inhibition of haemagglutination may be in the range between two concentrations corresponding to two adjacent wells of a dilution series on the microtiter plate. Nevertheless, the results obtained in this test were highly reproducible and the relative values and derived tendencies of binding potencies were identical for all tests performed.

The inhibition titres (ITs) are listed in Table 1. The monovalent reference compounds **1** and **2** have ITs in the mM range as also reported in the literature [24, 25]. Of those two, methyl α-D-mannoside (**2**) is generally taken as standard for relative calculations. The monovalent mannosylthiourea **4**, with an anomeric linkage analogous to the thiourea-bridged glycoclusters performed almost identically to **2**. The aromatic pNPMan (**3**) is known to be a much more potent inhibitor than **2**, with an IT in the μM range. Our focus was concentrated on whether non-aromatic multivalent α-mannosyl clusters could compensate for the effect of aromatic regions as present in pNPMan (**3**).

Indeed, the inhibition titres improved by up to a hundredfold, relative to **2**, using the di- and triantennary thiourea-bridged clusters **6–8** (Table 1). The divalent mannosyl derivative **7** with the long spacer showed better binding than **6**, the analogue with a shorter spacer. The trivalent thiourea-bridged cluster **8** showed further improved affinity, while the trivalent peptide-coupled cluster **10** had a remarkably higher IT than **8**.

Table 1. Inhibition of the haemagglutination of guinea pig erythrocytes by type 1 fimbriated *E. coli* HB101 pPKl4.

Compound tested	*Inhibition titer*[a] *[mM]*	*Relative inhibition titer (RIT)*	*RIT based on moles mannose*
monovalent references			
1 (D-mannose)	18	0.53	0.53
2 (methyl *α*-D-mannoside)	9.6	1	1
3 (pNP *α*-D-mannoside)	0.072	133	133
4 methylthiourylene *α*-D-mannoside	10.2	0.94	0.94
clusters			
6 divalent	0.32	30.6	15.3
7 divalent	0.13	74	37
8 trivalent	0.091	105.5	35.17
10 trivalent	0.97	9.9	3.3
11 tetravalent	0.26	39	9.75
12 tetravalent	0.26	39	9.75
13 tetravalent	0.26	39	9.75
15 hexavalent	0.091	105.5	17.58
16 octavalent	0.083	115.6	14.45
controls			
5 pNP-Glc	> 3.3[b]	no inhibition	
9 trivalent Glc	> 5[b]	no inhibition	
14 core	> 5[b]	no inhibition	

[a] average values from three independent tests
[b] higher concentrations were not tested

However, further increase in valency did not notably improve the binding potencies. The tetravalent clusters **11–13**, with different spacer lengths in the centre of the molecules, gave less advantageous values. The hexa- as well as octavalent clusters **15** and **16** reached the IT of the trivalent cluster **8**, all in the same range as pNPMan (**3**).

Non-specific binding effects could be due to parts of the molecule other than mannose. Therefore, control compounds **5** (which is the glucose analogue of **3**), **9** (which is the glucose analog of **8**) and **14** (resembling the functional features of the core molecules) were tested for their inhibitory potencies. None of the compounds showed inhibitory properties up to a concentration of 5 mM.

Discussion

The effect of oligoantennary saccharides has been investigated in the past with a number of high-mannose oligosaccharides [27, 28]. However, no clear conclusion could be

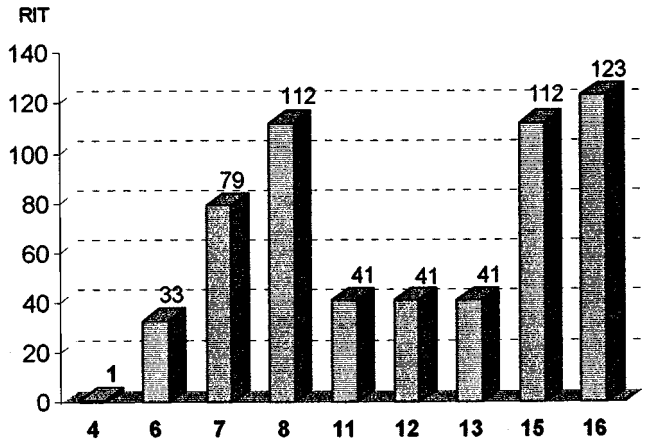

Figure 4. Relative inhibition titres (RITs) of the thiourea-bridged α-mannosyl clusters with the corresponding monomer **4** as standard (**4**: RIT ≡ 1).

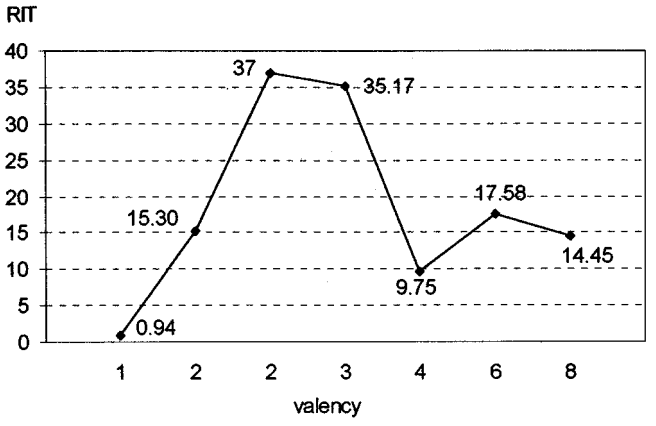

Figure 5. The effect of valency on binding potency

drawn from these results. From the data here, obtained with defined mannosyl cluster molecules, it can be concluded the clustering of α-mannosyl residues on a multivalent core molecule has a positive effect on binding up to a valency of three. In Figure 1 the relative inhibition potencies of all tested thiourea-bridged mannosyl clusters are compared, relative to the monovalent thiourea analog **4**. With the trivalent cluster **8** an increase in binding potency over 100-fold is achieved. This value can, however, not significantly be improved by the hexa- and octa-antennary mannose clusters **15** and **16**, while all tetra-antennary derivatives **11–13** even show reduced binding potencies compared to **8**. In Figure 2 the number of mannosyl residues in each tested derivative is taken into account for the calculation of RITs and this reflects the effect of carbohydrate valency in the glycoclusters. According to these data multivalency only effects binding potency of the molecules in the examined system up to a valency of three (compound **8**) with a 35-fold increase in RIT based on α-mannosyl moieties present in the molecule. Consequently, higher valent clusters show favorable results, most likely due to a statistical effect, such as a higher availability of α-mannosylresidues, while the effect of multivalency as shown in Figure 2 is limited.

For the interpretation of the data, non-specific interactions such as hydrophobic interactions and stacking effects (interactions of aromatic rings) have to be considered too. The known potent inhibitor of type 1 fimbriae mediated adhesion, p-nitrophenyl α-D-mannoside (**3**), binds approximately 100 times better than methyl α-D-mannoside (**2**). Also for other aromatic mannosides, such as the rather artificial p-methylumbelliferyl α-mannoside, excellent affinities have been determined [29]. Apparently, hydrophobic aromatic moieties of the inhibitor molecule can significantly contribute to binding. Together with other data this led to a model for the carbohydrate binding domain on type 1 fimbriae suggested by Ofek and Sharon, where the carbohydrate recognition domain has the approximate size of a trisaccharide with a hydrophobic region close to, or in, the binding pocket [30]. This model is partially supported by our results. If we consider an IT of 18 mM as the contribution of just one mannosyl residue (Table 1), the value 32×10^{-4} for the divalent cluster **6** seems to reflect binding of both mannosyl residues at the fimbrial carbohydrate recognition domain. Further improvement of binding in the case of the divalent molecule **7** might be due to additional hydrophobic interactions with the longer spacer chain. Consequently the data suggest that in the case of the trivalent glycocluster **8** not all three mannosyl residues were bound in the binding pocket. The binding potency of the tetravalent clusters **11–13** is in the range of the divalent clusters and this suggests that only two of the four α-mannosyl antennae are bound at the carbohydrate binding site of the fimbriae.

The significant IT-differences between the thiourea-bridged trivalent mannosyl cluster **8** and the peptide-coupled cluster **10** might be due to conformational differences of the molecules or other factors such as different abilities for hydrogen bonding. Molecular modeling should allow further insight into the conformational properties of **8** and **10** [31].

Conclusion

The results support the idea of a monovalent carbohydrate binding domain on type 1 fimbriae which does not accommodate bigger glycoclusters such as **15** or **16**. It might well have the approximate size of a trisaccharide or might recognize an ensemble of structural elements which is provided by certain trisaccharides as well as oligo-antennary glycoclusters such as **7** and **8**. Hydrophobic moieties in the inhibitor molecule add to the overall affinity, which may also be due to the binding to subsites, adjacent to the specific binding pocket. The non-carbohydrate core of the tested glycoclusters might contribute to non-specific bind-

ing in this way. A conclusive picture of the type 1 fimbrial carbohydrate recognition domain cannot yet be drawn. This topic appears to be currently under thorough investigation [32].

The pronounced effect of the p-nitrophenyl aglycon present in **3** can hardly be overcompensated by the tested non-aromatic glycoclusters. From the results shown it can be anticipated that the clustering of aromatic α-mannosides would lead to extremely potent inhibitors of haemagglutination with type 1 fimbriae. The employed chemistry can easily be modified to produce the suggested aromatic clusters. Work regarding this aspect is in progress [33].

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