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Functionalization of oligosaccharide mimetics and multimerization using squaric diester-mediated coupling

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Dedicated in memory of Professor Dr. Zoltán Györgydeák

Abstract—Functionalized carbohydrate-centered glycoclusters formed the starting material for the synthesis of tagged oligosaccharide and glycoconjugate mimetics, which were obtained by thiourea-bridging, peptide coupling and in particular squaric diester-mediated coupling. The latter method could also be utilized to provide new multivalent glycoconjugates, which were tested for their anti-adhesive properties in an ELISA with *Escherichia coli* bacteria.

Keywords: Multivalent neoglycoconjugates; Oligosaccharide mimetics; Fluorescence labeling; Squaric acid coupling

1. Introduction

Eukaryotic cells are covered by a complex macromolecular super-systems called the 'glycocalyx'.[1] This is made up by a highly diverse array of oligosaccharides, which are conjugated in glycolipids, glycoproteins and proteoglycans amongst others. To unravel the biological function of the glycocalyx and the details of the involved molecular mechanisms such as receptor–ligand interactions, functionalized model compounds are needed. With respect to the multivalency effects, which are important in glycobiology,[2] models of glycocalyx constituents have been designed as multivalent glycomimetics.[3]

Multivalent carbohydrate ligands are especially effective once they can be prepared and varied with relative easy whilst allowing mimicry of as many functional properties of the natural example structures as possible. Therefore, we have been interested in providing strategies for a straightforward and highly flexible preparation of oligosaccharide and glycoconjugate mimetics, and their multivalent analogs using a set of feasible building blocks.[4] In this context we have recently introduced carbohydrate-centered glycoclusters as oligosaccharide mimetics.[5] Utilizing the anomeric center as a site for regioselective modification of a basically multifunctional scaffold, the synthesis of tethered oligosaccharide mimetics such as the amino-functionalized cluster mannoside **1** (Scheme 1) was possible. Here we report on applications of **1** for the synthesis of fluorescence-labeled oligosaccharide mimetics, the synthesis of glycolipid mimetics and multimerization via squaric acid conjugation.

2. Results and discussion

2.1. Functionalization of the carbohydrate-centered glycocluster 1

It was our goal to use the amino group of the 6-aminohexyl glucoside **1** (Scheme 1), carrying four mannosyloxypropyl residues[5] for the introduction of fluorescence markers, as fluorescence labeling is a powerful tool to inspect and quantify biological processes.[6] Binding of glycoclusters to L-selectins, for example, has been made visible through fluorescing glycoclusters[7] and also dansyl derivatives are valuable marks in this regard[8] and rather inexpensive. Thus we selected dansylamino-

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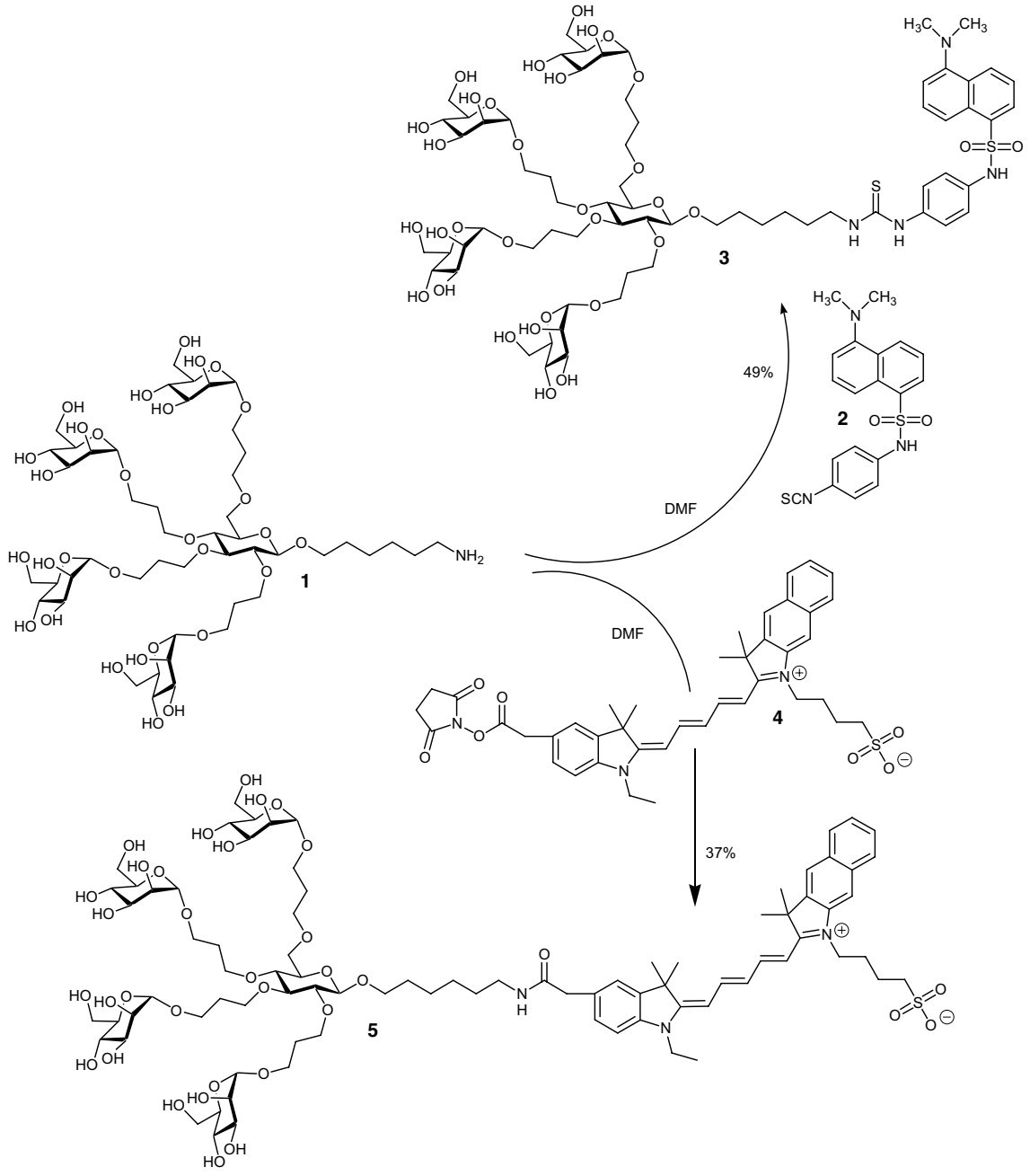


Scheme 1.

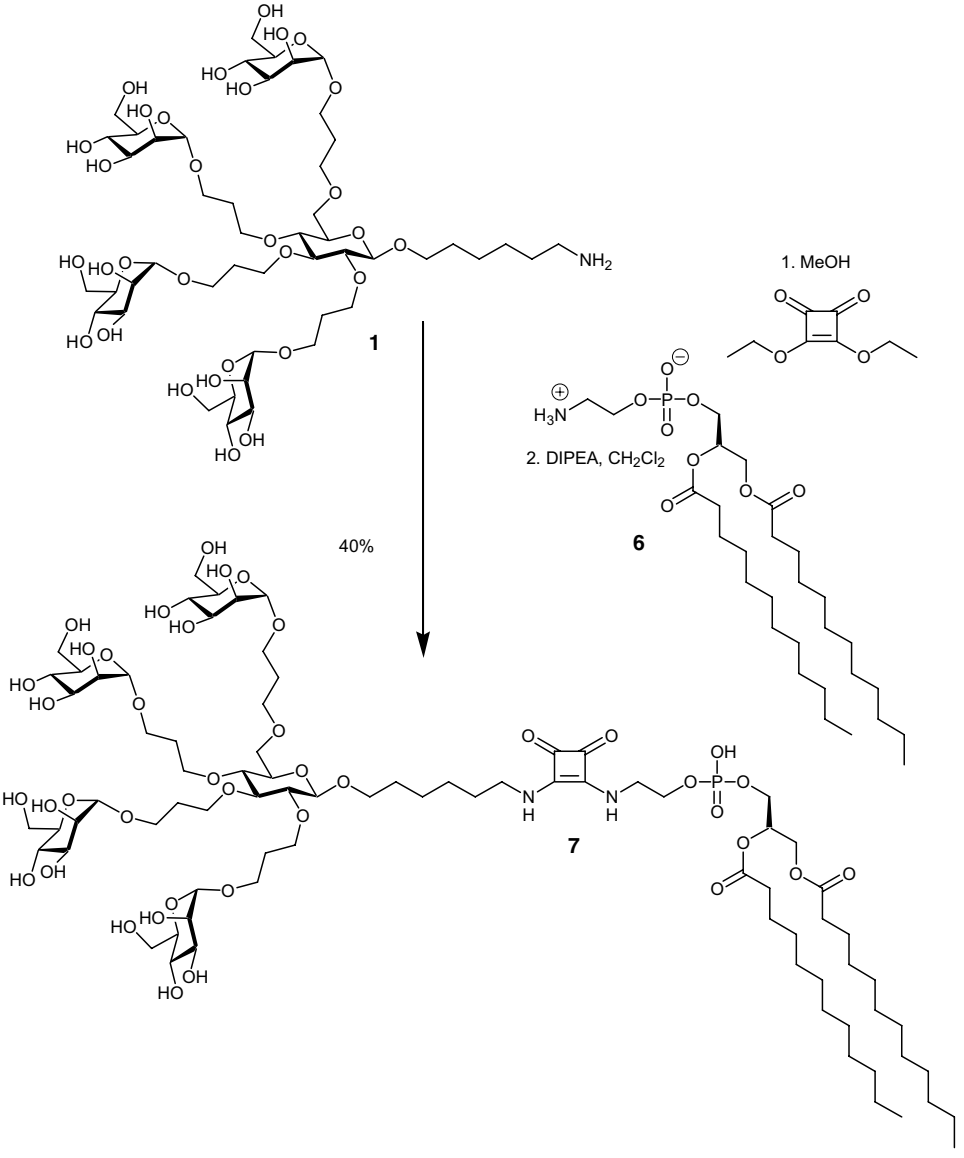
phenyl isothiocyanate (**2**) for the reaction with the amine **1**. Stirring in DMF solution gave direct access to the thiourea-bridged dansyl-labeled glycocluster **3**. Purification of the product by GPC was successful on Sephadex LH-20. The swelling behavior of this material in DMF or methanol, respectively, is very similar and therefore, small volumes of DMF solutions can be directly subjected to GPC on Sephadex even if elution is carried out with methanol.

As for competitive inhibition assays glycoclusters with different fluorescence markers are attractive biochemical tools, we envisaged an additional fluorescence marker for labeling of the amine **1**. The dansyl moiety shows fluorescence at 360 nm, whereas indocarbocyanine dyes absorb and fluoresce at higher wavelength.[9] Indodicarbocyanines are standard fluorescent probes for the investigation of for example, membrane structure and function and show absorption at 650 and emit at 670 nm. We have employed indodicarbocyanine activated as succinimidoylester **4**[10] to allow peptide coupling with glycocluster **1**. This led to the fluorescence-labeled peptide-coupled product **5** in one step only, however, in a somewhat moderate yield of 37% due to loss of material during purification.

To further extend the synthetic options for the modification of cluster mannoside **1**, we included squaric acid diethylester (1,2-diethoxycyclobutene-3,4-dione), as introduced by Tietze et al. in 1991.[11] The first ester function of squaric acid diester reacts at neutral pH, whereas the second ester group can be coupled to amines in basic medium. This allows subsequent coupling of two different amine components to obtain a distinct conjugate without the formation of mixtures. This method has already found broad application in glycobiology for the synthesis of various neoglycoconjugates and multivalent glycoligands.[12] With this in mind we approached the synthesis of glycolipid analogs using glycocluster **1** and the phosphatidylethanolamine **6** (lecithin). Squaric acid diester was added first to give the corresponding squaric acid monoamide and then the phospholipid was employed as solution in MeOH–dichloromethane to achieve the squaric acid-linked glycolipid analog **7** in 40% yield over two steps and after GPC purification (Scheme 2). Mass spectrometric analysis was performed in the negative mode.

2.2. Multimerization of the carbohydrate-centered glycocluster 1

To multimerize glycocluster **1** according to the same strategy, tris(2-aminoethyl)amine was chosen as trivalent scaffold molecule. Because **1** is a rather valuable starting material, squaric acid coupling to a triamine was first optimized using the squaric acid monoamide **8**,[13] which could be linked to tris(2-aminoethyl)amine



Scheme 2.

to deliver the trivalent model glycocluster **9** (Scheme 3). Optimization of this process led us to use an twofold excess of **8** per amino group, and this raised the yield from 36% (1.2 equiv of **8** per amino group) to an optimized yield of 68%.

Based on this know-how, synthesis of the dodecavalent neoglycoconjugate **10** was elaborated starting from the cluster mannoside **1**. Its reaction with diethyl squarate in methanol delivered the intermediate squaric acid monoamide, which was subsequently reacted with the branched tris(2-aminoethyl)amine in the presence of DIPEA to deliver the macromolecular glycoconjugate **10** in over 60% overall yield (Scheme 4). Three equivalents of the intermediate squaric acid monoester and a prolonged reaction time together with portion-wise addition of the core amine had to be employed.

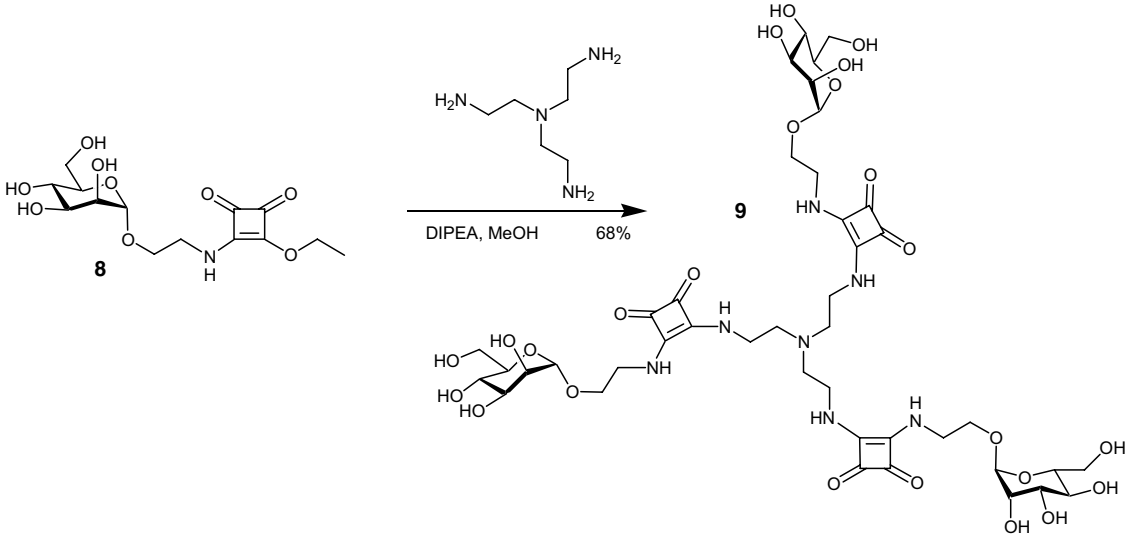
Preparation of the low-molecular weight glycocluster **9** offered the opportunity to compare the chemical and biological properties of the dodecacluster **10** with its smaller homolog. Thus, NMR analysis of **10** was greatly facilitated on the basis of the corresponding data for **9**. The biological properties of **9** and **10** were compared in the context of inhibition of bacterial adhesion.

2.3. Biological testing of cluster mannosides 9 and 10 as inhibitors of type 1 fimbriae-mediated bacterial adhesion

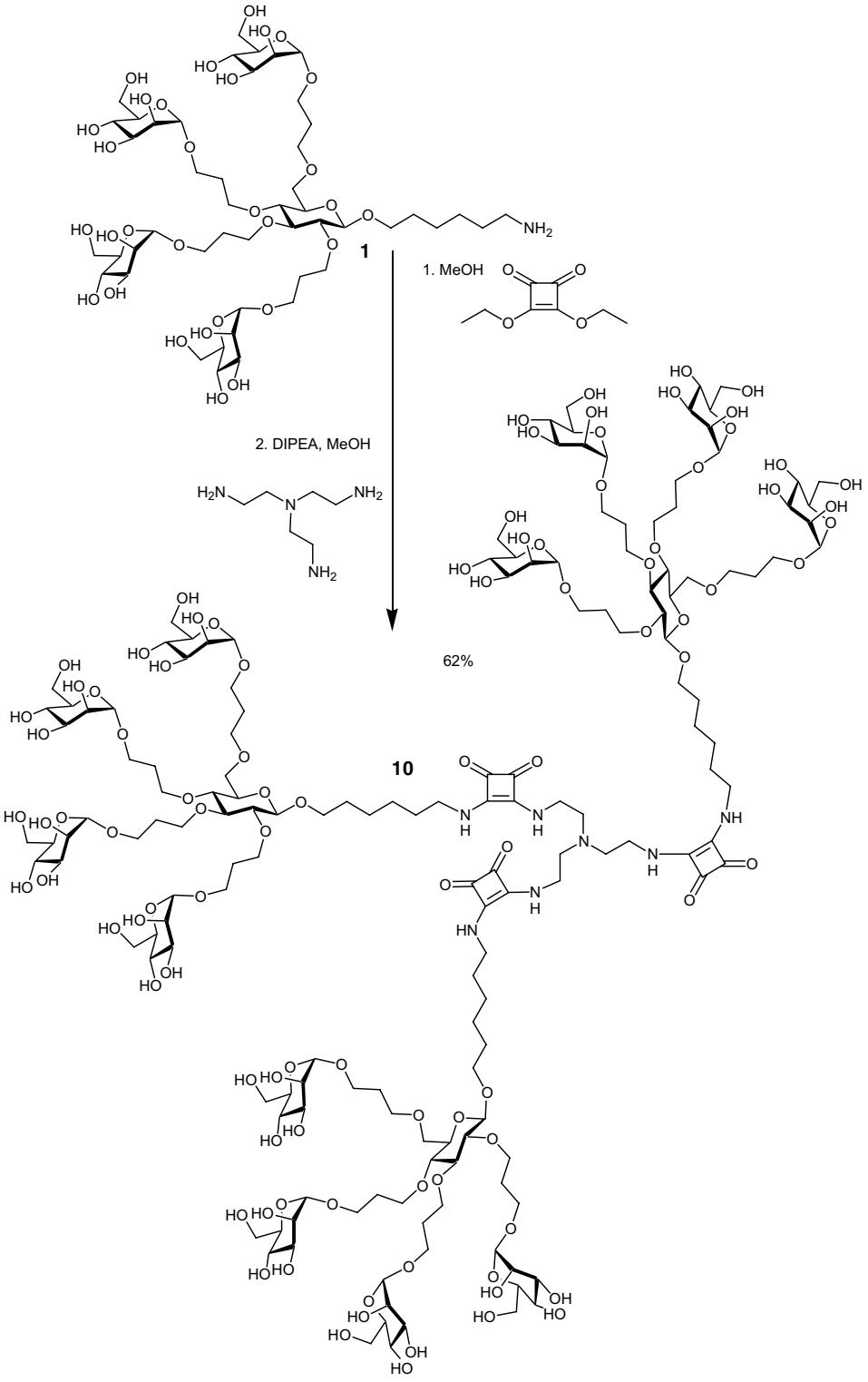
The prepared branched neoglycoconjugates **9** and **10** were investigated as inhibitors of mannose-specific bacterial adhesion. For the determination of their inhibitory potency, an enzyme-linked immunosorbent assay (ELISA) was used, which allows detection of the adhesion of type 1 fimbriated *Escherichia coli* bacteriae to a mannan-coated polystyrene surface. Inhibition of this bacterial adhesion is of interest to improve our understanding of the mechanisms underlying this process[14] as well as in a therapeutic context, such as for an anti-adhesion therapy against microbial diseases.[15] As type 1 fimbriae contain a lectin sub-unit, called FimH,[16] which has been shown to be specific for α-D-mannosyl units, the oligomannoside mimetics **9** and **10** are privileged candidates as inhibitors of type 1 fimbriae-mediated bacterial adhesion. Table 1 summarizes the results obtained by ELISA. The inhibitory potencies of **9** and **10** were compared to that of their monovalent carbohydrate analogs, namely methyl α-D-mannoside (MeMan). In case of the trivalent glycocluster **9** the inhibitory potential is increased in the range of one order of magnitude when compared to MeMan (RIP = 8.4), the much more complex dodecavalent cluster mannoside **10** on the other hand exceeds MeMan by two orders of magnitude, that is, its inhibitory potency is approximately 190 times higher than that of MeMan (RIP ≡ 1).

The IC_{50} value measured for the trivalent mannose cluster **9** is in agreement with studies published earlier.[17] However, the relative weak inhibitory potency of the high molecular weight compound **10** is somewhat unexpected. Owing to known multivalency effects in carbohydrate recognition we expected **10** to perform better. Possibly, intramolecular cluster formation occurs in the case of **10**, resulting in a diminished availability of the α-mannosyl residues for receptor binding.

To allow a deeper and more conclusive understanding of type 1 fimbriae-mediated bacterial adhesion, additional studies are required and this is currently underway in our laboratory. We will utilize the herein reported chemistry and glycoconjugates to further evaluate the principles of bacterial adhesion in a glycobiological context.



Scheme 3.



Scheme 4.

3. Experimental

3.1. General methods

3.1.1. General. Optical rotations were determined with a Perkin Elmer 241 polarimeter (10 cm cells, Na-D-line: 589 nm). NMR spectra were recorded at 500 MHz on DRX-500 instruments with Me_4Si ($\delta = 0$) as the internal standard. All reactions were monitored by TLC on silica gel FG_{254} (Merck) with detection by UV light and/or by charring with 10% ethanolic sulfuric acid. Flash column chromatography was performed on silica gel 60 (200–

Table 1. Inhibitory potencies in mannose-specific *E. coli* adhesion of the prepared carbohydrate-centered cluster mannosides as determined by ELISA

Compound tested	IC_{50} (μmol)	*S* (μmol)[a]	RIP[b] (*S*)	
MeMan	1600[c]	940[c]	1	—
9	570	110	8.2	0.39
10	8.4	2.4	190	54

IC_{50} values are average values from at least three independent assays and are listed together with their standard deviations (*S*). So-called relative inhibitory potencies (RIP) refer to the IC_{50} value of methyl α-D-mannopyranoside (MeMan), with RIP (MeMan) ≡ 1.

[a] *S* Standard deviation.

[b] RIP relative inhibitory potency.

[c] IC_{50} and *S* for MeMan are represented by a typical value.

400 mesh, Macherey Nagel & Co.). MPLC was performed using a Büchi apparatus and Merck Licroprep RP-18 columns. Gel permeation chromatography (GPC) was carried out on Sephadex LH-20 with MeOH as eluent if not otherwise stated. Methyl α-D-mannoside was purchased from Fluka, F-shaped 96-well microtiter plates from Sarstedt. Mannan from *Saccharomyces cerevisiae* was purchased from Sigma and was used in 50 mM aq Na_2CO_3 solution (1 mg mL^{-1}; pH 9.6). Peroxidase-conjugated goat anti-rabbit antibody (IgG, H+L) was purchased from Dianova. Skimmed milk was from Ulzena, Tween 20 from Roth, ABTS [2,2′-azidobis-(3-ethylbenzothiazoline-6-sulfonic acid)] from Fluka and thimerosal {2-[(ethylmercurio)thio]benzoic acid, sodium salt} was from Merck. A recominant type 1 fimbriated strain, *E. coli* HB101 (pPKl_4),[18] was used and cultured as described. Buffers were used as described earlier.[17]

3.1.2. ELISA. An ELISA protocol was used to determine the inhibitory potencies of the cluster mannosides **8** and **9** toward type 1 fimbriated *E. coli*. This was performed as described earlier[17] using polystyrene microtiter plates coated with mannan solution. ELISA plates were incubated at 37 °C. Optical densities (ODs) were measured on an AMP 400 COM ELISA reader at 405 nm with the reference read to 492 nm. The percentage inhibition was calculated as [OD (nI) − OD (I) × 100 × [OD (nI)] − 1 (nI: no inhibitor, I: with inhibitor). The IC_{50}-values were determined where the sigmoidal fit of a set of measured inhibitions crosses an imaginary 50%-line.

3.2. Synthesis of functionalized carbohydrate-centered glycoclusters

3.2.1. 6-[4-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}-amino)phenyl thiourea]-hexyl-2,3,4,6-tetra-*O*-[3-(α-D-mannopyranosyloxy)-propyl]-β-D-glucopyranoside (3). A solution of cluster mannoside **1** (23.7 mg, 0.020 mmol) and 4-dansylaminophenylisothiocyanate **2** (12 mg, 0.031 mmol) in DMF (6 mL) was stirred for 36 h and then subjected to GPC (Sephadex LH-20, MeOH) to furnish the title compound as third fraction (R_f (MeOH) 0.45, detection UV 366 nm) in the form of a yellowish red syrup. Yield: 15 mg, 0.0097 mmol, 49%. ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 8.55, 8.25 (each m, each 1H, $SO_2CCHCHCH$), 8.45 (m, 1H, $NMe_2CCHCHCH$), 7.65 (dd, 1H, $J = 7.7$ Hz, $J = 8.8$ Hz, $NMe_2CCHCHCH$), 7.55 (dd, 1H, $J = 7.3$ Hz, $J = 8.5$ Hz, $SO_2CCHCHCH$), 7.30 (d, 1H, $J = 7.5$ Hz, $NMe_2CCHCHCH$), 7.14, 7.03 (each m, each 2H, $2NHCCHCH$), 4.81, 4.80, 4.80, 4.78 (each d, 4H, $J_{1,2Man} = 1.5$ Hz, 4H-1_{Man}), 4.30 (d, 1H, $J_{1,2Glc} = 7.8$ Hz, H-1_{Glc}), 3.98–3.82 (m, 17H, 4H-2_{Man}, 4H-6_{Man}, 5(Glc)OC*H*H, 4(Man)OC*H*H), 3.79–3.52 (m, 29H, 4H-3_{Man}, 4H-4_{Man}, 4H-5_{Man}, 4H-$6'_{Man}$, H-6_{Glc}, H-$6'_{Glc}$, 5(Glc)OCH*H*, 4(Man)OCH*H*, CH_2NHCS), 3.36–3.32 (m, 1H+MeOH, H-5_{Glc}), 3.31–3.25 (m, 2H, H-3_{Glc}, H-4_{Glc}), 3.02 (m, 1H, H-2_{Glc}), 2.90 (m, 6H, $N(CH_3)_2$), 1.97–1.85 (m, 8H, $4OCH_2CH_2CH_2O$), 1.69–1.32 (m, 8H, (C-1_{Glc})$OCH_2CH_2CH_2CH_2CH_2CH_2NH$) ppm; ^{13}C NMR (125.76 MHz, MeOH-d_4, DEPT): δ_C 132.8, 132.6 ($SO_2CCHCHCH$), 130.5 ($NMe_2CCHCHCH$), 127.8 ($2NHCCH$), 125.4 ($SO_2CCHCHCH$), 123.1 ($2NHCCH$), 121.5 ($NMe_2CCHCHCH$), 117.7 ($NMe_2CCHCHCH$), 105.9 (C-1_{Glc}), 102.8 (4C-1_{Man}), 87.2 (C-3_{Glc}), 84.9 (C-2_{Glc}), 80.4 (C-4_{Glc}), 77.0 (C-5_{Glc}), 75.8 (4C-5_{Man}), 73.8 (4C-3_{Man}), 73.4 (4C-2_{Man}), 72.8, 72.0–71.8, 70.4 (5(Glc)OCH_2, C-6_{Glc}), 69.8 (3×), 69.7 (4C-4_{Man}), 66.8, 66.7, 66.5 (2×) (4(Man)OCH_2), 64.1 (4C-6_{Man}), 47.0 ($2NCH_3$, CH_2N), 32.9, 32.7 (2×), 32.1, 31.9, 31.2 ($4OCH_2CH_2CH_2O$, (C-1_{Glc})$OCH_2CH_2CH_2CH_2CH_2$), 28.8, 28.1 ((C-1_{Glc})$OCH_2CH_2CH_2CH_2$) ppm; MALDI-TOF MS: $m/z = 1566.7$ $[M+Na]^+$ (1566.7 calcd for $C_{67}H_{106}N_4O_{32}S_2$).

3.2.2. *N*-(2-(4,5-Benzo-1′-ethyl-3,3,3′3′-tetramethyl-1-(4-sulfobutyl)-indodicarbocyaninyl)-ethanoyl)-6-hexyl-2,3,4,6-tetra-*O*-[3-(α-D-mannopyranosyloxy)-propyl]-β-D-glucopyranoside (5). A solution of the amine **1** (9.0 mg, 0.0078 mmol), the indodicarbocyanine active ester **4** (6.0 mg, 0.0083 mmol) and $NaHCO_3$ (12 mg) was stirred for 12 h in DMF (6 mL) and purified by two subsequent GPCs to furnish the title compound as amorphous blue solid. Yield: 5.1 mg, 0.0029 mmol, 37%. ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 8.38 (t, 1H, $J = 13.2$ Hz, C=CHCH=CHC*H*=CHC), 8.28 (d, 1H, $J = 8.5$ Hz, aryl H), 8.26 (t, 1H, $J = 13.0$ Hz, C=CHC*H*=CHCH=CHC), 8.06 (d, 1H, $J = 8.8$ Hz, aryl H), 8.04 (d, 1H, $J = 8.3$ Hz, aryl H), 7.71–7.65 (m, 2H, aryl H), 7.53 (t, 1H, $J = 7.1$ Hz, aryl H), 7.47 (s, 1H, aryl H), 7.38 (dd, 1H, $J = 1.4$ Hz, $J = 8.3$ Hz, aryl H), 7.27 (d, 1H, $J = 8.1$ Hz, aryl H), 6.72 (t, 1H, 12.2 Hz, C=CHCH=C*H*CH=CHC), 6.46 (d, 1H, $J = 13.9$ Hz, C=CHCH=CHCH=C*H*C), 6.32 (d, 1H, $J = 13.6$ Hz, C=C*H*CH=CHCH=CHC), 4.82–4.77 (m, 4H, 4H-1_{Man}), 4.33 (m, 2H, $NCH_2CH_2CH_2CH_2SO_3$),

4.28 (d, 1H, $J_{1,2Glc} = 7.6$ Hz, H-1_{Glc}), 4.17 (m, 2H, NCH_2CH$_3$), 3.98–3.81 (m, 17H, 4H-2_{Man}, 4H-6_{Man}, 5(Glc)OCHH, 4(Man)OCHH), 3.80–3.50 (m, 29H, 4H-3_{Man}, 4H-4_{Man}, 4H-5_{Man}, 4H-$6'_{Man}$, H-6_{Glc}, H-$6'_{Glc}$, 5(Glc)OCHH, 4(Man)OCHH, CH_2CONH), 3.36–3.32 (m, 1H+MeOH, H-5_{Glc}), 3.31–3.21 (m, 4H, H-3_{Glc}, H-4_{Glc}, CH$_2$NHCO), 3.02 (m, 1H, H-2_{Glc}), 2.96 (t, 2H, $J = 7.0$ Hz, CH$_2$SO$_3$), 2.14–2.00 (m, 10H, NCH$_2$CH_2CH_2CH$_2$SO$_3$, N(CH$_3$)$_2$), 1.97–1.85 (m, 8H, 4OCH$_2$CH_2CH$_2$O), 1.78 (s, 6H, N(CH$_3$)$_2$), 1.68–1.37 (m, 11H, (C-1_{Glc})OCH$_2$CH_2CH_2CH_2CH_2, NCH$_2$CH_3) ppm; ^{13}C NMR (125.76 MHz, MeOH-d_4, DEPT): δ_C 156.1, 156.0 (C=CHCH=CHCH=CHC), 133.0, 132.3, 131.7, 129.9, 127.3, 125.4, 124.6 (aryl C), 128.1 (C=CHCH=CHCH=CHC), 113.5, 112.7 (2C-$7_{indole\ ring}$), 105.9 (C-1_{Glc}), 105.7, 104.9 (C=CHCH=CHCH=CHC), 102.8 (4C-1_{Man}), 87.2 (C-3_{Glc}), 84.9 (C-2_{Glc}), 80.5 (C-4_{Glc}), 77.1 (C-5_{Glc}), 75.8 (4C-5_{Man}), 73.8 (4C-3_{Man}), 73.4 (4C-2_{Man}), 72.8, 72.0–71.8 (4×), 70.5 (5(Glc)OCH$_2$, C-6_{Glc}), 69.8 (3×), 69.7 (4C-4_{Man}), 66.9, 66.8, 66.6 (2×) (4(Man)OCH$_2$), 64.1 (4C-6_{Man}), 52.9 (CH$_2$SO$_3$), 46.2 (NCH$_2$CH$_2$CH$_2$CH$_2$SO$_3$), 44.8 (NHCOCH$_2$), 41.8 (CH$_2$NHCO), 41.1 (NCH$_2$CH$_3$), 32.9, 32.8, 32.7, 32.1, 32.0, 31.6 ((C-1_{Glc})-OCH$_2C$H$_2$CH$_2$CH$_2C$H$_2$CH$_2$NH, 4OCH$_2C$H$_2$CH$_2$O), 29.2, 28.8 (2N(CH$_3$)$_2$), 28.9, 28.1 ((C-1_{Glc})OCH$_2$ CH$_2C$H$_2C$H$_2$), 28.7 (NCH$_2C$H$_2$), 24.7 (CH$_2$CH$_2$SO$_3$), 13.8 (NCH$_2C$H$_3$) ppm; ^{13}C NMR (BB): δ 173.6–175.3 (NHCO, 2C-$2_{indole\ ring}$) ppm; ^{1}H–^{13}C HMBC: δ 51.5 and 53.5 (2C-$3_{indole\ ring}$) ppm; MALDI-TOF MS: $m/z = 1791.1$ [M+Na]$^+$ (1767.8 calcd for $C_{85}H_{129}N_3O_{34}S$).

3.2.3. 3-[(2,3,4,6-Tetra-*O*-[3-(α-D-mannopyranosyloxy)-propyl]-β-D-glucopyranosyl-hexylamin]-3-[1,2-di-dodecanoyl-*sn*-glycero-3-phospho-ethanolamin]-3-cyclobuten-1,2-dione (7). The glycocluster **1** (12.5 mg, 0.0105 mmol) and diethylsquarate (0.0017 mL, 0.016 mmol) were stirred for 12 h in MeOH (8 mL). Then 1,2-didodecanoyl-*sn*-glycero-3-phospho-ethanolamine **6** (9 mg, 0.0155 mmol) in dichloromethane–MeOH (4:1, 8 mL) and DIPEA (0.3 mL) were subsequently added. The reaction mixture was stirred for 24 h and then the solvent was removed and the product purified by GPC to give **6** as colorless syrup. Yield: 7.7 mg, 0.0042 mmol, 40%. ^{1}H NMR (500 MHz, MeOH-d_4): δ_H 5.25 (m, 1H, POCH$_2$CH(OCOR)CH$_2$(OCOR)), 4.81, 4.80, 4.79, 4.78 (each d, each 1H, $J_{1,2Man} = 1.6$ Hz, 4H-1_{Man}), 4.47 (dd, 1H, $J = 3.5$ Hz, $J = 12.0$ Hz, CHH(OCOR)), 4.30 (d, H, $J_{1,2Glc} = 7.3$ Hz, H-1_{Glc}), 4.21 (dd, 1H, $J = 12.0$ Hz, $J = 6.6$ Hz, CHH(OCOR)), 4.06–3.55 (m, 52H, 4H-2_{Man}, 4H-6_{Man}, 10(Glc)OCHH, 8(Man)-OCHH, 4H-3_{Man}, 4H-4_{Man}, 4H-5_{Man}, 4H-$6'_{Man}$, H-6_{Glc}, H-$6'_{Glc}$, NCH_2CH_2OP(O$_2$)CH_2, CH_2NH), 3.30–3.33 (m, 3H, H-5_{Glc}, H-3_{Glc}, H-4_{Glc}), 3.03 (dd ≈ t, 1H, $J = 8.2$ Hz, H-2_{Glc}), 2.26 (m, 4H, 2CH_2CO$_2$), 1.98–1.84 (m, 16H, 8OCH$_2$CH_2CH$_2$O), 1.70–1.45 (m, 12H, (2CH_2CH$_2$CO$_2$, C-1_{Glc})OCH$_2$CH_2CH_2CH_2CH_2CH$_2$NH), 1.38–1.28 (m ≈ s, 32H, $H_{aliphat.\ chain}$), 0.95 (t, 6H, $J = 6.9$ Hz, CH$_3$) ppm; ^{13}C NMR (125.76 MHz, MeOH-d_4): δ_C 185.1, 184.8, 170.7, 170.4 (4$C_{squaric\ acid}$), 176.1, 175.8 (2NHCO$_{fatty\ acid}$), 105.9 (C-1_{Glc}), 102.8 (4C-1_{Man}), 87.2 (C-3_{Glc}), 85.0 (C-2_{Glc}), 80.5 (C-4_{Glc}), 77.1 (C-5_{Glc}), 75.8 (4C-5_{Man}), 73.8 (4C-3_{Man}), 73.4 (4C-2_{Man}), 73.1 (d, $J_{P,C} = 8.3$ Hz, POCH$_2C$H(O-COR)CH$_2$(OCOR)), 72.8, 72.0–71.8 (4×), 70.5 (5(Glc)OCH$_2$, C-6_{Glc}), 69.8 (3×), 69.7 (4C-4_{Man}), 66.9, 66.8, 66.6 (2×) (4(Man)OCH$_2$), 67.5, 66.0 (CH$_2$OP-(O$_2$)OCH$_2$), 64.8 (POCH$_2$CH(OCOR)CH$_2$(OCOR)), 64.1 (4C-6_{Man}), 48.2, 46.5 (2NHCH$_2$), 36.3, 36.1 (2CH$_2C$H$_2$CO$_2$), 34.3 (CH$_2$CH$_2$CO$_2$), 32.9, 32.8, 32.7, 32.1, 32.0–31.4 (14×) (4OCH$_2C$H$_2$CH$_2$O, (C-1_{Glc})OCH$_2$-CH$_2$CH$_2$CH$_2C$H$_2$, 12$C_{aliphat.\ chain}$), 28.4, 28.1 ((C-1_{Glc})OCH$_2$CH$_2C$H$_2C$H$_2$), 27.2 (2×), 25.0 (2×) (4$C_{aliphat.\ chain}$), 15.7 (2 CH$_3$) ppm; MALDI-TOF MS (negative mode): $m/z = 1815.8$ [M]$^-$ (1815.9 calcd for $C_{81}H_{144}N_2O_{40}P$).

3.3. Clustering by squaric acid-mediated coupling

3.3.1. Tris-2-{*N*-[4-*N*-(α-D-mannopyranosyloxyethyl)-2,3-dioxocyclobut-1-enyl]aminoethyl}amine (9). 4-Ethoxy-2,3-dioxocyclobut-1-enyl-amino-ethyl-α-D-mannopyranoside (**8**, 210 mg, 0.60 mmol), tris(2-aminoethyl)amine (15 μL, 0.10 mmol) and DIPEA (0.1 mL) were stirred in MeOH (10 mL) for 12 h at room temperature. Subsequent purification using MPLC and lyophilization gave the title compound as a white solid. Yield: 71 mg, 0.068 mmol, 67.6%. ^{1}H NMR (500 MHz, D$_2$O): δ_H 4.86 (3H, m, 3 × H-1), 4.08 (3H, m, 3 × H-2), 3.85 (3H, m, 3 × H-3), 3.78–3.61 (3H, m, 3 × H-5, 3 × H-6a, 3 × H-6b, 18 × ethyl), 3.61 (3H, m, 3 × H-4), 3.50 (6H, m, NCH$_2$CH$_2$N) ppm; ^{13}C NMR (125.76 MHz, D$_2$O): δ_C 184.44, 178.85 (C=O$_{squaric\ acid}$), 171.71, 169.98, (C=C$_{squaric\ acid}$), 101.94 (3 × C-1), 75.37 (3 × C-5), 72.95 (3 × C-3), 72.39 (3 × C-2), 68.99 (3 × C-4), 68.18 (3 × OCH$_2$CH$_2$NH$_2$), 65.43 (3 × NH$_2$CH$_2$CH$_2$N), 63.36 (3 × C-6), 46.29 (3 × OCH$_2$CH$_2$N), 41.18 (3 × NCH$_2$-CH$_2$N) ppm; MALDI-TOF MS: $m/z = 1072.57$ [M+Na]$^+$, (calcd 1072.4 for $C_{42}H_{63}N_7O_{24}$) (M = 1049.4), HRMS (ESI): $m/z = 1072.3826$ [M+Na]$^+$ (calcd 1072.3817 for $C_{18}H_{21}NO_9$).

3.3.2. Synthesis of tris-2-{*N*-[4-*N*-{6-aminohexyl-2,3,4,6-tetra-*O*-[3-(α-D-mannopyranosyloxy)-propyl]-β-D-glucopyranosyloxyhexyl}-2,3-dioxocyclobut-1-enyl]aminoethyl}-amine (10). Compound **1** (35.2 mg, 0.0296 mmol) and diethylsquarate (0.0031 mL, 0.030 mmol) in MeOH (2 mL) were stirred for 12 h. The resulting product was purified by GPC. To a solution of this adduct in a mixture of MeOH (2 mL) and DIPEA (0.5 mL) tris(2-aminoethyl)amine (two times 0.25 μL, 3.3 μmol) were added at the beginning of the reaction and after 24 h. This mix-

ture was stirred for 10 d at room temperature. Afterwards the solvent was removed and the product was purified using GPC. Yield: 8 mg, 2.06 μmol, 62.4%. ^{1}H NMR (500 MHz, D_2O): δ_H 4.86–4.83 (12H, m, 12H-1_{Man}), 4.38 (3H, d, 3H-1_{Glc}), 3.96–3.54 (141H, m, 12H-2_{Man}, 12H-3_{Man}, 12H-4_{Man}, 12H-5_{Man}, 12H-$6a_{Man}$, 12H-$6b_{Man}$, 3H-5_{Glc}, 3H-$6a_{Glc}$, 3H-$6b_{Glc}$, 15(Glc)OCH_2, 12Man-*O*-CH_2, $3CH_2CH_2CH_2N$, $3NCH_2CH_2N$), 3.37 (3H, m, 3H-4_{Glc}), 3.30 (3H, dd ≈ t, 3H-3_{Glc}), 3.09 (3H, m, 3H-2_{Glc}), 2.79 (6H, m, $3NCH_2CH_2N$), 2.00–1.86 (24H, m, $12OCH_2CH_2CH_2O$), 1.69–1.56 (12H, m, $3OCH_2CH_2CH_2CH_2CH_2CH_2NH$), 1.46–1.34 (12H, m, $3OCH_2CH_2CH_2CH_2CH_2CH_2NH$) ppm; ^{13}C NMR (125.76 MHz, D_2O): δ_C 104.9 (3C-1_{Glc}), 102.2 (12C-1_{Man}), 86.3 (3C-3_{Glc}), 84.0 (3C-2_{Glc}), 80.3 (3C-4_{Glc}), 76.2 (3C-5_{Glc}), 75.2 (12C-5_{Man}), 73.4 (3Glc-OCH_2), 73.1 (12C-3_{Man}), 73.0 (3Glc-OCH_2), 72.7 (3Glc-OCH_2), 72.6 (12C-2_{Man}), 72.26 (3Glc-OCH_2), 71.3 (3Glc-OCH_2), 70.6 (3C-6_{Glc}), 69.1 (12C-4_{Man}), 66.8 (12Man-OCH_2), 63.33 (12C-6_{Man}), 46.6 ($3OCH_2CH_2CH_2CH_2CH_2CH_2NH$), 32.6, 32.0, 31.9, 31.8, 31.2 ($12OCH_2CH_2CH_2O$, $3OCH_2CH_2CH_2$-$CH_2CH_2CH_2NH$, $3OCH_2CH_2CH_2CH_2CH_2CH_2NH$), 27.7, 27.3 ($3OCH_2CH_2CH_2CH_2CH_2CH_2NH$) ppm (squaric acid signals not detectable). MALDI-TOF MS: $m/z = 3915.3$ [M+Na^+] (calcd 3909.7 for $C_{164}H_{283}N_7O_{96}$) (M = 3886.8).

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