Gold nanoparticles coated with a pyruvated trisaccharide epitope of the extracellular proteoglycan of *Microciona prolifera* as potential tools to explore carbohydrate-mediated cell recognition

Adriana Carvalho de Souza, Johannes F. G. Vliegenthart and Johannis P. Kamerling*

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The species-specific cell adhesion in the marine sponge *Microciona prolifera* involves the interaction of an extracellular proteoglycan-like macromolecular complex, otherwise known as aggregation factor. In the interaction, two highly polyvalent functional domains play a role: a cell-binding and a self-interaction domain. The self-recognition has been characterized as a Ca^{2+}-dependent carbohydrate–carbohydrate interaction of repetitive low affinity carbohydrate epitopes. One of the involved epitopes is the pyruvated trisaccharide β-D-Gal*p*4,6(*R*)Pyr-(1→4)-β-D-Glc*p*NAc-(1→3)-L-Fuc*p*. To evaluate the role of this trisaccharide in the proteoglycan–proteoglycan self-recognition, β-D-Gal*p*4,6(*R*)Pyr-(1→4)-β-D-Glc*p*NAc-(1→3)-α-L-Fuc*p*-(1→O)$(CH_2)_3S(CH_2)_6SH$ was synthesized, and partially converted into gold glyconanoparticles. These mimics are being used to explore the self-interaction phenomenon for the trisaccharide epitope, *via* TEM aggregation experiments (gold glyconanoparticles) and atomic force microscopy (AFM) experiments (self assembled monolayers; binding forces).

Introduction

Sponges are the simplest multicellular animals living today. The complex extracellular matrix found in sponges suggests that the system mediating sponge cell motility and adhesion is the evolutionary ancestor to Metazoan cell adhesion and development mechanisms.[1] Therefore, they represent an ideal model system to study the molecular mechanisms that guide cell recognition and adhesion in higher Metazoans.[2] The species-specific cellular adhesion in the red-beard marine sponge *Microciona prolifera* depends on two functional domains in its proteoglycan-type aggregation factor: (i) an N-linked polysaccharide of 200 kDa molecular mass (g-200) for the Ca^{2+}-dependent self-interaction between cells, and (ii) an N-linked polysaccharide of 6 kDa molecular mass (g-6) for the Ca^{2+}-independent binding to cell surface receptors.[3,4] Two monoclonal antibodies prepared against the aggregation factor, called Block 1 and Block 2, were able to inhibit the Ca^{2+}-dependent self-aggregation process through the binding to repetitive carbohydrate epitopes on the g-200 glycan.[5,6] Isolation and characterization of these epitopes (Scheme 1) revealed two small oligosaccharide fragments, the pyruvated trisaccharide β-D-Gal*p*4,6(*R*)Pyr-(1→4)-β-D-Glc*p*NAc-(1→3)-L-Fuc*p* (Block 1) **1**,[7] and the sulfated disaccharide β-D-Glc*p*NAc3*S*-(1→3)-L-Fuc*p* (Block 2) **2**.[8]

To gain insight into the role of the different carbohydrate epitopes in the g-200 self-recognition, we started a challenging program involving the synthesis and interaction studies of the two g-200 oligosaccharide epitopes. Initially, the program was focused on the sulfated disaccharide element **2**. Surface plasmon resonance (SPR) spectroscopy of the amino-spacer-containing synthetic disaccharide β-D-Glc*p*NAc3*S*-(1→3)-α-L-Fuc*p*-(1→O)$(CH_2)_3S(CH_2)_2NH_2$, multivalently presented as a bovine serum albumin conjugate, indicated that the Ca^{2+}-dependent self-recognition of this epitope is one of the major forces behind the g-200 self-association.[9,10] These experiments showed that this interaction is highly Ca^{2+}-dependent, and not only based on electrostatic forces, as other negatively charged carbohydrates did not aggregate in the presence of Ca^{2+}-ions. Recently, transmission electron microscopy (TEM) aggregation experiments in the absence and presence of Ca^{2+}-ions using gold nanoparticles coated with the thiol-spacer-containing synthetic disaccharide β-D-Glc*p*NAc3*S*-(1→3)-α-L-Fuc*p*-(1→O)$(CH_2)_3S(CH_2)_6SH$, and structural variants of the synthetic disaccharide,[11] have given valuable information on the Ca^{2+}-mediated interaction mechanism of the disaccharide **2** self-recognition.[12] In summary, it turned out that the methyl group of Fuc, combined with the sulfate and *N*-acetyl groups of GlcNAc are essential for the self-recognition. Furthermore, the α-anomeric form of the L-Fuc*p* moiety results in larger aggregates than the β-form; in this context it should be noted that the g-200 polysaccharide contains only α-L-Fuc*p* units.[13]

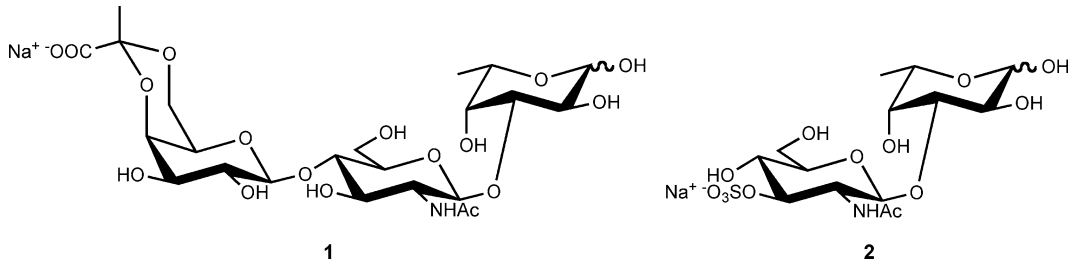
In order to understand the role of the pyruvated trisaccharide element **1**, we have started now a similar program as described for the sulfated disaccharide, and in this paper we report on the synthesis of gold nanoparticles decorated with β-D-Gal*p*4,6(*R*)Pyr-(1→4)-β-D-Glc*p*NAc-(1→3)-α-L-Fuc*p*-(1→O)$(CH_2)_3S(CH_2)_6SH$.

Results and discussion

Synthesis of the thiol-spacer-containing trisaccharide

As Fuc occurs in the α-anomeric configuration in the intact proteoglycan,[13] which turned out to be of high importance in

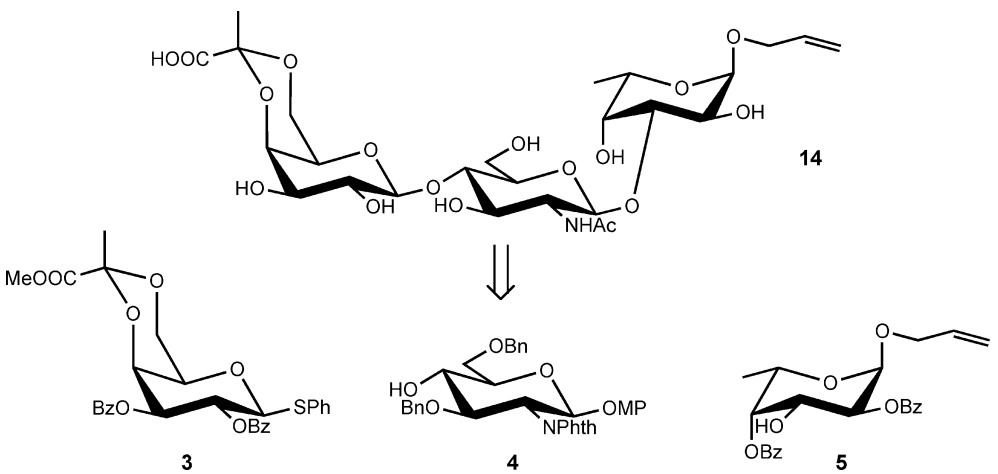
Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH, Utrecht, The Netherlands. E-mail: j.p.kamerling@uu.nl



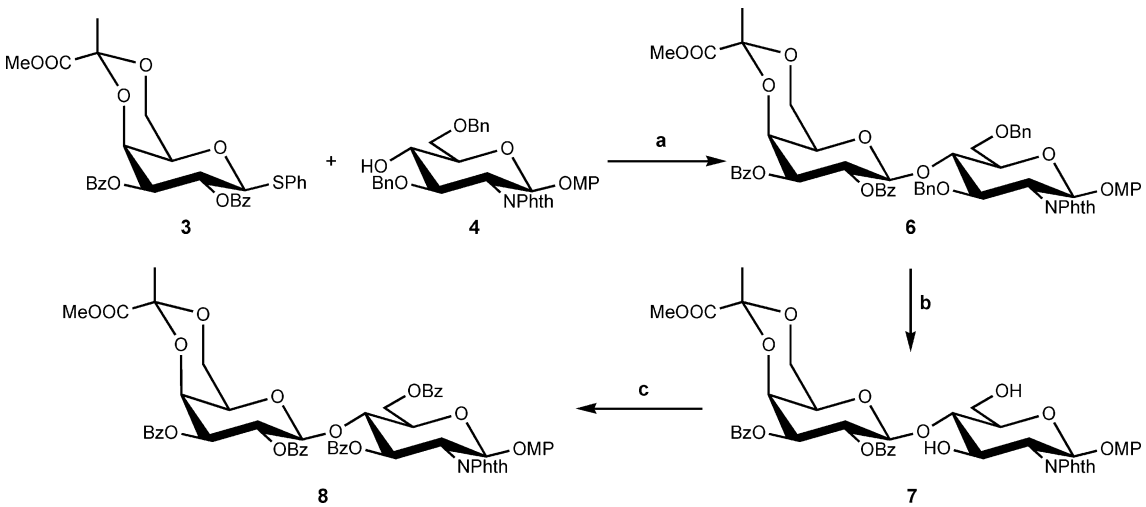
Scheme 1 Oligosaccharide epitopes isolated from the proteoglycan g-200 glycan of *Microciona prolifera*.

our previous interaction studies with the sulfated disaccharide,[12] the earlier reported synthetic methyl[14] and 5-aminopentyl[15] β-glycosides of the trisaccharide were expected to be less suited for the present interaction studies. In the synthetic route to allyl α-glycoside **14**, three monosaccharide building blocks were used, namely, **3**, **4**, and **5** (Scheme 2). The allyl α-fucoside acceptor **5** was previously applied to the synthesis of the allyl-spacer-containing sulfated disaccharide.[9] The 4,6-pyruvated galactosyl donor **3** was prepared according to the literature.[14] ^{13}C NMR analysis of this building block showed a signal with a chemical shift of 25.4 ppm, assigned to the $CH_3CCOOCH_3$ group in the desired (*R*)-pyruvate configuration.[7,16] Acceptor **4**, a product wherein the benzyl groups at O-3 and O-6 are essential for the activation of the non-reactive 4-OH group, was synthesized according to the literature.[17]

In a first step, coupling of donor **3** with acceptor **4**, promoted by *N*-iodosuccinimide (NIS) and a catalytic amount of triflic acid, generated disaccharide **6** in 74% yield (Scheme 3). Subsequent de-*O*-benzylation of **6**, using 10% Pd on charcoal and H_2, rendered



Scheme 2 Monosaccharide building blocks **3**, **4**, and **5**, used in the synthesis of the allyl-spacer-containing trisaccharide **14**.



Scheme 3 *Reagents and conditions*: **a**, NIS–HOTf in CH_2Cl_2, −30 °C, 30 min, 74%; **b**, Pd/C and H_2 in EtOAc–EtOH, 4 h, 94%; **c**, BzCl in CH_2Cl_2–pyridine, 3 h, 93%.

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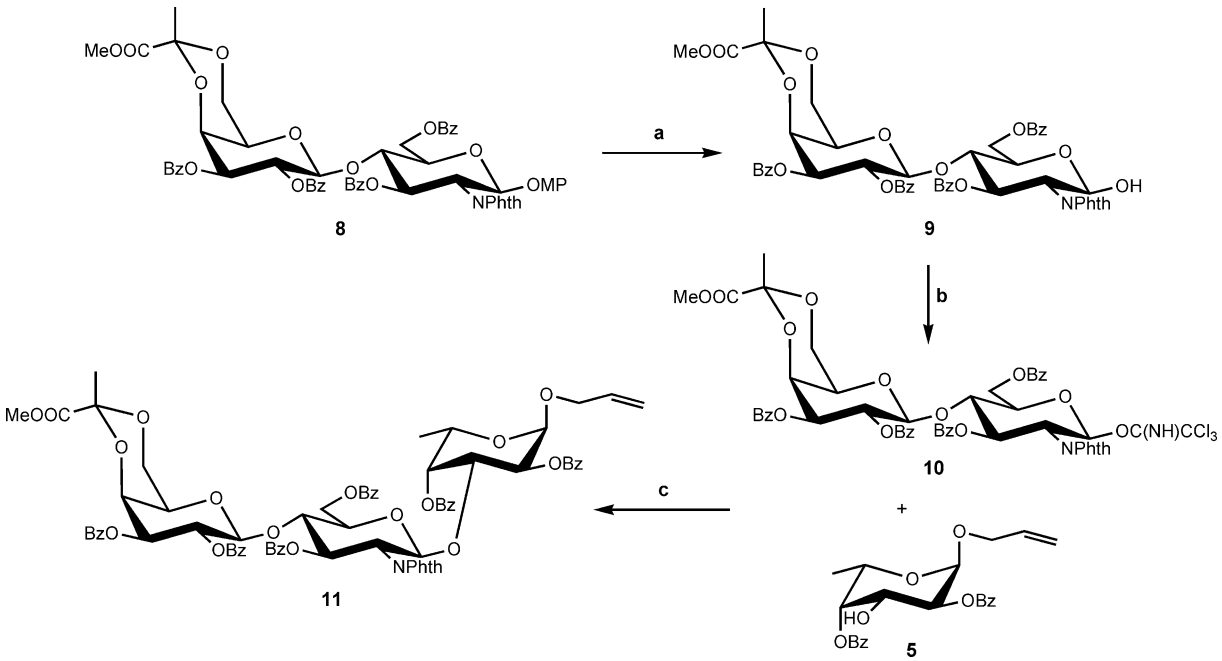
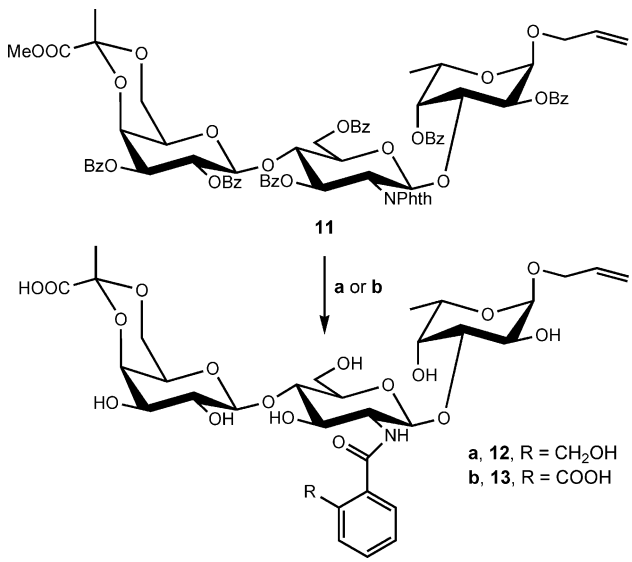


Scheme 4 *Reagents and conditions*: **a**, CAN in 9 : 1 acetonitrile–H_2O, 0 °C, 30 min, 77%; **b**, Cl_3CCN–DBU in CH_2Cl_2, 3 h, 73%; **c**, TMSOTf in CH_2Cl_2, 15 min, 0 °C followed by 15 min, rt, 70%.

disaccharide **7** (94%), of which the free 3-OH and 6-OH groups were benzoylated in dry dichloromethane using benzoyl chloride and pyridine (→ **8**, 93%). This deprotection/protection protocol was performed at the disaccharide level, to avoid hydrogenation of the allyl group that is present after coupling with the allyl α-fucoside acceptor **5**.

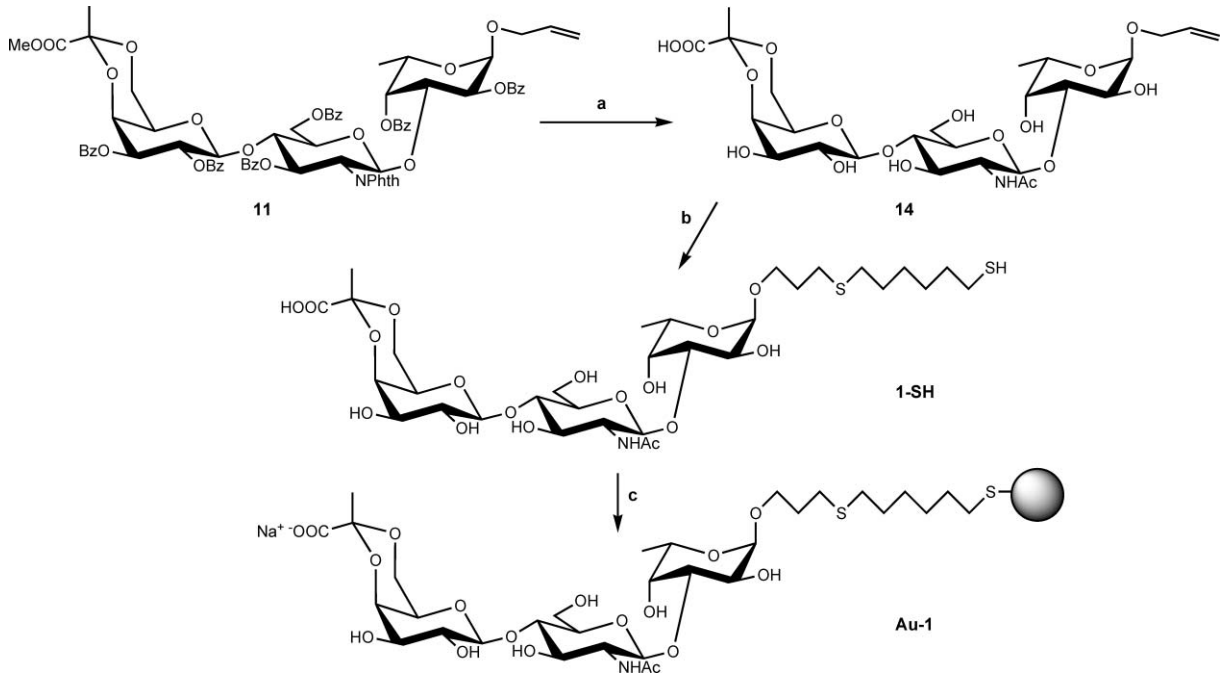
Oxidative removal of the anomeric 4-methoxyphenyl group, using ammonium cerium(IV) nitrate (CAN) (Scheme 4) in a 1 : 1 : 1 toluene–acetonitrile–water two-phase mixture, resulted in only 20% of the desired disaccharide **9**. Under these reaction conditions (incubation time 3 h) the removal of the 4,6-(1-methoxycarbonylethylidene) group was highly favoured. However, when the reaction was performed in 9 : 1 acetonitrile–water at 0 °C[18] for only 30 min, the reducing-end free disaccharide **9** was obtained in 77% yield. Imidation of **9**, using trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a promotor, gave disaccharide donor **10** (73%). Coupling of donor **10** with an excess of acceptor **5** (1.9 equiv.), in the presence of trimethylsilyl triflate as a promotor (0.05 equiv. based on **10**), gave finally trisaccharide **11** (70%).

In view of the presence of a 4,6-acetal group in the galactose unit of **11**, the deprotection of **11** to give **14** needs much care. At first instance, a near-neutral phthalimido deprotection protocol based on partial reduction of the *N*-phthalimide group using $NaBH_4$ in aq. isopropanol, followed by an acid-catalyzed cyclization to afford the free amine, was applied.[19] However, this de-*N*-phthaloylation procedure, followed by de-*O*-acetylation using Zemplén conditions[20] and saponification of the methyl ester of the pyruvate group, resulted only in trisaccharide **12** having a partially reduced phthalimide group (Scheme 5). Alternative attempts to achieve the cyclization of this intermediate and generate the free amine were not successful. To overcome this problem, we tried to selectively saponify the methyl ester in the presence of



Scheme 5 *Reagents and conditions*: **a**, (i) $NaBH_4$ in *i*-PrOH–H_2O, overnight, (ii) HOAc (pH 5), 80 °C, 5 h, (iii) NaOMe in MeOH, 2 h, (iv) 0.19 M NaOH in 1 : 1 MeOH–H_2O; **b**, (i) 3 M aq. NaOH in 5 : 1 MeOH–H_2O, 3 h, (ii) 33% ethanolic CH_3NH_2, 5 days, (iii) Ac_2O in MeOH, 3 h, 0 °C, or (i) 1 M aq. LiOH in acetonitrile, 1 h, (ii) 33% ethanolic CH_3NH_2, 5 days, (iii) Ac_2O in MeOH, 3 h, 0 °C.

the phthalimide group. However, the tested conditions, such as incubation with 1 M aq. LiOH in acetonitrile or 3 M aq. NaOH in methanol–water,[21] resulted only in the formation of the semi-hydrolyzed phthalimido product **13**. Under basic conditions, the phthalimide group is partially opened, yielding an intermediate that could not be further deprotected. In addition, a milder saponification method using $CaCl_2$ in 1 M LiOH in 70% aq.



Scheme 6 *Reagents and conditions*: **a**, (i) LiI in EtOAc, boil under reflux, 16 h, (ii) 33% ethanolic CH_3NH_2, 5 days, (iii) Ac_2O in MeOH, 0 °C, 2 h, 60% overall yield; **b**, $HS(CH_2)_6SH$, MeOH, UV-light, 2 h, 40%; **c**, 25 mM aq. $HAuCl_4$, 1 M aq. $NaBH_4$, MeOH, 2 h.

isopropanol[22,23] was not able to cleave the methyl ester of the pyruvate group. Finally, the methyl ester cleavage in **11** was realized under neutral conditions with LiI in refluxing ethyl acetate (16 h), followed by de-*N*-phthaloylation/de-*O*-acetylation with ethanolic 33% methyl amine (5 days), and *N*-acetylation with acetic anhydride in methanol at 0 °C,[24] yielding allyl glycoside **14** in 60% yield. Then, the allyl group of **14** was elongated with 1,6-hexanedithiol,[11] and spacer-containing trisaccharide **1-SH** was obtained in 40% yield (Scheme 6).

Preparation of gold glyconanoparticles

Gold glyconanoparticles **Au-1** were prepared by a modification of Brust's method,[25] following the same procedures as used for the preparation of the gold glyconanoparticles coated with the sulfated disaccharide and its structural variants.[11] Accordingly, tetrachloroauric anion was reduced in the presence of the thiol-spacer-containing trisaccharide **1-SH** by the careful addition of an excess of $NaBH_4$. The water-soluble gold glyconanoparticles **Au-1** were purified by centrifugal filtration and characterized by ^{1}H NMR spectroscopy, monosaccharide analysis, and TEM.

The ^{1}H NMR spectrum of **Au-1** (Fig. 1a) showed line broadening of the carbohydrate signals, this being typical in the spectra of gold glyconanoparticles.[11] The broad peaks matched those of the corresponding thiol-spacer-containing trisaccharide **1-SH** (Fig. 1b). Monosaccharide analysis of **Au-1** revealed the expected molar ratio of Fuc:GlcNAc:Gal = 1 : 1 : 1, and a 53% weight-percentage of carbohydrate. As is evident from Fig. 2, the TEM micrographs of **Au-1** (0.1 mg cm^{-3}) in water showed uniformly dispersed nanoparticles throughout the grid surface. The size distribution was calculated from approximately 1000 particles in different micrographs, giving a mean diameter of 1.59 ± 0.5 nm (116 Au atoms).[26] Combining the results of the TEM size distribution and the weight-percentage of carbohydrate, it was calculated that the surface coverage amounts to 41%, which corresponds to 32 trisaccharide molecules per nanoparticle.[27,28]

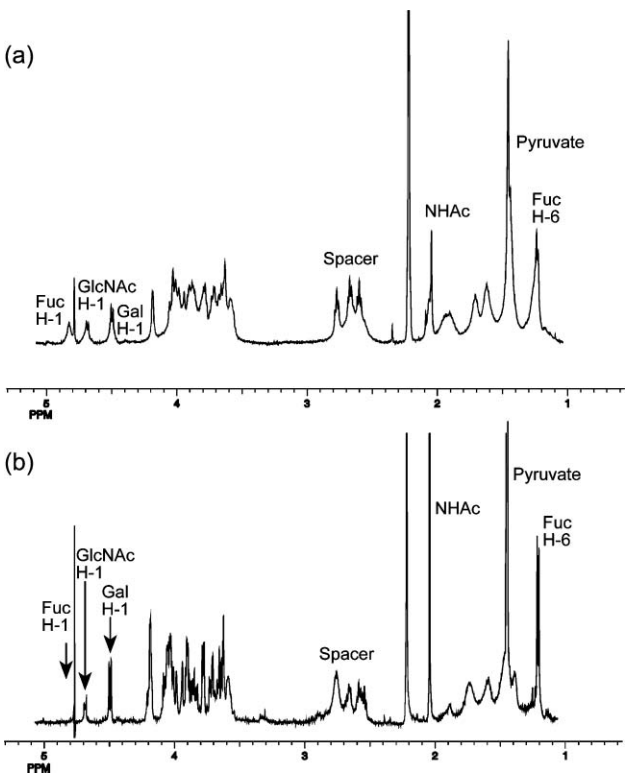


Fig. 1 The ^{1}H NMR spectra of (a) gold nanoparticles decorated with **1-SH** (**Au-1**) and (b) **1-SH** in D_2O.

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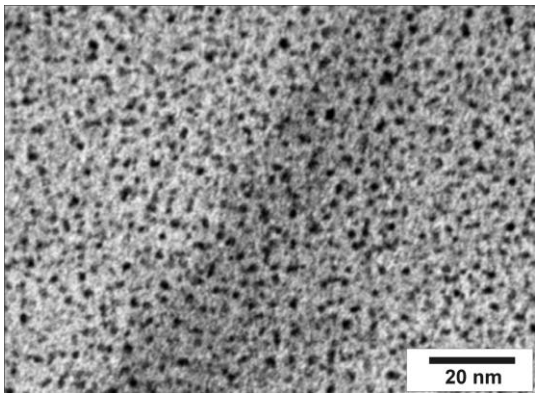

Fig. 2 TEM image of gold nanoparticles decorated with **1-SH** (**Au-1**) in H_2O (0.1 mg cm^{-3}); scale bar 20 nm.

The gold glyconanoparticles **Au-1** have been used to investigate the pyruvated trisaccharide self-recognition on the molecular level *via* TEM, carried out in the absence and presence of Ca^{2+} ions. Furthermore, the thiol-spacer-containing trisaccharide **1-SH** has been used to create self-assembling monolayers for atomic force microscopy (AFM) experiments. The results of these studies will be described elsewhere.

Conclusion

In biological recognition and adhesion processes, protein–protein, carbohydrate–protein, and carbohydrate–carbohydrate interactions play key roles. Carbohydrate–carbohydrate interactions are characterized by extremely low affinities, and so far, only a few examples have been described. In biological systems, these low affinities are compensated by multivalent presentation of the ligands. In the Ca^{2+}-dependent species-specific cellular adhesion of marine sponges, a large number of polysaccharide chains of 200 kDa, linked to specific protein domains of the extracellular proteoglycan-type aggregation factor, are responsible for the self-recognition among the cells. The multivalency is reached *via* highly repetitive oligosaccharide epitopes in these polysaccharides. The structural knowledge of two of these epitopes, a sulfated disaccharide and a pyruvated trisaccharide, in the marine sponge *M. prolifera* has opened new analytical opportunities to explore multivalent epitope systems as mimics. By using UV, SPR, TEM, AFM, NMR, and MC as technologies, the carbohydrate–carbohydrate molecular self-recognition on the epitope level can be investigated. To carry out such studies, the availability of the synthetic oligosaccharide epitopes is a prerequisite, as has been demonstrated recently by us for the sulfated disaccharide. With the successful synthesis of the pyruvated trisaccharide described in this study, now it will be possible to visualize the interaction picture in more detail, and it is expected that molecular models at the epitope level can be extrapolated to the polysaccharide level, leading to an interaction model of a phenomenon that was observed in 1901, for the first time. Additionally, the fundamental results of these studies will assist in a further understanding on the molecular level of carbohydrate–carbohydrate phenomena observed in human embryogenesis, metastasis, and other cellular proliferation processes.

Experimental

General procedures

All chemicals were of reagent grade, and were used without further purification. Reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck); after examination under UV-light, compounds were visualized by heating with orcinol (1 mg cm^{-3}) in 5% (v/v) methanolic H_2SO_4, or ninhydrin (1.5 mg cm^{-3}) in 38 : 1.75 : 0.25 1-BuOH–H_2O–HOAc. In the work-up procedures of reaction mixtures, organic solutions were washed with appropriate amounts of the indicated aqueous solutions, then dried with $MgSO_4$, and concentrated under reduced pressure at 30–50 °C in a water bath. Column chromatography was performed on Silica Gel 60 (Merck, 0.040–0.063 mm). ^{1}H NMR spectra were recorded at 300 K with a Bruker AMX 500 (500 MHz) spectrometer; δ_H values are given in ppm relative to the signal for internal Me_4Si ($\delta_H = 0$, $CDCl_3$) or internal acetone ($\delta_H = 2.22$, D_2O). Two-dimensional ^{1}H-^{1}H TOCSY (mixing times 7 and 100 ms) and ^{1}H–^{13}C-correlated HSQC spectra were recorded at 300 K with a Bruker AMX 500 spectrometer; δ_C values are given in ppm relative to the signal of $CDCl_3$ ($\delta_C = 77.1$, $CDCl_3$) or internal acetone ($\delta_C = 30.9$, D_2O). Exact masses were measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager-DE Pro (Applied Biosystems) instrument in the reflector mode at a resolution of 5000 FWHM. 2,4-Dihydroxybenzoic acid in 1 : 1 acetonitrile–H_2O (5 mg cm^{-3}) was used as a matrix. A ladder of maltose oligosaccharides (G3-G13) was added as internal calibration.

4-Methoxyphenyl 2,3-di-*O*-benzoyl-4,6-*O*-[(*R*)-1-methoxycarbonylethylidene]-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 6. A solution of 4-methoxyphenyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside[17] (**4**; 367 mg, 0.61 mmol) and phenyl 2,3-di-*O*-benzoyl-4,6-*O*-[(*R*)-1-methoxycarbonylethylidene]-1-thio-β-D-galactopyranoside[14] (**3**; 618 mg, 1.10 mmol) in dry CH_2Cl_2 (20 cm^3), containing activated molecular sieves (4 Å, 2 g), was stirred for 45 min at rt. The mixture was cooled down to −30 °C, and, after the addition of NIS (376 mg, 1.65 mmol) and a catalytic amount of triflic acid, stirred for 30 min at −30 °C, when TLC (95 : 5 CH_2Cl_2–acetone) showed the formation of **6** ($R_f = 0.32$). After neutralization with pyridine and filtration, the solution was washed with saturated aq. $Na_2S_2O_3$, dried, filtered, and concentrated. Column chromatography (95 : 5 CH_2Cl_2–acetone) of the residue gave **6**, isolated as a yellow solid (478 mg, 74%); δ_H(500 MHz; $CDCl_3$) 1.56 (3 H, s, $CH_3CCOOCH_3$), 3.32 (1 H, bs, H-5′), 3.48 (1 H, m, H-5), 3.57 (1 H, dd, $J_{H\text{-}5,H\text{-}6a}$ <1, $J_{H\text{-}6a,H\text{-}6b}$ 10.9, H-6a), 3.63 (3 H, s, $CH_3CCOOCH_3$), 3.66 (3 H, s, $C_6H_4OCH_3$), 3.71 (1 H, dd, $J_{H\text{-}5,H\text{-}6b}$ 3.2, H-6b), 3.95 and 4.17 (each 1 H, 2 × m, H-6′a, H-6′b), 4.21 (1 H, bt, H-4), 4.36, 4.59, 4.68, and 5.02 (each 1 H, 4 × d, 2 × $CH_2C_6H_5$), 4.46 (1 H, bd, $J_{H\text{-}3',H\text{-}4'}$ 3.4, $J_{H\text{-}4',H\text{-}5'}$ <1, H-4′), 4.84 (1 H, d, $J_{H\text{-}1',H\text{-}2'}$ 8.1, H-1′), 5.05 (1 H, dd, $J_{H\text{-}2',H\text{-}3'}$ 10.4, H-3′), 5.52 (1 H, d, $J_{H\text{-}1,H\text{-}2}$ 7.8, H-1), 5.81 (1 H, dd, H-2′), 6.62 and 6.74 (each 2 H, 2 × m, $C_6H_4OCH_3$), 6.84, 7.08, 7.36, 7.53, 7.90, and 7.98 (2 H, 2 H, 10 H, 4 H, 1 H, 1 H, 6 × m, 2 × $CH_2C_6H_5$ and 2 × COC_6H_5), 7.67 and 7.79 (each 2 H, 2 × m, Phth); δ_C(125.76 MHz; $CDCl_3$) 25.7 ($CH_3CCOOCH_3$), 52.4 ($CH_3CCOOCH_3$), 55.7 ($C_6H_4OCH_3$), 55.9 (C-2), 65.1 (C-6′), 65.7 (C-5′), 67.8 (C-6), 69.1 (C-4′), 69.8 (C-2′), 72.9 (C-3′), 73.6

and 75.3 (2 × $CH_2C_6H_5$), 74.9 (C-5), 77.2 (C-3), 78.2 (C-4), 97.7 (C-1), 100.6 (C-1′), 114.6 and 118.9 ($C_6H_4OCH_3$), 123.6 and 133.9 ($N(CO)_2C_6H_4$), 127.3, 128.1, 128.2, 128.6, 128.7, 130.0, 130.2, and 133.5 (2 × $CH_2C_6H_5$ and 2 × COC_6H_5); *m/z* (HR MALDI-TOF) 1072.339 ([M + Na]$^+$, $C_{59}H_{55}NNaO_{17}$ requires 1072.337).

4-Methoxyphenyl 2,3-di-*O*-benzoyl-4,6-*O*-[(*R*)-1-methoxycarbonylethylidene]-β-D-galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-β-D-glucopyranoside 7. To a solution of **6** (478 mg, 0.45 mmol) in 1 : 1 EtOAc–EtOH (20 cm^3) was added 10% Pd/C (0.93 g), and the mixture was stirred for 4 h, while H_2 was bubbled through. After filtration over Celite and concentration, the residue was purified by column chromatography (9 : 1 CH_2Cl_2–acetone → 9 : 1 CH_2Cl_2–MeOH), yielding **7**, isolated as a white solid (380 mg, 94%); δ_H(500 MHz; $CDCl_3$) 1.54 (3 H, s, CH_3CCOOCH$_3$), 3.54 (1 H, m, H-5), 3.55 and 3.61 (each 1 H, 2 × m, H-6a, H-6b), 3.63 (3 H, s, CH$_3$CCOOCH_3), 3.67 (1 H, bs, H-5′), 3.69 (3 H, s, $C_6H_4OCH_3$), 4.87 (1 H, bt, H-4), 4.05 (2 H, m, H-6′a, H-6′b), 4.37 (1 H, dd, $J_{H\text{-}1,H\text{-}2}$ 8.6, $J_{H\text{-}2,H\text{-}3}$ 10.9, H-2), 4.53 (1 H, bd, $J_{H\text{-}3',H\text{-}4'}$ 3.7, $J_{H\text{-}4',H\text{-}5'}$ <1, H-4′), 4.58 (1 H, dd, $J_{H\text{-}3,H\text{-}4}$ 8.6, H-3), 4.86 (1 H, d, $J_{H\text{-}1',H\text{-}2'}$ 8.0, H-1′), 5.19 (1 H, dd, $J_{H\text{-}2',H\text{-}3'}$ 10.4, H-3′), 5.77 (1 H, d, H-1), 5.84 (1 H, dd, H-2′), 6.68 and 6.77 (each 2 H, 2 × m, $C_6H_4OCH_3$), 7.41, 7.52, 7.98, and 8.04 (6 H, 2 H, 1 H, 1 H, 4 × m, 2 × COC_6H_5), 7.71 and 7.85 (each 2 H, 2 × m, Phth); δ_C(125.76 MHz; $CDCl_3$) 25.5 (*C*H$_3$CCOOCH$_3$), 52.5 (CH$_3$CCOO*C*H$_3$), 55.7 ($C_6H_4OCH_3$), 56.0 (C-2), 60.5 (C-6), 64.7 (C-6′), 66.2 (C-5′), 68.8 (C-4′), 68.9 (C-2′), 69.5 (C-3), 72.6 (C-3′), 74.5 (C-5), 81.0 (C-4), 97.5 (C-1), 101.8 (C-1′), 114.7 and 118.5 ($C_6H_4OCH_3$), 123.7 and 134.3 ($N(CO)_2C_6H_4$), 128.6, 128.7, 129.9, and 130.1 (2 × COC_6H_5); *m/z* (HR MALDI-TOF) 892.243 ([M + Na]$^+$, $C_{45}H_{43}NNaO_{17}$ requires 892.215).

4-Methoxyphenyl 2,3-di-*O*-benzoyl-4,6-*O*-[(*R*)-1-methoxycarbonylethylidene]-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 8. To a solution of **7** (470 mg, 0.54 mmol) in dry CH_2Cl_2 (8 cm^3) and dry pyridine (1 cm^3) was added benzoyl chloride (1 cm^3). The mixture was stirred for 3 h, when TLC (95 : 5 CH_2Cl_2–acetone) showed the complete conversion into **8** (R_f = 0.56). After dilution with CH_2Cl_2, the organic phase was washed with saturated aq. $NaHCO_3$, dried, filtered, and concentrated. Column chromatography (98 : 2 CH_2Cl_2–acetone → 95 : 5 CH_2Cl_2–acetone) of the residue yielded **8**, isolated as a white foam (545 mg, 93%); δ_H(500 MHz; $CDCl_3$) 1.39 (3 H, s, CH_3CCOOCH$_3$), 2.71 (1 H, bs, H-5′), 3.53 (2 H, m, H-6′a, H-6′b), 3.54 (3 H, s, CH$_3$CCOOCH_3), 3.66 (3 H, s, $C_6H_4OCH_3$), 4.07 (1 H, m, H-5), 4.19 (1 H, bd, $J_{H\text{-}3',H\text{-}4'}$ 3.5, $J_{H\text{-}4',H\text{-}5'}$ <1, H-4′), 4.21 (1 H, bt, H-4), 4.34 (1 H, dd, $J_{H\text{-}5,H\text{-}6b}$ 5.5, $J_{H\text{-}6a,H\text{-}6b}$ 11.8, H-6b), 4.60 (1 H, dd, $J_{H\text{-}1,H\text{-}2}$ 8.6, $J_{H\text{-}2,H\text{-}3}$ 10.4, H-2), 4.64 (1 H, dd, $J_{H\text{-}5,H\text{-}6a}$ <1, H-6a), 4.79 (1 H, d, $J_{H\text{-}1',H\text{-}2'}$ 8.1, H-1′), 4.98 (1 H, dd, $J_{H\text{-}2',H\text{-}3'}$ 10.4, H-3′), 5.72 (1 H, dd, H-2′), 5.90 (1 H, d, H-1), 6.23 (1 H, dd, $J_{H\text{-}3,H\text{-}4}$ 8.7, H-3), 6.57 and 6.77 (each 2 H, 2 × m, $C_6H_4OCH_3$), 7.22, 7.33, 7.42, 7.45, 7.56, 7.89, and 7.93 (3 H, 6 H, 3 H, 2 H, 2 H, 1 H, 3 H, 7 × m, 4 × COC_6H_5), 7.68 (4 H, m, Phth); δ_C(125.76 MHz; $CDCl_3$) 25.5 (*C*H$_3$CCOOCH$_3$), 52.3 (CH$_3$CCOO*C*H$_3$), 55.1 (C-2), 55.6 ($C_6H_4OCH_3$), 62.7 (C-6), 64.2 (C-6′), 65.8 (C-5′), 68.6 (C-4′), 69.4 (C-2′), 72.7 (C-3′), 72.8 (C-3), 73.0 (C-5), 77.6 (C-4), 97.7 (C-1), 101.4 (C-1′), 114.5 and 119.2 ($C_6H_4OCH_3$), 123.7 and 134.3 ($N(CO)_2C_6H_4$), 128.4, 128.5, 128.6, 129.8, 129.9, 133.1, 133.4, 133.5, and 134.3 (4 × COC_6H_5); *m/z* (HR MALDI-TOF) 1100.287 ([M + Na]$^+$, $C_{59}H_{51}NNaO_{19}$ requires 1100.295).

2,3-Di-*O*-benzoyl-4,6-*O*-[(*R*)-1-methoxycarbonylethylidene]-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranose 9. To a solution of **8** (545 mg, 0.51 mmol) in acetonitrile (13.5 cm^3) and water (1.5 cm^3) was added, at 0 °C, ammonium cerium(IV) nitrate (2.79 g, 5.1 mmol). The mixture was vigorously stirred for 30 min at 0 °C, when TLC (95 : 5 CH_2Cl_2–acetone) showed the appearance of **9** (R_f = 0.14). After dilution with EtOAc, the organic phase was washed with saturated aq. $NaHCO_3$, dried, filtered, and concentrated. Column chromatography (95 : 5 CH_2Cl_2–acetone → 9 : 1 CH_2Cl_2–acetone) of the residue yielded **9**, isolated as a yellow solid (380 mg, 77%); δ_H(500 MHz; $CDCl_3$) 1.39 (3 H, s, CH_3CCOOCH$_3$), 2.69 (1 H, bs, H-5′), 3.47 (1 H, dd, $J_{H\text{-}5',H\text{-}6'b}$ 2.0, $J_{H\text{-}6'a,H\text{-}6'b}$ 12.9, H-6′b), 3.53 (3 H, s, CH$_3$CCOOCH_3), 3.55 (1 H, dd, $J_{H\text{-}5',H\text{-}6'a}$ 1.2, H-6′a), 4.02 (1 H, m, H-5), 4.18 (1 H, bd, $J_{H\text{-}3',H\text{-}4'}$ 3.6, $J_{H\text{-}4',H\text{-}5'}$ <1, H-4′), 4.23 (1 H, bt, H-4), 4.29 (1 H, dd, $J_{H\text{-}1,H\text{-}2}$ 8.4, $J_{H\text{-}2,H\text{-}3}$ 10.7, H-2), 4.35 (1 H, dd, $J_{H\text{-}5,H\text{-}6b}$ 4.0, $J_{H\text{-}6a,H\text{-}6b}$ 12.0, H-6b), 4.69 (1 H, dd, $J_{H\text{-}5,H\text{-}6a}$ 1.9, H-6a), 4.79 (1 H, d, $J_{H\text{-}1',H\text{-}2'}$ 8.1, H-1′), 4.97 (1 H, dd, $J_{H\text{-}2',H\text{-}3'}$ 10.5, H-3′), 5.72 (1 H, dd, H-2′), 5.75 (1 H, d, H-1), 6.22 (1 H, dd, $J_{H\text{-}3,H\text{-}4}$ 8.7, H-3), 7.16, 7.32, 7.40, 7.45, 7.55, 7.86, and 7.91 (3 H, 6 H, 3 H, 2 H, 2 H, 1 H, 3 H, 7 × m, 4 × COC_6H_5), 7.68 (4 H, m, Phth); δ_C(125.76 MHz; $CDCl_3$) 25.4 (*C*H$_3$CCOOCH$_3$), 52.3 (CH$_3$CCOO*C*H$_3$), 56.5 (C-2), 62.3 (C-6), 64.1 (C-6′), 65.8 (C-5′), 68.5 (C-4′), 69.3 (C-2′), 72.6 (C-3), 72.7 (C-3′), 73.0 (C-5), 77.1 (C-4), 92.6 (C-1), 101.4 (C-1′), 123.7 and 134.3 ($N(CO)_2C_6H_4$), 128.3, 128.4, 128.5, 128.6, 129.7, 129.9, 133.1, 133.4, 133.6, and 134.3 (4 × COC_6H_5); *m/z* (HR MALDI-TOF) 994.226 ([M + Na]$^+$, $C_{52}H_{45}NNaO_{18}$ requires 994.253).

Allyl 2,3-di-*O*-benzoyl-4,6-*O*-[(*R*)-1-methoxycarbonylethylidene]-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→3)-2,4-di-*O*-benzoyl-α-L-fucopyranoside 11. To a solution of **9** (380 mg, 0.39 mmol) in CH_2Cl_2 (12 cm^3) and trichloroacetonitrile (0.4 cm^3, 3.9 mmol) was added, at 0 °C, DBU (10.3 mm^3, 39 μmol). The mixture was stirred for 3 h at rt, when TLC (95 : 5 CH_2Cl_2–acetone) showed the conversion of **9** into imidate **10** (R_f = 0.45). After concentration, column chromatography (95 : 5 CH_2Cl_2–acetone) of the residue gave **10**, isolated as a yellow foam (320 mg, 73%).

A solution of **10** (70 mg, 62 μmol) and allyl 2,4-di-*O*-benzoyl-α-L-fucopyranoside[9] (**5**; 48.7 mg, 118 μmol) in dry CH_2Cl_2 (3 cm^3), containing activated molecular sieves (4 Å, 0.3 g), was stirred for 45 min at rt, then TMSOTf (13.5 mm^3, 6.2 μmol) was added at 0 °C. The mixture was stirred for 15 min at 0 °C and 15 min at rt, when TLC (95 : 5 CH_2Cl_2–acetone) showed the formation of a new product (R_f = 0.38). After neutralization with pyridine and filtration, the solution was washed with 10% aq. NaCl, dried, filtered, and concentrated. Column chromatography (95 : 5 CH_2Cl_2–acetone) of the residue afforded **11**, isolated as a white solid (60 mg, 70%); δ_H(500 MHz; $CDCl_3$) 1.04 (3 H, d, $J_{H\text{-}5,H\text{-}6}$ 6.6, 3 × H-6), 1.33 (3 H, s, CH_3CCOOCH$_3$), 2.59 (1 H, bs, H-5″), 3.43 (2 H, m, H-6″a, H-6″b), 3.55 (3 H, s, CH$_3$CCOOCH_3), 3.92 and 4.05 (each 1 H, 2 × m, OCH_2CH=CH$_2$), 3.99 (1 H, m, H-5′), 4.00 (1 H, bt, H-4′), 4.12 (1 H, m, H-5), 4.13 (1 H, bd, $J_{H\text{-}3'',H\text{-}4''}$ 3.7, $J_{H\text{-}4'',H\text{-}5''}$ <1, H-4″), 4.20 (1 H, dd, $J_{H\text{-}1',H\text{-}2'}$ 8.4, $J_{H\text{-}2',H\text{-}3'}$ 10.5, H-2′), 4.01 and 4.43 (each 1 H, 2 × m, H-6′a, H-6′b), 4.53 (1 H, dd, $J_{H\text{-}2,H\text{-}3}$ 10.4, $J_{H\text{-}3,H\text{-}4}$ 3.4, H-3), 4.60 (1 H, d,

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$J_{H\text{-}1'',H\text{-}2''}$ 7.9, H-1″), 4.85 (1 H, dd, $J_{H\text{-}2'',H\text{-}3''}$ 10.4, H-3″), 4.98 and 5.14 (each 1 H, 2 × m, $OCH_2CH{=}CH_2$), 5.10 (1 H, d, $J_{H\text{-}1,H\text{-}2}$ 3.8, H-1), 5.37 (1 H, bd, $J_{H\text{-}4,H\text{-}5}$ <1, H-4), 5.39 (1 H, dd, H-2), 5.64 (1 H, dd, H-2″), 5.65 (1 H, m, $OCH_2CH{=}CH_2$), 5.69 (1 H, d, H-1′), 6.05 (1 H, dd, $J_{H\text{-}3',H\text{-}4'}$ 8.3, H-3′), 7.09, 7.19, 7.29, 7.44, 7.45, 7.75, 7.88, and 7.97 (3 H, 3 H, 6 H, 8 H, 4 H, 2 H, 2 H, 2 H, 8 × m, 6 × COC_6H_5), 7.55 (4 H, m, Phth); δ_C(125.76 MHz; $CDCl_3$) 16.2 (C-6), 25.3 ($CH_3CCOOCH_3$), 52.1 ($CH_3CCOOCH_3$), 55.0 (C-2′), 63.4 (C-6′), 64.0 (C-6″), 65.6 (C-5″), 68.3 (C-4″), 68.4 (C-5), 68.7 ($OCH_2CH{=}CH_2$), 69.2 (C-2), 69.3 (C-2″), 71.9 (C-4), 72.4 (C-3′), 72.6 (C-5′), 72.8 (C-3″), 73.8 (C-3), 77.0 (C-4′), 95.8 (C-1), 96.9 (C-1′), 101.0 (C-1″), 117.4 ($OCH_2CH{=}CH_2$), 123.7 and 134.0 ($N(CO)_2C_6H_4$), 133.7 ($OCH_2CH{=}CH_2$), 128.2, 128.3, 128.4, 128.5, 128.6, 129.5, 129.8, 130.0, 132.9, 133.0, 133.3, 133.4, and 133.5 (6 × COC_6H_5); *m/z* (HR MALDI-TOF) 1388.416 ([M + Na]$^+$, $C_{75}H_{67}NNaO_{24}$ requires 1388.395).

Allyl 4,6-*O*-[(*R*)-1-carboxyethylidene]-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-α-L-fucopyranoside 14. To a solution of **11** (96 mg, 71 μmol) in EtOAc (10 cm^3) was added anhydrous LiI (472 mg, 3.55 mmol). The mixture was boiled under reflux, in the dark and overnight, when TLC (95 : 5 CH_2Cl_2–acetone) showed the disappearance of **11**. After dilution with EtOAc, the mixture was washed with 1% aq. HCl, saturated aq. $NaHCO_3$, and saturated aq. $Na_2S_2O_3$, dried, filtered, and concentrated. A solution of the residue in 33% ethanolic CH_3NH_2 (10 cm^3) was stirred for 5 days, during which time the mixture was twice concentrated and fresh 33% ethanolic CH_3NH_2 (10 cm^3) was added. After co-concentration with toluene, to a solution of the residue in dry MeOH (5 cm^3) at 0 °C was added acetic anhydride (0.5 cm^3). The mixture was stirred for 2 h at 0 °C, then concentrated. Size-exclusion chromatography (Bio-Gel P-2, 100 mM NH_4HCO_3) of the residue afforded **14**, isolated after lyophilization from water, as a white amorphous powder (27 mg, 60%); δ_H(500 MHz; D_2O) 1.20 (3 H, d, $J_{H\text{-}5,H\text{-}6}$ 6.6, 3 × H-6), 1.45 (3 H, s, CH_3CCOOD), 2.04 (3 H, s, $NDCOCH_3$), 3.59 (1 H, bt, H-3′), 3.62 (1 H, bt, H-2″), 3.65 (1 H, m, H-5″), 3.67 (1 H, m, H-5′), 3.72 (1 H, dd, $J_{H\text{-}2'',H\text{-}3''}$ 9.9, $J_{H\text{-}3'',H\text{-}4''}$ 3.7, H-3″), 3.77 (1 H, bt, H-2′), 3.78 (1 H, bt, H-4′), 3.85 (1 H, dd, $J_{H\text{-}5',H\text{-}6'b}$ 5.2, $J_{H\text{-}6'a,H\text{-}6'b}$ 12.4, H-6′b), 3.88 (1 H, bt, H-2), 3.89 (1 H, bd, H-4), 3.92 and 4.04 (each 1 H, 2 × m, H-6″a, H-6″b), 4.00 (1 H, bd, $J_{H\text{-}5',H\text{-}6'a}$ <1, H-6′a), 4.03 (1 H, bt, H-3), 4.04 (1 H, m, H-5), 4.07 and 4.18 (each 1 H, 2 × m, $OCH_2CH{=}CH_2$), 4.08 (1 H, bd, $J_{H\text{-}3'',H\text{-}4''}$ 2.8, $J_{H\text{-}4'',H\text{-}5''}$ <1, H-4″), 4.49 (1 H, d, $J_{H\text{-}1'',H\text{-}2''}$ 8.0, H-1″), 4.68 (1 H, d, $J_{H\text{-}1',H\text{-}2'}$ 7.7, H-1′), 4.95 (1 H, d, $J_{H\text{-}1,H\text{-}2}$ 3.9, H-1), 5.26 and 5.35 (each 1 H, 2 × m, $OCH_2CH{=}CH_2$), 5.97 (1 H, m, $OCH_2CH{=}CH_2$); δ_C(125.76 MHz; D_2O) 15.9 (C-6), 22.8 ($NDCOCH_3$), 25.7 (CH_3CCOOD), 55.9 (C-2′), 60.7 (C-6′), 65.6 (C-6″), 66.9 (C-2″), 67.0 (C-2), 67.3 (C-5), 69.4 ($OCH_2CH{=}CH_2$), 69.8 (C-4), 70.1 (C-4″), 71.1 (C-5″), 72.1 (C-3″), 72.9 (C-4′), 75.6 (C-3′), 77.5 (C-3), 79.6 (C-5′), 98.1 (C-1), 99.6 (C-1′), 103.6 (C-1″), 118.9 ($OCH_2CH{=}CH_2$), 134.4 ($OCH_2CH{=}CH_2$); *m/z* (HR MALDI-TOF) 662.223 ([M + Na]$^+$, $C_{26}H_{41}NNaO_{17}$ requires 662.227).

3-(6-Mercaptohexylthio)propyl 4,6-*O*-[(*R*)-1-carboxyethylidene]-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-α-L-fucopyranoside 1-SH. To a solution of **14** (10 mg, 15.6 μmol) in MeOH (1.5 cm^3) was added 1,6-hexanedithiol (25 cm^3, 156 μmol), and the mixture was irradiated for 2 h in a quartz vial using a VL-50C Vilber Lourmat UV lamp. After concentration, the excess of 1,6-hexanedithiol was separated from carbohydrate by column chromatography (9 : 1 CH_2Cl_2–MeOH → MeOH). The carbohydrate-containing fractions were concentrated, and a solution of the residue in water was loaded on a C-18 Extract-Clean™ column. After elution of remaining **14** with water (3 × 3 cm^3), **1-SH** was eluted with MeOH (3 × 3 cm^3), then concentrated *in vacuo*, and obtained, after lyophilization from water, as a white amorphous powder (5 mg, 40%); δ_H(500 MHz; D_2O) 1.21 (3 H, d, $J_{H\text{-}5,H\text{-}6}$ 6.5, 3 × H-6), 1.45 (3 H, s, CH_3CCOOD), 1.48, 1.60, 2.58, and 2.61 (each 3 H, 4 × m, $O(CH_2)_3S(CH_2)_6SH$), 1.75 (2 H, m, $OCH_2CH_2CH_2S(CH_2)_6SH$), 2.04 (3 H, s, $NDCOCH_3$), 2.75 (2 H, m, $OCH_2CH_2CH_2S(CH_2)_6SH$), 3.58 and 3.78 (each 1 H, 2 × m, $OCH_2CH_2CH_2S(CH_2)_6SH$), 3.59 (1 H, bt, H-3′), 3.62 (1 H, bt, H-2″), 3.64 (1 H, m, H-5″), 3.67 (1 H, m, H-5′), 3.72 (1 H, bt, H-3″), 3.78 (2 H, m, H-2′, H-4′), 3.85 and 3.99 (each 1 H, 2 × m, H-6′a, H-6′b), 3.88 (1 H, bt, H-2), 3.90 (1 H, bd, H-4), 3.94 and 4.04 (each 1 H, 2 × m, H-6″a, H-6″b), 4.01 (1 H, bt, H-3), 4.03 (1 H, m, H-5), 4.18 (1 H, bd, $J_{H\text{-}3'',H\text{-}4''}$ 2.8, $J_{H\text{-}4'',H\text{-}5''}$ <1, H-4″), 4.49 (1 H, d, $J_{H\text{-}1'',H\text{-}2''}$ 7.9, H-1″), 4.69 (1 H, d, $J_{H\text{-}1',H\text{-}2'}$ 8.0, H-1′), 4.91 (1 H, d, $J_{H\text{-}1,H\text{-}2}$ 3.9, H-1); δ_C(125.76 MHz; D_2O) 15.9 (C-6), 22.8 ($NDCOCH_3$), 23.8, 24.3, 28.8, 29.2, 29.3, 31.9, 33.5, and 39.7 ($OCH_2CH_2CH_2S(CH_2)_6SH$), 25.8 (CH_3CCOOD), 55.9 (C-2′), 60.7 (C-6′), 65.6 (C-6″), 66.8 (C-2″), 67.1 (C-2), 67.2 (C-5), 67.3 ($OCH_2CH_2CH_2S(CH_2)_6SH$), 69.8 (C-4), 71.1 (C-4″), 71.5 (C-5″), 72.1 (C-3″), 72.9 (C-4′), 75.5 (C-3′), 77.9 (C-3), 79.8 (C-5′), 98.9 (C-1), 99.6 (C-1′), 103.6 (C-1″); *m/z* (HR MALDI-TOF) 812.287 ([M + Na]$^+$, $C_{32}H_{55}NNaO_{17}S_2$ requires 812.809).

Preparation of gold glyconanoparticles Au-1

A solution of the thiol-spacer-containing trisaccharide **1-SH** in MeOH (10 mM, 5 equiv.) was added to a solution of tetrachloroauric acid in water (25 mM, 1 equiv.). Then, an aqueous solution of $NaBH_4$ (1 M, 22 equiv.) was slowly added with vigorous stirring. The obtained black suspension was stirred for 2 h at rt. After concentration, a solution of the residue in water (10 cm^3) was loaded on a 30 kDa Nalgene centrifugal filter, and washed with water (5 × 15 cm^3). After lyophilization from water, the gold glyconanoparticles **Au-1** were obtained as a brown amorphous powder. The gold glyconanoparticles were characterized by 500 MHz ^{1}H NMR spectroscopy in D_2O, monosaccharide analysis, and TEM.

Monosaccharide analysis

Samples were subjected to methanolysis (1 M methanolic HCl, 24 h, 85 °C), followed by re-*N*-acetylation and trimethylsilylation. The trimethylsilylated methyl glycosides were analyzed by GLC on an EC-1 capillary column (30 m × 0.32 mm, Alltech) using a Chrompack CP 9002 gas chromatograph (temperature program, 140–240 °C at 4 °C min^{-1}). The identification of the monosaccharide derivatives was confirmed by gas chromatography–mass spectrometry on a Fisons Instruments GC 8060/MD 800 system (Interscience) equipped with an AT-1 capillary column (30 m × 0.25 mm, Alltech), using the same temperature program.[29]

Transmission electron microscopy

Examinations of **Au-1** samples (0.1 mg cm^{-3}) were performed with a Philips Tecnai12 microscope at 120 kV accelerating voltage. Aqueous aliquots (1 mm^3) were placed onto copper grids coated with carbon film (QUANTIFOIL on 200 square mesh copper grid, hole shape R 2/2). The grids were left to dry at rt for several hours. The particle size distribution of the gold glyconanoparticles was automatically determined from several micrographs of the same sample, using analySIS® 3.2 (Soft Imaging System GmbH).

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