Glycoconj J
DOI 10.1007/s10719-010-9300-7

Synthesis of PEGylated lactose analogs for inhibition studies on *T.cruzi* trans-sialidase

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Received: 4 May 2010 / Revised: 29 June 2010 / Accepted: 30 June 2010

Abstract *Trypanosoma cruzi,* the agent of Chagas disease, expresses a unique enzyme, the trans-sialidase (TcTS) involved in the transfer of sialic acid from host glycoconjugates to mucins of the parasite. The enzyme is shed to the medium and may affect the immune system of the host. We have previously described that lactose derivatives effectively inhibited the transfer of sialic acid to *N*-acetyllactosamine. Lactitol also prevented the apoptosis caused by the TcTS, although it is rapidly eliminated from the circulatory system. In this paper we report covalent conjugation of polyethylene glycol (PEG) with lactose, lactobionolactone and benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**) with the hope to improve the bioavailability, though retaining their inhibitory properties. Different conjugation methods have been used and the behavior of the PEGylated products in the TcTS reaction was studied.

Keywords Trans-sialidase · PEGylation · Inhibitors · *Trypanosoma cruzi*

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Introduction

The need to maintain drugs in blood is one of the main goals in therapeutic applications, which are often hampered by the short *in vivo* half-life of administered compounds. Many factors are involved in the removal of substances from the circulation, being renal excretion and inactivation reactions by blood enzymes the main causes for the brief plasma residence time of low-molecular mass non-protein drugs [1]. Modification of biological molecules by covalent conjugation with polyethylene glycol (PEG) has been used to improve the bioavailability of drugs [2]. PEG is a linear polyether diol with many useful properties such as biocompatibility, solubility in aqueous and organic media, lack of toxicity and very low immunogenicity. Probably, the most important feature of PEG modification is that it greatly extends the half-life ($t_{1/2}$) of most proteins, and results in a greatly increased plasma presence. This can be attributed, in part, to the increase in molecular weight of the conjugate beyond the limits of renal filtration and reduced proteolysis of the conjugate [3]. PEG has been coupled to antibodies or antibody fragments to prolong the circulating half-lives *in vivo* [4]. Selective alkylation and acylation of amino groups in a somatostatin analogue using two different PEG reagents has been described [5]. Also, low molecular-weight drugs have been PEGylated in order to prolong the *in vivo* action [6] or for targeting drug delivery [7].

There are few reports on the PEGylation of carbohydrate molecules and these have been focused on polysaccharides or on carbohydrates linked to proteins. Thus, the linear glycan chitosan was coupled by amide bond formation between the aminogroups and a carboxylic acid functionalized PEG [8]. *N*-hydroxysuccinimide (NHS) esters are frequently used for derivatization of primary amino groups.

Published online: 20 July 2010

A heterodifunctional PEG with a protected amino group at one end and a NHS ester at the other was used to locate active molecules at the distal end of the PEG chain linked to chitosan. An amino functionalized α-mannoside was introduced in this way [9]. Chitosan partially amidated with lactobionic acid was PEGylated for DNA carrier studies [10]. These PEG-chitosan copolymers have shown improved biocompatibility. Specific PEGylation of the carbohydrate of ricin A-chain was accomplished by periodate oxidation followed by hydrazide formation with a hydrazine derivative of PEG [11]. The method was applied to proteins previously glycosylated with oligosaccharides [12]. Also, a protein was modified by sequential enzymatic transfer of galactose and PEGylated sialic acid from the respective nucleotides [13]. However, few reports describe direct PEGylation of small oligosaccharides. Diels-Alder chemistry was used to immobilize several different monosaccharides bearing a cyclopentadiene group, attached through a polyethylene glycol (PEG) linker, to a monolayer presenting benzoquinone groups on a gold surface [14]. A recent paper [15] describes the preparation of monosaccharide-capped PEGylated quantum dots (PEG-QDs) by reaction of the PEG-QD with a glycosidic thiol.

Glycans have been recognized as candidates for chemotherapy [16]. In this respect, *Trypanosoma cruzi,* the agent of American trypanosomiasis [17], expresses a unique enzyme, the trans-sialidase (TcTS) involved in the transfer of sialic acid from host glycoconjugates to mucins of the parasite [18, 19]. This is the only way trypanosomes may incorporate sialic acid, instead of using the corresponding nucleotide sugar as donor. Oligosaccharides of the mucins have been synthesized and their acceptor and inhibitory properties were studied [20, 21]. Recombinant TcTS has also been used for the preparation of sialylated oligosaccharides [22]. Sialic acid on the surface of the parasite is involved in host cell invasion and in protection of the parasite against complement [23] and from killing by anti α-galactosyl antibodies [24]. Also, in *Trypanosoma brucei*, the agent of sleeping sickness, incorporation of sialic acid through a trans-sialidase is essential for the parasite to survive in the insect vector [25].Trans-sialidase, being a virulence factor in the mammal and/or insect stages of trypanosomes and being specific for the parasite, with no equivalent in the human host, is an interesting target for drug design. The 3D structure of TcTS shows two (sub)sites in the active center: the sialic acid-binding site and the galactose-binding site [26, 27]. Potential inhibitors are usually classified depending on the active site region they target. In the last years several laboratories have been active in seeking inhibitors for TcTS, mainly directed to the sialic acid binding site [28, 29]. In particular, some natural products or synthetically modified natural products proved to be good inhibitors *in vitro* of the TcTS but the authors do not report studies on toxicity in animals [30, 31]. Inhibitors of TcTS directed to the β-galactosyl acceptor site should be more specific, as other sialidases lack this interaction. Lactose derivatives effectively inhibited the transfer of sialic acid to *N*-acetyllactosamine, lactitol being the best [32]. The lactose analogs are only moderate inhibitors. However, they are non-toxic to animals [33]. In fact, Mucci *et al*. proved that intravenous administration of 10 mg of lactitol inhibited TcTS in mice blood by 95–98%. Fast clearance from blood was solved using lactitol releasing pellets which prevented apoptosis of spleen cells.

Here, we report PEGylation of lactose derivatives and of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**) with the hope to improve the bioavailability, though retaining their inhibitory properties. Different conjugation methods have been used and the behavior of the PEGylated products in the TcTS reaction was studied.

Materials and methods

Materials and general methods

Methoxypolyethylene glycol amine 750 (MW: 750)(CH_3O-PEG750-NH_2), Methoxypolyethylene glycol amine 5000 (MW: 5000)(CH_3O-PEG5000-NH_2) and Methoxypolyethylene glycol 5000 acetic acid *N*-hydroxysuccinimidyl ester (MW: 5000)(CH_3O-PEG5000-NHS ester) were purchased from Fluka (Steinheim, Germany), Methoxypolyethylene glycol *N*-hydroxysuccinimidyl ester (MW: 685.71) (CH_3O-PEG_{12}-NHS ester) was purchased from Pierce Biotechnology (Rockford, USA). Lactobionic acid, lactose and 3'-sialyllactose were purchased from Sigma Chemical Co. [D-glucose-1-^{14}C] lactose was purchased from Amersham Biosciences. NMR spectra were recorded with a Bruker AM 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) at 30°C. Assignments were supported by 2D HSQC experiments. High resolution electrospray ionization mass spectra (ESI-TOF) were recorded on BRUKER microTOF-Q II ESI-Qq-TOF spectrometer. MALDI-TOF spectra were collected on a PE BIOSYSTEMS DE-STR MALDI-TOF spectrometer. Optical rotations were measured with Perkin-Elmer 343 polarimeter. Analytical TLC was performed on 0.2 mm Silica Gel 60 F254 (Merck) aluminium supported plates. Detection was effected by spraying with 10% (v/v) sulfuric acid in ethanol containing 0.5% p-anisaldehyde and charring. Column chromatography was performed on Silica Gel 60 (230–400 mesh, Merck). Melting points were determined with a Fisher-Johns apparatus. FT-IR spectra were determined with a 510 P Nicolet FT-IR spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the KBr disk method, at 4,000−250 cm^{-1}; 32–

64 scans were taken with a resolution of 2–4 cm^{-1}. Radioactivity was measured on WinSpectral 1414 liquid scintillation counter (Wallac).

Synthesis of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**).

Synthesis of benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**2**). To a solution of *N*-acetyl-D-glucosamine (3 g, 13,56 mmol) in benzyl alcohol (21 mL), BF_3 diethyl ether complex (320 μl, 2.7 mmol) was added and the mixture was heated with stirring at 80°C for 4 h. After this time, TLC showed disappearance of the starting compound and the product was precipitated by addition of diethyl ether (100 mL). The benzyl glycosides were obtained in 83% yield as a mixture of α and β anomers (8:2 ratio) (R_f 0.66 and 0.60 respectively, nPrOH-EtOH-H_2O, 7:1:2). ^{1}H NMR (D2O, 500 MHz):δ anomeric region 4.83 (d, 0.8 H, *J*= 3.5 Hz, H-1α), 4.42(d, 0.2 H, J=8.5 Hz, H-1β) Pure benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside was obtained by fractional crystallization from ethanol. The melting point and optical rotation were in agreement with those previously reported [34].

*Synthesis of benzyl (2,3,4,6-tetra-*O*-benzoyl-*β*-D-galactopyranosyl)-(1→6)-2-acetamido-2-deoxy-*α*-D-glucopyranoside (**3**)*.To an externally cooled (0°C) solution of 1,2,3,4,6-penta-*O*-benzoyl-β-D-galactopyranose [35] (1.67 g, 2.36 mmol) in dry Cl_2CH_2 (6 mL) under argon, tin(IV) chloride (0.27 mL, 2.31 mmol) was added. After 15 min of stirring at 0°C, benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside (0.60 g, 1.42 mmol) and dry CH_3CN (0.6 mL) were added, and stirring was continued for 16 h at room temperature. TLC showed a main compound of R_f 0.69, minor amounts of less polar compounds and remaining starting material (15% CH_3OH in CH_2Cl_2). The mixture was diluted with Cl_2CH_2 (40 mL) and poured into cold saturated aqueous $NaHCO_3$ with vigorous stirring. The aqueous layer was extracted with Cl_2CH_2 (2×50 mL), and the combined organic solutions were washed with water until pH 7, dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The product was purified by column chromatography (toluene-EtOAc,1:15) to give compound **3** (731 mg, 42.4%), mp 118–120°C; [α]D:+86,8º (c1, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 8.05-7.2 (m, 25 H, aromatic), 6.0 (dd,1H, $J_{3',4'}$=3.5 Hz, $J_{4',5'}$=1 Hz, H-4'), 5.91 (d, 1H, *J*=8.5 Hz, N*H*), 5.85 (dd, 1H, $J_{2',3'}$=10.5 Hz, $J_{1',2'}$= 7.8 Hz, H-2'), 5.64 (dd, 1H, H-3'), 4.93 (d, 1H, H-1'), 4.7 (d, 1H, $J_{1,2}$=3.5 Hz, H-1), 4.67, 4.42 (2dd, 2H, $J_{6'a,6'b}$= 11.5 Hz, $J_{6'a,5'}$=6.5 Hz, $J_{6'b,5'}$=6.5 Hz, H-6′a, H-6′b), 4.57, 4.19 (2d, 2H, *J*=11.7 Hz, PhCH_2), 4.35 (ddd, 1H, H-5′), 4.27 (dd, 1H, $J_{5,6a}$=8.5 Hz, $J_{6a,6b}$=11.5 Hz, H-6a), 3.88 (ddd, 1H, $J_{4,5}$=9 Hz, $J_{5,6b}$=4 Hz, H-5), 3.83-3.77 (2dd, 2H, H-2, H-6), 3.58 (dd, 1H, $J_{2,3}$=10.2, $J_{3,4}$=9 Hz, H-3), 3.39 (dd, 1H, H-4), 1.90 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ 172.1(*C*ONH), 166.1-165.3 (Ph*C*O), 136-128 (aromatic), 102.4 (C-1'), 96.2 (C-1), 74.3 (C-3), 71.5-71.4 (C-4, C-3′, C-5′), 70.5 (C-2), 69.8 (C-2′), 69.6 (C-6), 69.2 (Ph*C*H_2), 68.1 (C-4′), 61.9 (C-6'), 53.6 (C-5), 23.1 (*C*H_3CO). Anal. Calcd for $C_{49}H_{47}NO_{15}$: C, 66.13; H, 5.32; N, 1.57. Found: C, 65.74; H, 5.31; N, 1.68. ESI-TOF MS m/z: calcd for $[M + H]^+$: 890.3018, found: 890.3036. Calcd for $[M + Na]^+$: 912.2838, found: 912.2847.

Synthesis of benzyl β-D-*galactopyranosyl-(1→6)-2-acetamido-2-deoxy-*α*-D-glucopyranoside* (**4**). To a solution of compound **3** (731 mg, 0.82 mmol) in anhydrous MeOH (10 mL) at 0°C, 0.5 M NaOMe in MeOH (10 mL) was added. After stirring for 1 h at 0°C and 2 h at room temperature, TLC examination showed only one compound more polar than **3**. The solution was passed through a column of Amberlite IR 120 (plus) H^+ resin eluting the product with methanol. The solvent was evaporated and the remaining methyl benzoate was removed by successive co-evaporations with water, to afford 291 mg of **4** as a white solid (75 % yield). Crystallization from EtOH gave mp 234-235°C. The physical constants and spectra were the same as previously reported [35].

Synthesis of benzyl β-*D-galactopyranosyl-(1→6)-2-amino-2-deoxy-*α*-D-glucopyranoside (**1**).* Compound **4** (203 mg, 0.43 mmol) was dissolved in 1.2 mL of 1 M KOH and heated at 100°C for 16 h. After this time, TLC showed that the reaction was complete, (R_f 0.36, n-PrOH-EtOH-H_2O, 7:1:2). The solution was passed through a column (1.5 cm×6 cm) containing Amberlite IR 120 (plus) H^+ resin and eluted with 0.1 N HCl to afford 110 mg of **1** (0.29 mmol, 67% yield).[α]D:+45,4º (c1, H_2O). ^{1}H NMR (D_2O): δ 7.47 (m, 5H, aromatic), 5.24 (d, 1H, $J_{1,2}$=3.7 Hz, H-1), 4.81, 4.66 (2d, 2H, *J*=11.6 Hz, PhCH_2), 4.43 (d, 1H, $J_{1',2'}$=7.9 Hz, H-1'), 4.10 (dd, 1H, $J_{6a,6b}$=11.2 Hz, $J_{5,6a}$= 1.7 Hz, H-6a), 3.93 (dd, 1H, $J_{4',5'}$=0.7 Hz, $J_{3',4'}$=3.5 Hz, H-4′), 3.89 (dd, 1H, $J_{2,3}$=10.5 Hz, $J_{3,4}$=9.1, H-3), 3.89 (dd, 1H, $J_{5,6b}$=4.3 Hz, H-6b), 3.87 (ddd, 1H, $J_{4,5}$=9.5 Hz, H-5), 3.79 (dd, 1H, $J_{5',6'a}$=7.9 Hz, $J_{6'a,6'b}$=11.7 Hz, H-6′a), 3.75 (dd, 1H, $J_{5',6'b}$=4.3 Hz, H-6′b), 3.7 (ddd, 1H, H-5′), 3.66 (dd, 1H, $J_{2',3'}$=10.0 Hz, H-3′), 3.64 (dd, 1H, H-4), 3.56 (dd, 1H, H-2′), 3.37 (dd, 1H, H-2). ^{13}C NMR (D_2O): δ 136.5, 128.8, 128.6, 128.5 (aromatic), 103.4 (C-1'), 94.5 (C-1), 75.1 (C-5'), 72.7 (C-3′), 71.2 (C-5), 70.7 (C-2′), 70.0 (Ph*C*H_2), 69.9 (C-3), 69.1 (C-4), 68.6 (C-4′), 67.8 (C-6), 60.9 (C-6'), 53.8 (C-2). ESI-TOF MS m/z: calcd for $[M + H]^+$:464.1684 found:432.1863. Calcd for $[M + Na]^+$: 454. 1684 found: 454.1677.

PEGylation of compound **1**

Preparation of benzyl β-*D-galactopyranosyl-(1→6)-2-*CH_3O*-*PEG_{12}*amido-2-deoxy-*α*-D-glucopyranoside (**5**).* A solution of benzyl β-D-galactopyranosyl-*(1→6)*-2-amino-2-deoxy-α-D-glucopyranoside (**1**) (38 mg, 0.09 mmol) and

CH_3O-PEG_{12}-NHS ester (50 mg, 0.07 mmol) in 1 mL phosphate buffer (50 mM, pH 8) was left at room temperature for 24 h . The reaction was monitored by TLC. The PEGylated compound **5** (R_f 0.33, EtOH-H_2O-NH_4OH(c), 9:1: 0.15) appeared as a black spot by spraying with the sulfuric acid reagent and heat charring. Unreacted PEG is not detected with this reagent and compound **1** had R_f 0.51. The product was purified by passing through a RP-18 column (2 g, Strata, Phenomenex). Some non-reacted compound **1** and salts were eluted with water and compound **5** was eluted with methanol. After evaporation of methanol under vacuum followed by lyophilization, compound **5** was obtained as a white powder (59 mg, 67%). ^{1}H NMR (D_2O): δ 7.44 (m, 5 H, aromatic), 4.95 (d, 1H, *J*= 3.6 Hz, H-1), 4.54 (d, 1H, *J*=12 Hz, PhC*H*$_2$), 4.43 (d, 1H, *J*=7.8 Hz, H-1'), 4.15 (d, 1H, *J*=9.6 Hz, N*H*), 3.96-3.88 (m, 5H, sugar protons), 3.72-3.67 (m, 44H, OC*H*$_2$C*H*$_2$O), 3.66-3.56 (m, sugar protons), 3.37 (s, 3H, OC*H*$_3$), 2.52 (dt, 2H, *J*=1,7 Hz, *J*=6 Hz, C*H*$_2$CO). ^{13}C NMR (D_2O): δ,174.2 (CO), 137.0, 128.7, 128.4 (aromatic), 103.4 (C-1′), 96.1 (C-1), 75.1, 72.7, 71.1, 70.9, 70.8, 70.7, 68.6, 68.3, 61.0, (sugar carbons), 69.6 (O*C*H$_2$*C*H$_2$O), 58.0 (O*C*H$_3$), 53.6 (C-2), 35.8 (*C*H$_2$CO). MALDI-TOF MS m/z: calcd for [M (12 oxyethylene) + Na]$^+$: 1,024.49; found: 1,024.4953.

Preparation of benzyl β-*D-galactopyranosyl-(1→6)-2-CH_3O-PEG5000amido-2-deoxy-*α-*D-glucopyranoside* (**6**). Compound **1** (26 mg, 0.04 mmol) was conjugated with CH_3O-PEG5000-NHS ester (150 mg, 0.024 mmol) as described for **5**. After 24 h, analysis by TLC showed a product close to the origin of the plate. Excess of disaccharide and salts were removed by dialysis against water (cut off 3500). After lyophilization compound **6** (148 mg, 97%) was obtained as a white powder. ^{1}H NMR (D2O): δ 7.44 (m, aromatic), 4.98 (d, 3.7 Hz, H-1), 4.55 (d, *J*=11.8 Hz, PhC*H*$_2$), 4.44 (d, *J*=7.8 Hz, H-1'), 4.18 (d, *J*= 9.5, N*H*), 4.09-3.9 (m, sugar protons); 3.87-3.56 (m, OC*H*$_2$C*H*$_2$O); 3.37 (s, OC*H*$_3$). ESI-TOF MS m/z: calcd for [M (104 oxyethylene) + NH_4]$^+$: 5,099; found M_n 4992.95, M_w 5007.41, M_p 5097.

PEGylation of lactobionolactone

Lactobionolactone (**7**) was prepared from lactobionic acid as previously described [36]. Analysis by TLC showed disappearance of lactobionic acid, R_f 0.38, and formation of the lactone, R_f 0.78 (EtOH-H_2O-HAcO, 60:25:15). IR (CO): 1,741 cm^{-1}

Preparation of N*-(CH_3O-PEG750)-lactobionamide* (**8**). Lactobionolactone **7** (100 mg, 0.29 mmol) and CH_3O-PEG750-NH_2 (300 mg, 0.4 mmol) were dissolved in anhydrous dimethylformamide (1.5 mL) under argon and the reaction mixture was heated at 120°C. The reaction was monitored by TLC, detecting the PEGamine reagent with ninhydrine and the appearance of the PEGylated sugar amide **8** with the sulfuric acid reagent (R_f 0.35, EtOH-H_2O-HAcO, 80:5:15). The reaction was completed after 6 h. The excess of CH_3O-PEG750-NH_2 was removed by passing through a cationic exchange resin. The product was eluted with water and chromatographed on a Sephadex G10 column (1.5 cm×7 cm). Conjugate **8** was recovered in the first 40 mL of water. After evaporation a white solid was obtained (209 mg, 66% yield). IR (C=O) 1,654 cm^{-1}. ^{1}H NMR (D_2O): δ 4.55 (d, 1H, *J*=7.8 Hz, H-1'), 4.42 (d, 1H, *J*=2.75 Hz, H-2), 4.2 (m, 1H), 3.98 (dd, 1H, *J*=4.15 Hz, *J*= 6.45 Hz), 3.95-3.74 (m, sugar protons), 3.73-3.6 (m, 60H, OCH_2CH_2O), 3.56 (dd, 2H, *J*=8 Hz, *J*=9.7 Hz, NHCH_2C*H*$_2$), 3.46 (m, 2H, NHC*H*$_2$), 3.38 (s, 3H, OC*H*$_3$). ^{13}C NMR: 174.4 (C-1), 103.5 (C-1′), 81.0, 75.3, 72.5, 72.4, 71.4, 70.4, 68.8, 68.6, 61.9, 61.0 (sugar carbons), 71.0, 70.9, 69.6, 69.4 (O*C*H$_2$*C*H$_2$O), 58.0 (O*C*H$_3$), 38.6 (NH*C*H$_2$). MALDI-TOF MS m/z: calcd. for [M (16 oxyethylene) + Na]$^+$: 1,098.55; found: M_n 1,130.9, M_w 1,143.0, M_p 1,098.55.

Preparation of N-(CH_3O-PEG 5000)-lactobionamide (**9**). Lactobionolactone **7** (40.8 mg, 0.12 mmol) and CH_3O-PEG5000-NH_2 (200 mg, 0.04 mmol) were dissolved in anhydrous dimethylformamide (2 mL) under argon and the reaction mixture was heated at 100°C for 15 h. After this time CH_3O-PEG5000-NH_2 was not longer detected by ninhydrine on a TLC plate. The solution was extensively dialyzed (cut-off 3500) and lyophilized yielding a white powder (136 mg, 65.5%). IR (C = O) 1,672 cm^{-1}. ^{1}H NMR (D_2O): δ 4.55 (d, *J*=7.8 Hz, H-1'); 4.41 (d, *J*=2.75 Hz, H-2), 4.36 (m, 1H), 4.2-3.88 (m, sugar protons), 4.18 (m, 1H, N*H*), 3.88-3.5 (m, OCH_2CH_2O), 3.43 (m, NHC*H*$_2$), 3.37 (s, OC*H*$_3$). ESI-TOF MS m/z: calcd. for [M (95 oxyethylene) + NH_4]$^+$: 4,569; found M_n 4,586.6, M_w 4611.5, M_p 4,569.

PEGylation of lactose by reductive amination

Preparation of 1-amino-(CH_3O-PEG750)-lactitol (**10**). Lactose (100 mg, 0.3 mmol) and CH_3O-PEG750-NH_2 (75 mg, 0.1 mmol) were dissolved in 3 mL of water, the pH was adjusted to 8 with 10% acetic acid and $NaBH_3CN$ (30 mg, 0.5 mmol) was added. The reaction was kept at 50°C for 96 h and monitored by TLC which showed disappearance of CH_3O-PEG750-NH_2 with the ninhydrine reagent whereas conjugate **10** was detected with the sulfuric acid reagent (R_f 0.16, EtOH-H_2O, 8:2). The solution was evaporated under vacuum, boric acid was removed by successive co-evaporations with methanol and finally the product was purified by passage through a cationic resin. The excess of lactose was eluted with water and compound **10** was eluted with HCl 1 N. Lyophilization afforded 78 mg (71.3% yield) of a white powder. ^{1}H NMR (D_2O) δ 4.54 (d, 1H, *J*=8 Hz, H-1'), 4.24 (m,1H, N*H*), 3.97-3.77 (m, 12H,

sugar protons), 3.76-3.64 (m, 48 H, OCH_2CH_2O), 3.59-3.56 (dd, 2H, NH-CH_2CH_2O), 3.44-3.31 (m, 3H, NH-CH_2CH_2O and H-6), 3.39 (s, 3H, OCH_3), 3.22 (m, 1H, $CH_{2(a)}NH$), 3.02 (m, 1H, $CH_{2(b)}NH$). ^{13}C NMR: 102.8 (C-1′), 78.6, 75.3, 72.4, 70.9, 70.4, 68.7, 67.7, 65.3, 61.9, 61.2 (sugar carbons), 71.0, 69.6, 69.4 (OCH_2CH_2O), 58.1 (OCH_3), 49.5 (C-1), 47.1 ($NHCH_2$). ESI-TOF MS m/z: calcd. for [M (13 oxyethylene) + Na]$^+$: 952.49; found M_n 980.46, M_w 985.91, M_p 952.49.

Competitive radioactive assay for TS activity

Trans-sialidase activity was measured as the transfer of sialic acid from 1 mM of 3'-sialyllactose to 12 μM [D-glucose-1-^{14}C] lactose (55 mCi/mmol) by 0.5 ng of purified TcTS enzyme in 30 μl of 20 mM Hepes-Na (pH 7.5), 0.2% BSA, 30 mM NaCl. After 60 min at room temperature, the reaction was stopped by dilution with 1 ml of water. QAE-Sephadex (Amersham Pharmacia Biotech) was added and the resin was washed twice with water. Negatively charged compounds were eluted with 800 μl of 1 M NaCl and quantified in a WinSpectral 1414 liquid scintillation counter. When required, the purified enzyme was diluted in the reaction buffer before use (0.5 ng of TcTS611/2 rendered about 4,000 cpm per hour).

Increasing concentrations (0.2 to 2.0 mM) of the PEGylated sugars were preincubated for 20 min with the enzyme at room temperature prior to the addition of 1 mM of 3'-sialyllactose and [D-glucose-1-^{14}C] lactose.

Results are expressed in percentage of inhibition of trans-sialidase activity. Radioactivity measured without addition of any inhibitor was considered as 0% of inhibition (100% of enzymatic activity).

Assay of PEG-conjugates in blood

Adult mice (C3H strain) were intravenously injected with 200 μl of sterile PBS containing 30 mg (29.5 mM) of compound **6**, 28 mg of CH_3O-PEG5000-NHS ester (29.5 mM,), 44 mg (50 mM) of compound **9** or 40 mg of CH_3O-PEG5000-NH_2 (50 mM). Blood was extracted from the tail at different times and serum was used to assay free or combined PEG.

The colorimetric test described by Nag *et al* [37] was adapted to assay the PEG-conjugates in blood. Briefly, 10 μl of sera, 0.5 mL of $CHCl_3$ and 0.5 mL of the ferrothiocyanate reagent (prepared by dissolving 16.2 g anhydrous ferric chloride ($FeCl_3$) and 30.4 g ammonium ferrothiocyanate (NH_4SCN) in 1 L of water) were vigorously shaken for 30 min and later centrifuged at 5,000 rpm for 2 min. Absorbances from chloroformic layers were recorded at 510 nm. A calibration curve using CH_3O-PEG5000-NHS ester was performed.

Results and Discussion

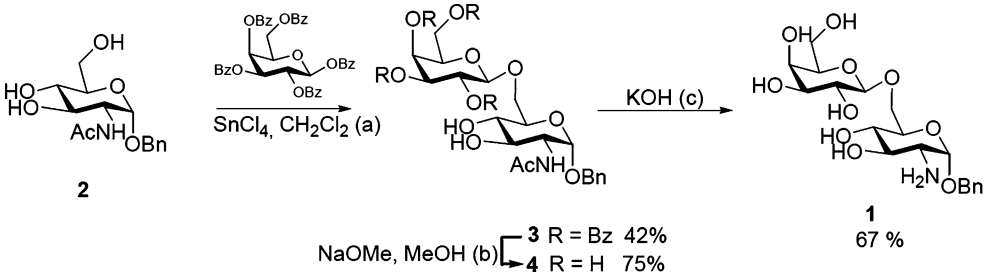
Synthesis of PEG-conjugates

Three different chemical approaches were used for PEGylation of disaccharide derivatives containing β-D-Gal*p* as non-reducing unit. 1. Amide formation between the amino group of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**) and a succinimidyl activated ester of PEG (PEG-NHS ester). 2. Amide formation between the carboxyl group of lactobionic acid and an amino-derivatized PEG (PEGamino). 3. Reductive amination of lactose with a PEGamino in the presence of $NaBH_3CN$. The conjugates were characterized by NMR and mass spectrometry, MALDI-TOF or ESI-TOF were used. Except for the monodisperse CH_3O-PEG_{12}-NHS ester, all PEGylations were performed with polydisperse PEGs and the values for M_n (number average molecular weight), M_w (weight average molecular weight) and M_p (molecular weight at peak top) were obtained. In all cases the PDI value (polydispersity index) was close to 1. The match between the experimental and calculated values confirmed the identity of the conjugates. Our purpose was to study the influence of PEGylation on the inhibitory properties of the above mentioned disaccharides in the TcTS reaction.

PEGylation of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside

We have previously shown that the disaccharide β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-α-D-glucose as well as its benzyl glycoside **4**, are good acceptors for sialic acid in the reaction catalyzed by TcTS [20]. They are also competitive inhibitors when 3'-sialyllactose was used as the sialic acid donor and *N*-acetyl-lactosamine as the acceptor. A simplified preparation of compound **4**, that relies on the selective glycosylation at 6-*O* of benzyl α-D-GlcNAc (**2**) is now presented. Although the total yield is only moderate (42%), the present synthesis of **4** avoids the cumbersome protection and deprotection steps. Deacylation to afford the reactive amino group was accomplished by alkaline hydrolysis (Scheme 1).

Monomethoxy polyethyleneglycol activated as the *N*-hydroxysuccinimide ester (CH_3O-PEG-NHS ester) was used for amidation of the 2-amino group in the sugar with the PEG chain (Scheme 2). Conjugation was performed using two different molecular weight PEG derivatives, a low molecular weight PEG, which introduces 12 oxyethylene groups (CH_3O-PEG_{12}-NHS ester) and a high molecular weight, polydisperse PEG (CH_3O-PEG5000-NHS ester). The reaction was mild. Amidation took place in buffer pH 8 at room temperature and was monitored by



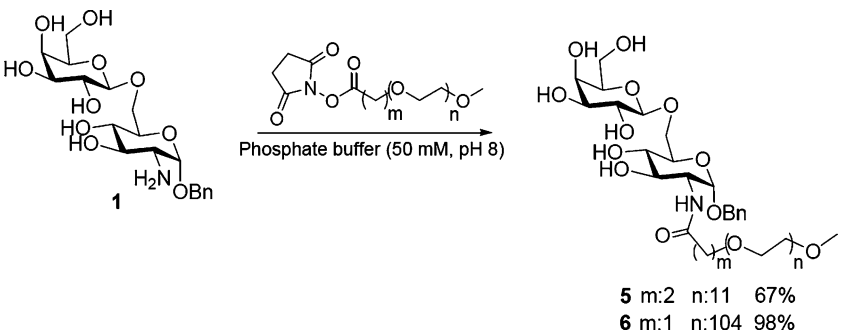
Scheme 1 Synthesis of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**). Conditions: **a** 16 h, r.t.; **b** 1 h, 0°C→2 h r.t.; (c) 1 M KOH, 16 h, 100°C

TLC being detected as a black spot after heating with the sulfuric acid reagent. The starting CH_3O-PEG-NHS ester did not stain with this reagent. After purification by passing through a RP-18 cartridge or by dialysis against water and lyophilization, in the case of the high molecular weight product, compounds **5** and **6** were obtained as white powders in 61% and 97% yield, respectively. The ^{1}H NMR spectrum of **5** (Fig. 1a) indicated PEGylation with one equivalent of PEG, by integration of the only OCH_3 signal, which appeared as a singlet at δ 3.37 ppm and the two anomeric signals at δ 4.95 ppm ($J_{1,2}$=3.6 Hz) for the α-GlcN and at δ 4.43 ppm ($J_{1',2'}$=7.8 Hz) due to the β-Gal*p*. One of the protons of the PhCH_2, substituent in the sugar, was shown at δ 4.54 ppm with the characteristic high coupling constant *J*=12 Hz, the other proton was suppressed together with the DHO signal. The PEG oxymethylene protons appeared as a strong signal at δ 3.70 ppm, which integrated to about 11 oxyethylene groups. The methylene linked to the amide carbonyl appeared at higher field (δ 2.52 ppm). The ^{13}C NMR spectrum (Fig. 2a) showed among others the anomeric signals at δ 103.4 ppm (β-Gal*p*) and δ 96.1 ppm (α-GlcN), and the C-2 signal of the amino sugar at δ 53.6 ppm. For the PEG moiety the OCH_3 signal appeared at 58.0 ppm, the methylene next to the carbonyl sugar amide appeared at 35.8 ppm and the oxymethylene groups of the PEG chain appeared as a strong peak at 69.6 ppm. MALDI-TOF mass spectral analysis showed a main peak of $[M + Na]^+$ at 1024 corresponding to the conjugate **5** with 12 oxyethylene groups.

Accordingly, the higher molecular weight PEG-conjugate **6** showed signals with similar chemical shifts (not shown). ESI-TOF mass spectral analysis of **6** revealed a series of 44 Da (OCH_2CH_2) spaced peaks corresponding to the NH_4^+ adducts. In this case, ammonium acetate was added to help ionization. A main peak of $[M + NH_4]^+$ at 5,097 was observed. This value corresponded to conjugate **6** with 104 oxyethylene groups.

PEGylation of lactobionolactone

We had previously determined that lactobionic acid was a good acceptor of sialic acid and inhibitor of TcTS [32]. In this work we took advantage of the carboxyl group already present in this disaccharide to form an amide linkage with an amino functionalized PEG (Scheme 3). Again, we used two different molecular weight derivatives, CH_3O-PEG750-NH_2 and CH_3O-PEG5000-NH_2. In the case of the high MW PEG, a molecular excess of the sugar was used since it could be easily removed by dialysis after reaction. The lactobionic acid was activated as lactobionolactone [36] and then heated with the corresponding PEGamino in anhydrous dimethylformamide. The reaction could be monitored by TLC. Whereas the PEGamino starting material was detected with the ninhydrin reagent, the sugar conjugate was only revealed with the sulfuric acid reagent. In the case of the lower MW conjugate **8,** purification from excess PEGamino was accomplished by cation exchange chromatography, which retained excess reagent, followed by Sephadex G-10 column chromatography. After lyophiliza-



Scheme 2 Synthesis of *N*-PEGylated derivatives of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside. Conditions: 24 h, r.t

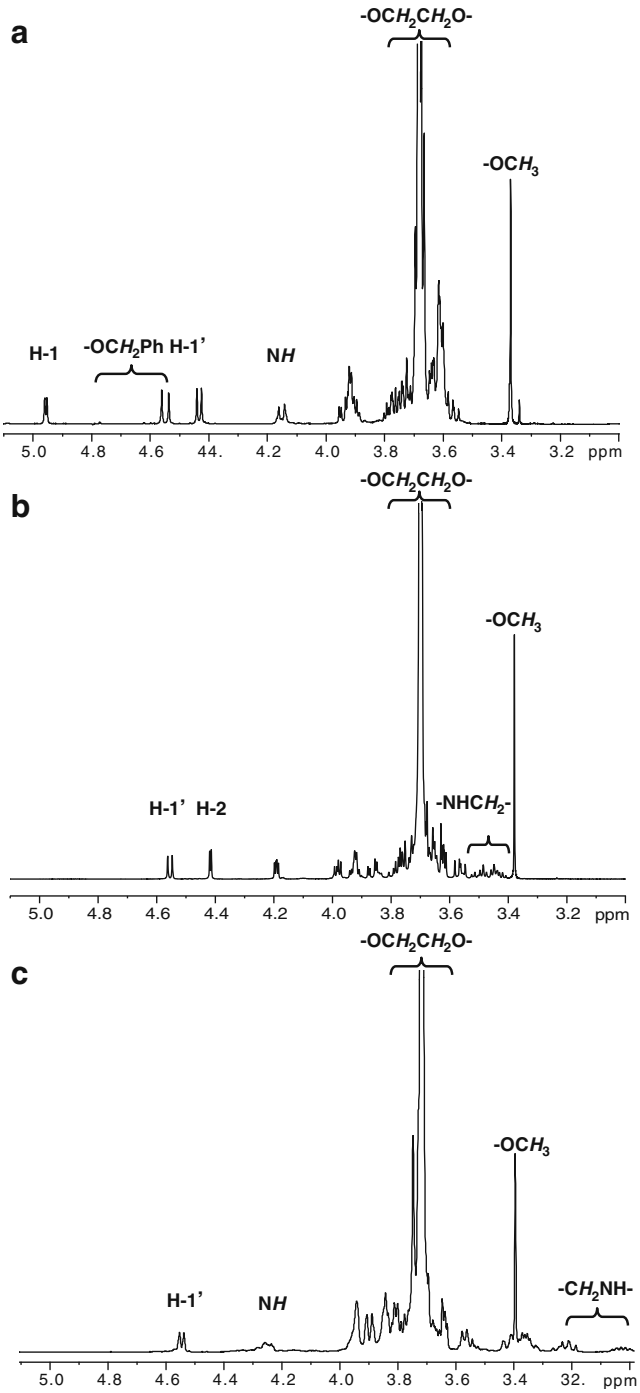


Fig. 1 ^{1}H NMR spectra of PEGylated sugar derivatives in D_2O (500 MHz). **a**. benzyl β-D-galactopyranosyl-(1→6)-2-CH_3O-PEG_{12}a-mido-2-deoxy-α-D-glucopyranoside. **b**. *N*-(CH_3O-PEG750)-lactobio-namide. **c**. *N*-(CH_3O-PEG750)-lactitol

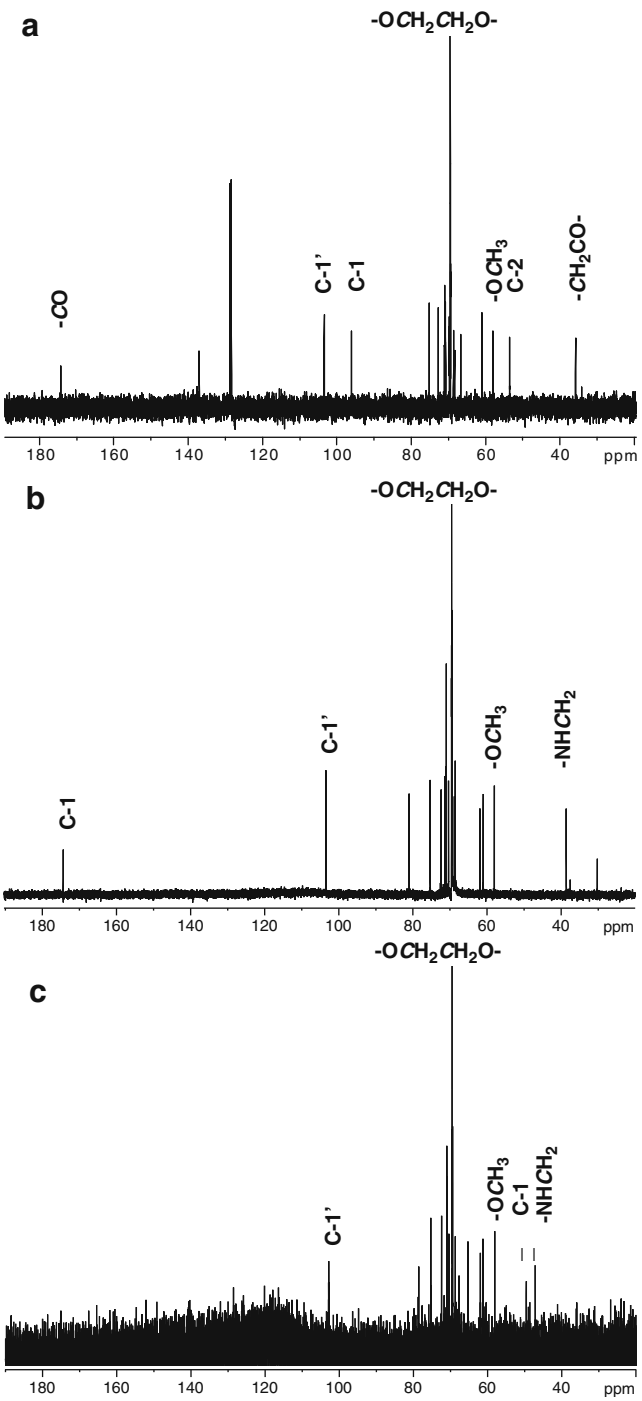
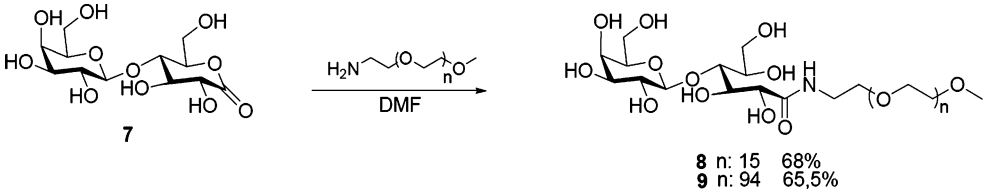


Fig. 2 ^{13}C NMR spectra of PEGylated sugar derivatives in D_2O (500 MHz). **a**. benzyl β-D-galactopyranosyl-(1→6)-2-CH_3O-PEG_{12}a-mido-2-deoxy-α-D-glucopyranoside. **b**. *N*-(CH_3O-PEG750)-lactobiona-mide. **c**. *N*-(CH_3O-PEG750)-lactitol

tion, compound **8** was obtained as a white powder in 66% yield. The IR spectrum of the conjugate showed the amide carbonyl at 1,654 cm^{-1} and disappearance of the lactone carbonyl at 1,740 cm^{-1}.

The ^{1}H NMR spectrum of **8** (Fig. 1b) indicated PEGylation with one equivalent of PEG, by integration of the OCH_3 signal, which appeared as a singlet at δ 3.38 ppm and the only anomeric signal at δ 4.55 ppm (J=7.8 Hz) due to the β-Gal*p*. A characteristic doublet at δ 4.42 ppm (J= 2.8 Hz) due to the H-2 of the derivatized lactobionamide was also observed. The oxymethylene groups of the repeating units gave a strong signal centered at δ



Scheme 3 Lactobionolactone amidation with PEG-NH_2. Conditions: Dimethylformamide (DMF), 6 h, 120°C for **8** and 15 h, 100°C for **9**

3.70 ppm. The ^{13}C NMR spectrum (Fig. 2b) showed among others the carbonyl signal of the amide at δ 174.4 ppm and the anomeric signal at δ 103.4 ppm (β-Gal*p*). For the PEG moiety the OCH_3 signal appeared at 58.0 ppm, the methylene next to the amide appeared at 38.6 ppm and the oxymethylene groups of the chain appeared as strong peaks at 69.6-69.4 ppm. MALDI-TOF mass spectral analysis revealed a series of 44 Da spaced peaks corresponding to the Na^+ adducts. A main peak of $[M + Na]^+$ at 1,098 was observed corresponding to conjugate **8** with 16 oxyethylene groups.

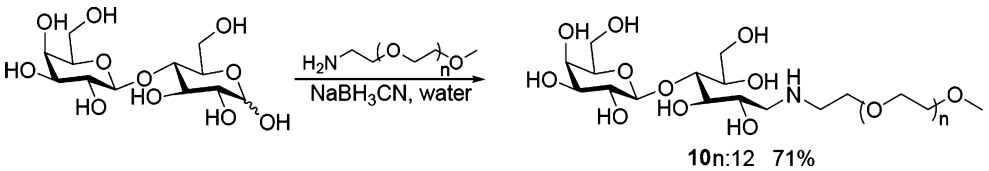
For the isolation of the higher MW conjugate **9** the excess of lactobionolactone was removed by dialysis and, after lyophilization, **9** was obtained as a white powder in 66% yield. IR spectroscopy confirmed the presence of the amide group by showing its characteristic absortion band at 1,672 cm^{-1} and the disappearance of the lactone band above 1,700 cm^{-1}. The ^1H NMR spectrum of **9** (not shown) was similar to the spectrum of **8**. ESI-TOF mass spectral analysis revealed a series of 44 Da spaced peaks corresponding to the $[NH_4]^+$ adducts, accompanied by lower intensity peaks from the $[H]^+$ adducts. A main peak of $[M + NH_4]^+$ at 4,569 was observed corresponding to conjugate **9** with 95 oxyethylene groups.

PEGylation of lactose

Previous results [32] indicated that lactitol was the preferred acceptor in comparison with lactose and *N*-acetyllactosamine, suggesting that better binding with the enzyme takes place with the open chain alditol. Lactitol also prevented parasite sialylation and partially inhibited cell infection. PEGylation of lactose with a PEGamino derivative by reductive amination retains the open chain structure of lactitol, while providing a stable amino-bond between the PEG derivative and the disaccharide (Scheme 4). The reaction was performed incubating lactose with CH_3O-PEG750-NH_2 in the presence of $NaBH_3CN$ at pH 8, conditions under which the selective reduction of the imine takes place. The reaction was monitored by TLC, which showed disappearance of the starting PEGamino compound when revealing with the ninhydrin reagent. Purification of the reaction mixture was performed by means of a cation exchange resin that retained the non-reacted PEGamino. Boric acid was then eliminated by co-evaporations with methanol. A white solid of CH_3O-PEG750-NH-lactitol (**10**) was obtained with 71% yield. The ^1H NMR spectrum of **10** (Fig. 1c) indicated PEGylation with one equivalent of PEG, by integration of the only OCH_3 signal which appeared as a singlet at δ 3.39 ppm and the anomeric signal at δ 4.54 ppm (J=8.0 Hz) due to the β-Gal*p*. Characteristic signals at δ 4.24 ppm due to the NH of the secondary amine and at δ 3.22 and δ 3.02 ppm due to the methylene protons obtained by reduction of the lactose anomeric carbon were also observed. The ^{13}C NMR spectrum (Fig. 2c) showed among others the only anomeric signal at δ 102.8 ppm (β-Gal*p*) and the -CH_2NHCH_2- signals at δ 49.6 ppm and 47.2 ppm. For the PEG moiety the OCH_3 signal appeared at 58.1 ppm and the oxymethylene groups of the chain appeared as a strong peak at 69.5 ppm. ESI-TOF mass spectral analysis revealed a series of 44 Da spaced peaks corresponding to the Na^+ adducts. A main peak of $[M + Na]^+$ at 952.49 was observed corresponding to the conjugate **10** with 13 oxyethylene groups.

Inhibition of sialylation of *N*-acetyllactosamine by PEG-conjugates

To find out whether the PEG derivatives used in this study retain the ability of inhibiting sialylation of lactose, different concentrations of compounds **5**,**6**,**8**,**9** and **10** were tested in transfer reactions containing 1 mM of 3'-sialyllactose as a donor, 12 μM [D-glucose-1-^{14}C]-lactose as an acceptor, and TcTS (Table 1). Inhibition of sialylation was quantified by measuring the radioactivity bound to an anion exchange resin after incubation and comparing with



Scheme 4 Lactose modification by reductive amination with PEG-NH_2 Conditions: pH 8, 96 h, 50°C

Table 1 TcTS inhibition by lactose analogs and their PEGylated derivatives using the radioactive inhibition assay

Inhibitor[a]	% Inhibition	IC_{50} (mM)
Lactitol	90	0.21
Latobionic Acid	86	0.33
1	60	0.70
5	82	0.34
6	61	0.73
8	21	2.70
9	56	0.61
10	3.5	4.52

[a] 1 mM Concentration of each compound and the donor 3′-sialyllactose were used

the radioactivity bound in the absence of inhibitor. The PEGylated amides (**5** and **6**) of the aminodisaccharide **1** showed inhibition values similar to those of the original disaccharide, even better for the shorter PEG conjugate. These results indicate that the presence of a polyoxyethylene chain attached to the glucoside unit do not impair their ability to interact with the enzyme. However, the PEG derivatives of the open chain sugars (**8**-**10**) gave lower inhibition values for the TcTS reaction than the corresponding precursors, being insignificant for the PEGylated amine **10**.

Stability in blood

To determine if the insertion of the long chain of PEG in the disaccharides increased the permanence in blood of the conjugates, the PEG derivatives **6** or **9** were injected in mice and a sample of blood was taken at different times after injection (0-120 min). Controls were injected with equivalent amounts of PEG. Compounds **6** and **9** were chosen because of the larger molecular weight of the PEG. Quantification was performed using a colorimetric method based on partitioning a chromophore present in the ammonium ferrothiocyanate reagent from an aqueous to a chloroform phase in the presence of PEG. This method was previously reported for evaluation of PEG-protein conjugates [37]. The concentration of compound **6** in a blood sample taken immediately after injection was 4 mg/mL and diminished to 1 mg /ml after 30 min. This value remained constant for 2 h (Fig. 3a). A similar behavior was observed for compound **9** (Fig. 3b) and for controls injected with CH_3O-PEG5000-NHS ester or CH_3O-PEG5000-NH_2, respectively. The recovery of PEGylated conjugate in urine was estimated for **9.** A value of 3.5 mg of compound **9** in 100 μL of urine was found after 30 min of injection, decreasing to 1 mg /100 μL after 60 min. Although total recovery values were not determined it was shown that rapid clearance from blood takes place by kidney filtration, probably due to the low MW (5,000 Da) of the polymer used for conjugation to the sugar. The results suggest the use of homobifunctional or multi-arm PEGs for coupling with lactose analogs. These PEG derivatives, having higher molecular weights and carrying several active groups, might prolong blood circulation though maintaining the inhibitory properties. Multiarm (star) PEG derivatization has been successfully used to improve the efficacy of small drugs like *N*-acetyl cysteine [38].

The methods described in the present work for the preparation, purification and characterization of the PEG-conjugates provide useful data for conjugation of sugar derivatives to the higher MW PEGs.

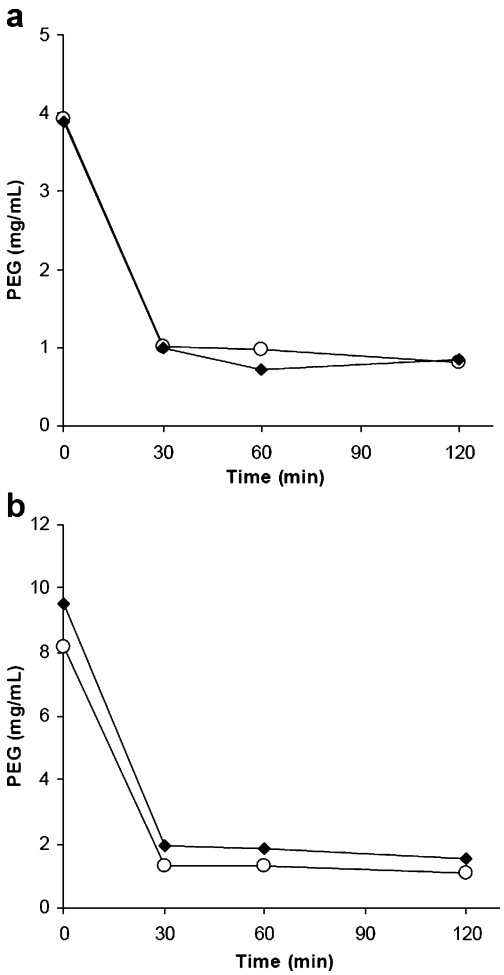


Fig. 3 Plasma clearance of PEG derivatives. Adult mice were intravenously injected with 200 μl of sterile PBS containing: **a**. 30 mg of compound **6** (♦—♦) or 28 mg of CH_3O-PEG5000-NHS ester (o—o); **b**, 44 mg of compound **9** (♦—♦) or 40 mg of CH_3O-PEG5000-NH_2 (o—o). Blood was extracted from the tail at different times and 10 μL serum was used to assay free or combined PEG by a colorimetric assay [37]. Time 0 refers to samples taken immediately after injection

The PEG-conjugates are potentially useful for studies on inhibition of other galactose binding proteins. In this respect, amino derivatives of lactulose have been synthesized and their function as galectin inhibitors has been described [39].

Acknowledgments This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), National Research Council (CONICET) and Universidad de Buenos Aires. A. C. C. Frasch, R. M. de Lederkremer and R. Agustí are research members of CONICET and M. E. Giorgi is a fellow from CONICET. The work of A. C. C. Frasch was partially supported by International Research Scholar grants from the Howard Hughes Medical Institute.

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