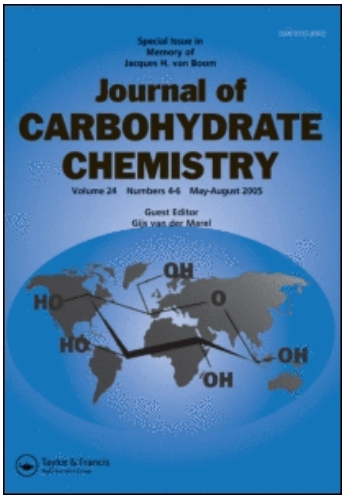

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CONVERSION OF COMPLEX SIALOOLIGOSACCHARIDES INTO POLYMERIC CONJUGATES AND THEIR ANTI-INFLUENZA VIRUS INHIBITORY POTENCY

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ABSTRACT

To investigate the specificity of various influenza virus strains we have prepared polyacrylic type conjugates of undecasaccharide (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1)$_2$-3,6Manβ1-4GlcNAcβ1-4GlcNAc (YDS), and trisaccharides 6`-sialyl-*N*-acetyllactosamine (6`SLN), 6`-sialyllactose (6`SL), and 3`-sialyllactose (3`SL). Free oligosaccharides were transformed to glycosylamine-1-*N*-glycyl derivatives by sequential action of NH_4HCO_3, chloroacetic anhydride, and aqueous NH_3. The known derivatization protocol has been optimized for these sialooligosaccharides. Coupling of obtained amino-spacered derivatives with poly(4-nitrophenyl acrylate) gave rise to two types of conjugates, namely with polyacrylic acid and polyacrylamide backbones; the conversion proceeded quantitatively and without destruction of the oligosaccharides. The content of oligosaccharides in the conjugates was 10, 20, and 30% mol for 3`SL, 6`SL, 6`SLN, and 2, 5 and 10% mol for YDS. Free oligosaccharides and the glycoconjugates were tested as inhibitors of influenza virus adhesion, and also as blockers of virus infectivity in MDCK cell culture. Biantennary YDS demonstrated similar activity to trisaccharide 6`SLN both as the free form and neoglycoconjugate.

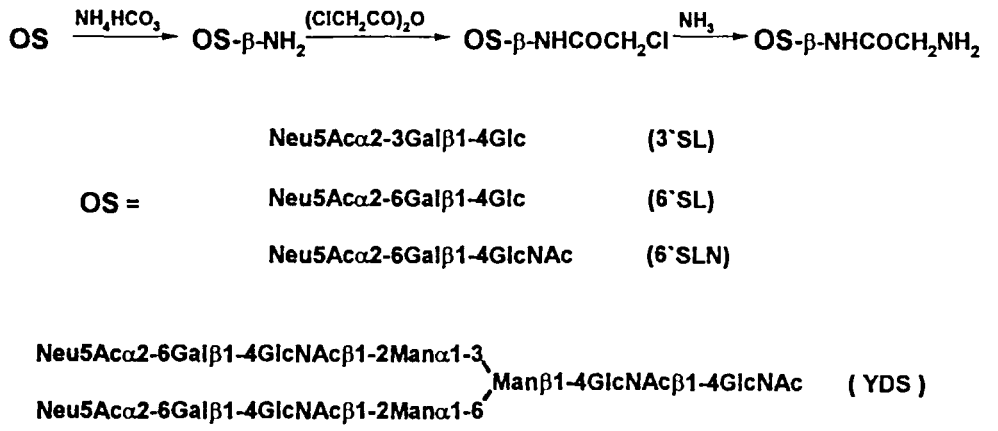
INTRODUCTION

Influenza virus infection is initiated by virus attachment to terminal sialooligosaccharide receptor determinants of cell surface glycoproteins and/or gangliosides.[1] Human influenza viruses preferentially bind to Neu5Acα2-6Gal-containing molecules and more weakly to the Neu5Acα2-3Gal- motif (reviewed in reference 1), the two major moieties expressed on the cell surface of human respiratory epithelium and on human respiratory mucins, respectively.[2] Human influenza A and B viruses that are present in clinical material cannot grow in the allantoic cavity of embryonic chicken eggs. Adaptation of these viruses to growth in chicken eggs always results in a selection of variants with amino acid substitutions around the receptor-binding site of hemagglutinin (HA). Unlike egg-adapted variants, human viruses isolated in Madin-Darby canine kidney (MDCK) cell cultures are usually homogeneous and identical to viruses replicating in humans, at least in their HA.[3] We have shown[4,5] that all non-egg-adapted human influenza H1, H3, and B viruses, which were isolated and grown solely in MDCK cells, share a common high binding affinity for 6`-sialyl-*N*-acetyllactosamine (6`SLN). Glycoprobes containing a 6`SLN ligand are necessary for further, more detailed studies of viral strains. In this paper we describe in detail a convenient and simple synthetic protocol for these probes starting from natural trisaccharides 6`SL, 6`SLN, and undecasaccharide, containing the motif 6`SLN in both antennas.

RESULTS AND DISCUSSION

To synthesize spacer connected glycosides from natural sialooligosaccharides we used, with some modifications, known procedures described for the preparation of glycosylamines and their *N*-glycyl derivatives from neutral oligosaccharides.[6,7] For the 3`SL example, three variants of glycosylamine synthesis were compared: (a) the reaction under the mildest conditions - saturated NH_4HCO_3 at 20 °C for 5-10 days; (b) saturated NH_4HCO_3 at 40 °C for 2-3 days; (c) heating in concentrated aqueous ammonia with catalytic amounts of $(NH_4)_2CO_3$.[8]

It was found that variant (a) was the most suitable; variant (b) required repeated (up to 5 in the case of 1 g or more oligosaccharide treated) freeze-drying procedures to completely remove excess ammonia salts, and led to partial 2-epimerisation of the reducing moiety; a colored by-product was formed in variant (c). Taking into consideration



Scheme 1. Synthesis of sialooligosaccharides 1-*N*-glycyl derivatives

the formation of by-products we used mainly variant (a) for the derivatization of other oligosaccharides.

The *N*-chloroacetylation was performed using chloroacetic anhydride or chloroacetyl chloride in presence of 1M $NaHCO_3$. The last variant is less convenient because of partial glycosylamine hydrolysis. The optimal procedure is described in "Experimental".

The following ammonolysis of 1-*N*-chloroacetyl into the target 1-*N*-glycyl group using ammonium carbonate[7] proceeds slowly. In contrast, the reaction with 10% aqueous ammonia is faster, at the same time giving no by-products.

The purification of *N*-glycyl derivatives was performed taking advantage of their acid-base properties. For monosialylated oligosaccharides the most convenient purification method was ion-exchange chromatography using H^+-resin. Elution with water removed completely unreacted reducing saccharide and all neutral and acidic impurities. The following elution with aqueous pyridine yielded *N*-glycyl derivatives without any cationic material. Only in the case of 3`SL was purification on $H^+$-resin complicated with some lactonisation occurring (~5%). The lactone was not eluted with aqueous pyridine, but was eluted with aqueous $Et_3N$, resulting in hydrolysis of the lactone to the required 3`SL *N*-glycyl derivative. Elution of the lactone with aqueous ammonia led to the corresponding amide. Purification of YDS *N*-glycyl derivative using H^+-resin failed due to its high acidity, but was successful when performed on DEAE Sepharose. Attachment of *N*-

glycyl derivatives to polymer was performed exactly by the method as described earlier,[9] giving rise both to the derivatives of polyacrylamide and polyacrylic acid in almost quantitative yields. No problem due to solubility or stability of sialooligosaccharides during the attachment was observed. Compositions of the neoglycoconjugates were confirmed after mild acid hydrolysis and HPLC quantitative determination of *N*-acetylneuraminic acid (*N*-propionylneuraminic acid as an internal standard was used) in the form of quinoxaline derivatives,[10] and corresponded to the calculated ones within the accuracy of the analysis.

Inhibition of influenza virus isolates from different hosts using polymeric conjugates of 3`SL, 6`SL, and 6`SLN was described in our previous paper.[4] The comparison of inhibition potency for these sialoglycoconjugates with YDS derivatives is shown in Table. Two parameters were tested, the first one, IC_{50} reflects antiadhesion properties. IC_{50} was measured using a solid-phase assaying system. The second parameter is SC_{90} showing the potency of the substance to block virus replication in a MDCK cell system. The antiviral effect (SC_{90}) of the polymeric sialosides correlated with their affinity (IC_{50}) to the corresponding virus strain. In accord with our previous data[5] 3`SL-polymer inhibited adhesion and decreased the infectivity only for the avian but not for human virus. Human H3N2 virus recognized the polymers bearing both 6`SL and 6`SLN-terminated moieties, and these conjugates effectively decreased the virus infectivity. H1N1 virus recognized and was neutralized only by the polymers bearing 6`SLN-terminated moieties (6`SLN-polymer and YDS-polymers). Type B virus did not discriminate between 6`SL and 6`SLN-terminated polymers. It should be mentioned that the comparison of inhibition potency for sialoglycocojugates prepared from trisaccharide 6`SLN and biantennary undecasaccharide YDS demonstrated similar activity of these compounds as inhibitors of virus adhesion both as free form and as neoglycoconjugate. The same is true for suppression of virus infectivity in all human viruses.

EXPERIMENTAL

Materials and methods. Undecasaccharide YDS was obtained as described.[11] Trisaccharides 6`SL and 6`SLN were from Sigma. Trisaccharide 3`SL was a gift of NEOSE Corp. (USA). NH_4HCO_3 (extra pure) was from Merck (Germany), Sephadexes G-25 F, G-10, A-25 DEAE, LH-20 were from Pharmacia (Sweden), Dowex AG 50W-X4 was from Bio-Rad (USA).

Table. Inhibition of influenza virus adhesion and virus infectivity by free sialooligosaccharides and sialoglycoconjugates. Concentration of 50% inhibition (IC_{50}) and concentration of 90% suppressing of virus infectivity in MDCK cell culture (SC_{90}), μM Neu5Ac.

Inhibitor	Avian strain		Human strains					
	H9N2 A/Mallard/3/82		B B/NIB/15/88 M***		H1N1 A/NIB/23/89 M		H3N2 A/NIB/3/90 M	
	IC_{50}	SC_{90}	IC_{50}	SC_{90}	IC_{50}	SC_{90}	IC_{50}	SC_{90}
Glycoconjugates								
3'SL-pA*(10%)**	0.2	4	>20	ND	>20	ND	>20	ND
6'SL-pA(10%)	>50	ND	0.1	1	>20	>20	0.05	0.2
6'SLN-pA(10%)	>50	ND	0.1	1	0.1	0.5	0.05	0.2
YDS-pA(2%)	>50	ND	1	ND	0.5	ND	0.5	ND
YDS-pA(5%)	>50	ND	0.2	ND	0.2	ND	0.2	ND
YDS-pA(10%)	>50	ND	0.1	1	0.1	0.5	0.05	0.2
YDS-PAA(10%)	>50	ND	0.1	1	0.1	0.5	0.05	0.2
Free oligosaccharides								
3'SL	50	ND	200	ND	700	ND	400	ND
6'SL	>2000	ND	100	ND	700	ND	100	ND
6'SLN	>2000	ND	100	ND	100	ND	100	ND
YDS	>2000	ND	200	ND	300	ND	400	ND

* pA - polyacrylic acid, PAA- (*N*-2-hydroxyethyl polyacrylamide)

** mol %, e.g., percent of acrylic residues substituted by oligosaccharide

*** M means that virus strain was cultured solely on MDCK cells, without embryonated eggs stage.

Poly(4-nitrophenyl acrylate) was synthesized by radical polymerization of the monomer in benzene as described.[9] The molecular weight of its derivative poly(*N*-2-hydroxyethylacrylamide, PAA) was estimated on Sephacryl S-300 Superfine column (0.2 M NaCl) calibrated with globular proteins. Mean MW of the polymer was 45 kDa.

Thin-layer chromatography was performed using Kieselgel 60 (Merck, Art. 5724) plates with detection by charring after 7% H_3PO_4 soaking. The eluents used: 2-propanol/acetone/water 4:3:2 (A); 2-propanol/ethyl acetate/water 4:3:2 (B); methanol/acetonitrile/water 3:3:2 (C) or 3:3:1 (D).

^{1}H NMR spectra were recorded on a Bruker 500 MHz spectrometer.

1-*N*-Glycyl derivatives of trisaccharides

A. Synthesis of glycosylamines, OS-β1-NH$_2$ (method of Likhosherstov et al.[6]). The oligosaccharide (0.1 mmol) was dissolved in saturated aqueous NH_4HCO_3, some excess of solid NH_4HCO_3 was added, and the mixture was kept for 10 days at 20 °C or 3 days at 40 °C. The reaction progress was monitored by TLC, and conversion of starting material to glycosylamine was 80-90%. The mixture was diluted with two volumes of water, frozen and then lyophilized; in case the removal of NH_4HCO_3 was not complete (weight control), this procedure was repeated.

B. Synthesis of 1-*N*-chloroacetyl derivatives OS-β1-NHCOCH$_2$Cl (modification of the method described[7]). The glycosylamine was dissolved in cold 1M $NaHCO_3$ (2.5 mL) and the solution of chloroacetic anhydride (171 mg, 1 mmol) in 2.5 mL ethyl acetate was added at 0 °C. The mixture was stirred for 1 h, then 50 μL of AcOH was added and the mixture was concentrated to dryness. The residue was dissolved in a minimum volume of water, passed through a Sephadex G-25 F 2x50 cm column in 0.2% AcOH and the salts-free sugar-containing fraction (TLC) was concentrated to dryness.

C. Synthesis of 1-*N*-glycyl derivatives OS-β1-NHCOCH$_2$NH$_2$. The 1-*N*-chloroacetyl derivative (containing 10-20% of starting reducing oligosaccharide) was dissolved in 4 mL 10% aqueous NH_3 and kept at room temperature for 24 h. After the ammonolysis was complete (monitored by TLC), the solution was concentrated to dryness, and the residue was dissolved in 1 mL 5% AcOH and kept overnight to destroy glycosylamine formed from reducing oligosaccharide. The solution was applied to a 3 mL Dowex AG 50W-X4 (H^+) column, the reducing oligosaccharide was eluted with ~20 mL of water, and the target 1-*N*-glycyl derivative was eluted with 10% aqueous pyridine. The

pyridine eluate was concentrated to dryness, the residue was dissolved in water, frozen and lyophilized. The yield of 1-*N*-glycyl derivatives was 60-70% based on starting reducing oligosaccharide.

TLC - the R_f values were the following (eluents A, B, C): 3`SL 0.68[A], 0.46[B]; 3`SLβ1-NH_2 0.61[A], 0.39[B]; 3`SLβ1-$NHCOCH_2Cl$ 0.77[A], 0.53[B]; 3`SLβ1-$NHCOCH_2NH_2$ 0.49[C]; 6`SL 0.67[A], 0.44[B]; 6`SLβ1-NH_2 0.49[A]; 6`SLβ1-$NHCOCH_2Cl$ 0.62[B]; 6`SLβ1-$NHCOCH_2NH_2$ 0.57[C]; 6`SLN 0.65[A]; 6`SLNβ1-NH_2 0.52[A]; 6`SLNβ1-$NHCOCH_2Cl$ 0.76[A]; 6`SLNβ1-$NHCOCH_2NH_2$ 0.53[C].

^{1}H NMR, selected chemical shifts (D_2O, 303 K, δ, ppm):

3`SLβ1-$NHCOCH_2NH_2$: 1.82 (dd, 1H, J_4 = 12 Hz, H-3_{ax} Neu5Ac), 2.06 (s, 3H, NAc), 2.79 (dd, 1H, J_{3ax} = 12.4 Hz, J_4 = 4.6 Hz, H-3_{eq} Neu5Ac), 3.48 (m, 1H, J_3 = 9 Hz, H-2 Glc), 3.61 (dd, 1H, H-2 Gal), 3.99 (dd, 1H, H-4 Gal), 4.14 (dd, 1H, J_2 = 9.8 Hz, J_4 = 3.1 Hz, H-3 Gal), 4.57 (d, 1H, J_2 = 7.8 Hz, H-1 Gal), 5.09 (d, 1H, J_2 = 9.3 Hz, H-1 Glc);

6`SLβ1-$NHCOCH_2NH_2$: 1.72 (dd, 1H, J_4 = 12 Hz, H-3_{ax} Neu5Ac), 2.01 (s, 3H, NAc), 2.70 (dd, 1H, J_{3ax} = 12.4 Hz, J_4 = 4.6 Hz, H-3_{eq} Neu5Ac), 3.47 (dd, 1H, J_3 = 9 Hz, H-2 Glc), 4.42 (d, 1H, J_2 = 7.9 Hz, H-1 Gal), 5.04 (d, 1H, J_2 = 9.4 Hz, H-1 Glc);

6`SLNβ1-$NHCOCH_2NH_2$: 1.75 (dd, 1H, J_4 = 12 Hz, H-3_{ax} Neu5Ac), 2.075, 2.069 (2s, 6H, 2NAc), 2.72 (dd, 1H, J_{3ax} = 12.4 Hz, J_4 = 4.6 Hz, H-3_{eq} Neu5Ac), 4.50 (d, 1H, J_2 = 7.9 Hz, H-1 Gal), 5.22 (d, 1H, J_2 = 9.4 Hz, H-1 GlcNAc).

1-*N*-Glycyl derivative of YDS. YDS (50 mg, 24.7 μmol) was dissolved in 1 mL of saturated aqueous NH_4HCO_3 and some excess of solid NH_4HCO_3 was added. The solution was kept 10 days at 20 °C, diluted with 1 mL of water, freeze-dried, dissolved in water and freeze-dried again. The residue (52 mg) was dissolved in 1 mL of cooled (0 °C) 1 M $NaHCO_3$ solution and chloroacetic anhydride (85 mg, 0.5 mmol) in 0.85 mL of ethyl acetate was added to the solution at 0 °C and stirred for 1 h. The reaction mixture was neutralized with AcOH and concentrated. The residue was dissolved in 1 mL of water, applied to a Sephadex G-10 column and eluted with 0.1 M AcOH. Fractions containing salts-free oligosaccharide (TLC) were collected, concentrated, and the residue was freeze-dried. The chloroacetyl derivative YDSβ1-$NHCOCH_2Cl$ (containing about 10% of unreacted YDS) was dissolved in 3 mL 10 M aqueous NH_3, kept 2 days, concentrated to dryness, dissolved in 1 mL of 10% AcOH, kept overnight and then concentrated to dryness. The residue was applied to a Sephadex A-25 DEAE column equilibrated pre-

viously with 0.1 M Py/AcOH. First eluted with 0.1 M Py/AcOH was target compound YDSβ1-NHCOCH$_2$NH$_2$, yield 44.7 mg (79%). The second compound eluted with 1 M Py/AcOH was unreacted YDS, yield 6 mg (11%).

TLC, R_f values: YDS 0.58, YDSβ1-NH$_2$ 0.42, YDSβ1-NHCOCH$_2$Cl 0.64 (eluent D); YDSβ1-NHCOCH$_2$NH$_2$ 0.69 (eluent C).

^{1}H NMR data for YDSβ1-NHCOCH$_2$NH$_2$ (D$_2$O, 303 K, δ, ppm): 1.767 (t, 2H, J_{3eq}~J_4 = 12.3 Hz, H-3$_{ax}$ Neu5Ac-7,7`), 2.030 (s, 3H, NAc), 2.059 (s, 6H, 2 NAc), 2.093 (s, 6H, 2 NAc), 2.108 (s, 3H, NAc), 2.694 (dd+dd, 2H, H-3$_{eq}$ Neu5Ac-7,7`), 4.143, 4.221 and 4.279 (~s, 1H, H-2 Man-3,4,4`), 4.474 (d+d, 2H, $J_2$ = 7.8 Hz, H-1 Gal-6,6`), 4.639 (d+d+d, 3H, H-1 GlcNAc-2,5,5`), 4.801 (~s, 1H, H-1 Man), 4.975 (~s, 1H, H-1 Man), 5.148 (d, 1H, J_2 = 10 Hz, H-1 GlcNAc-1), 5.159 (~s, 1H, H-1 Man).

Neoglycoconjugates

Polyacrylic acid conjugates of 3`SL, 6`SL, 6`SLN (10, 20 and 30% mol oligosaccharide content) were prepared by the method described earlier.[9,12] OS-β1-NHCOCH$_2$NH$_2$ (5, 10 or 15 μmol) was dissolved in 100 μL of DMSO, and to the solution was added poly(4-nitrophenyl acrylate) (9.66 mg, 50 μmol) in 500 μL DMF followed by Et$_3$N (20 μL). The mixture was kept at 40 °C for 24 h and the resulting conjugate was then modified by saponification with 2 mL of 0.1M aqueous NaOH for 24 h at 20 °C. The solution was neutralized with 100 μL 1M HCl and applied to a Sephadex LH-20 column (1x30 cm); elution with acetonitrile/H$_2$O (1:1 by vol) followed by concentration and freeze-drying gave rise to polyacrylic acid conjugates, yields 90-95%.

PAA conjugates of 3`SL, 6`SL, 6`SLN (10, 20 and 30% mol oligosaccharide content). OS-β1-NHCOCH$_2$NH$_2$ (5, 10 or 15 μmol) was dissolved in 100 μL of DMSO, and to the solution was added poly(4-nitrophenyl acrylate) (9.66 mg, 50 μmol) in 500 μL DMF followed by Et$_3$N (20 μL). The mixture was kept at 40 °C for 24 h and the resulting conjugate was then modified by amidation with 60 μL of ethanolamine for 24 h at 20 °C. Gel-filtration on a Sephadex LH-20 column (1x30 cm); elution with acetonitrile/H$_2$O (1:1 by vol) followed by concentration and freeze-drying gave rise to PAA conjugates, yields 90-95%.

Polyacrylic acid and PAA conjugates of YDS (2, 5 and 10% mol YDS content) were prepared by the same methods, except for that 1, 2.5 or 5 μmol of YDS-β1-NHCOCH$_2$NH$_2$ were coupled to 50 μmol of poly(4-nitrophenyl acrylate), yields 90-95%.

Inhibition of influenza virus adhesion. Influenza virus receptor-binding inhibition assay was performed as described.[13] Briefly: viruses, diluted with PBS to an HA titre of 1:50-1:200 were incubated in the wells of the fetuin-precoated polystyrene 96-well microplates (0.1 mL/well) for 2 h at 4 °C. The plates were washed with PBS-Tween 20 (0.01%), 0.1 mL PBS-Tween diluted mixture of horseradish peroxidase-conjugated fetuin[13] (final concentration 2 x 10^{-8} M with respect to fetuin) together with inhibitor (sialooligosaccharide or sialoglycoconjugate, see Table) were added to the wells. After incubation for 2 h at 4 °C the plate was washed, peroxidase activity in the wells was assayed (*o*-phenylenediamine - H_2O_2 reagent, absorbance at 492 nm), and the binding in the presence of the inhibitor was compared to the binding in its absence. The data are presented as dissociation constant values of virus-inhibitor complex.[13] The lower the constant value the higher the inhibitor potency.

Inhibition of viral infectivity. MDCK cells were grown in 96-well microplates, washed with minimal essential medium (MM). Serial dilutions (0.15 mL) of sialoglycoconjugates samples (see Table) in MM were inoculated onto monolayers, and equal volumes of influenza virus adjusted to give a final control count of 250-1000 infected cells per well. After incubation of the plates for 14-18 h at 35 °C in 5% CO_2 atmosphere, the medium was removed, the cells were fixed with 0.02% glutaraldehyde for 30 min and washed with PBS. The fetuin-peroxidase conjugate solution (0.05 mL per well) in PBS supplemented with 0.01% Tween 20 was added, the plates were incubated for 1 h at 4 °C and washed with PBS-Tween. The fetuin-HRP conjugate bound to the virus hemagglutinin of infected cells was visualized by incubation with 0.1 mL substrate solution (0.05% 3-amino-9-ethylcarbazole, 0.01% H_2O_2 in 0.05 M sodium acetate buffer, pH 5.7) per well. Stained cells were counted with an inverted microscope.

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