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Ligand Recognition by Influenza Virus

THE BINDING OF BIVALENT SIALOSIDES*

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Infection by influenza virus is initiated by a cellular adhesion event that is mediated by the viral protein, hemagglutinin, which is exposed on the surface of the virion. Hemagglutinin recognizes and binds to cell surface sialic acid residues. Although each individual ligand binding interaction is weak, the high affinity of influenza virus for cells that bear sialic acid residues is thought to result from a multivalent attachment process involving many similar recognition events. To evaluate such binding we have synthesized three series of compounds, each containing two sialic acid residues separated by spacers of different length, and have tested them as ligands for influenza hemagglutinin. No increased binding to the bromelain-released hemagglutinin ectodomain was seen for any of the bivalent compounds as determined by ^{1}H NMR titration. In contrast, however, a spacer length between sialic acid residues of ~55 Å sharply increases the binding of these bidentate species *to whole virus* as determined by hemagglutination inhibition assays. The most effective compound containing glycines in the linking chain displayed 100-fold increased affinity for whole virus over the paradigm monovalent ligand, Neu5Acα2Me.

Prevention of influenza infection by conventional methods, such as vaccination, is complicated by antigenic drift and shift of the viral RNA that encodes the major surface antigen of the influenza virus, hemagglutinin (HA)[1] (Laver and Kilburn, 1966). The virus evades the host immune system by continually changing its surface antigens while retaining infectivity. Hemagglutinin is a membrane-spanning glycoprotein and it has been estimated that a single virus particle contains some 500–1000 HA molecules (Ruigrok *et al.*, 1984). The other protein that is exposed on the viral membrane, the enzyme neuraminidase, is present at lower levels, of perhaps 100–200 copies per virion (Laver, 1973; Schulze, 1973). Besides being the major antigenic component of influenza virus, HA is also responsible for binding to target cells (Hirst, 1942). Furthermore, HA is involved in the fusion event that follows binding and uptake of the virus, and results in the release of viral RNA into the cell (reviewed in White *et al.*, 1983; Wiley and Skehel, 1987).

Cell recognition and binding by influenza virus occur as a consequence of the affinity of HA for the sialic acid (SA) residues that terminate a number of the pendant oligosaccharides of cell surface glycoproteins and glycolipids (Paulson, 1985; Suzuki *et al.*, 1985; Bergelson *et al.*, 1982; Paulson *et al.*, 1979). The affinity of influenza virus for monomeric sialosides in solution has been studied in several laboratories. Paulson's group, using inhibition of hemagglutination as an indicator of binding affinity, has demonstrated that the binding of monovalent sialosides is relatively weak and is largely independent of the structure to which the sialic acid fragment is attached (Paulson, 1985). An atomic model for the recognition and binding process was provided by Weis and co-workers (1988), who determined the crystal structure of α(2,6)-sialyllactose bound to BHA (hemagglutinin that has been cleaved from the virus by treatment with the protease, bromelain). BHA is a homotrimeric protein of subunit molecular weight 70,000 (Wiley *et al.*, 1977). The molecule projects about 135 Å from the viral membrane, and each protomer contains a single SA-binding site at the membrane-distal end of the molecule (Wilson *et al.*, 1981). Comparison of the amino acid sequences of natural isolates of HA from various sources shows that the amino acid residues that comprise the SA-binding site are highly conserved (Wiley *et al.*, 1981), providing further support for the view that it is the binding of SA to HA that is responsible for viral recognition, this event leading to membrane fusion and, ultimately, infection.

Monomeric sialosides such as Neu5Acα2Me or α(2,3)-sialyllactose bind to HA with dissociation constants of about 2 mM, whether this value is based upon inhibition of hemagglutination by whole virus, or whether binding to BHA is studied directly by nuclear magnetic resonance analysis (Sauter *et al.*, 1989; Pritchett *et al.*, 1987). These results prompt the question: how does such a low affinity interaction account for the apparently strong binding of influenza virus to target cells? The obvious explanation is that the virus takes advantage of several simultaneous binding events (Fazekas de St. Groth and Gottschalk, 1963). Such a polyvalent interaction is supported by the observation that glycoproteins (*e.g.* fetuin, orosomucoid, and α_2-macroglobulins) and synthetic polymers having high contents of SA, are good inhibitors of hemagglutination (Spaltenstein and Whitesides, 1991; Matrosovich *et al.*, 1990; Hanaoka *et al.*, 1989; Pritchett and Paulson, 1989; Barclay *et al.*, 1969; Whitehead and Winzler, 1968; Morawiecki and Lisowska, 1965).

If the adhesion of influenza virus to cells involves multi-

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[1] The abbreviations used are: HA, hemagglutinin; BHA, bromelain-released HA; SA, sialic acid; QSL, quasi-elastic light-scattering.

point attachment, compounds that contain more than one SA residue, suitably tethered to permit binding to more than one site on the viral surface, should display tighter binding than simple monovalent sialosides. This increased affinity would derive from the additivity of the intrinsic SA-HA binding free energies reduced only by the loss of independent translational and rotational entropy. This is simply a restatement of the well known chelate effect (Jencks, 1981; Eilat and Chaiken, 1979; Gopalakrishnan and Karush, 1974; Page and Jencks, 1971).

A similar approach to the production of tight binding inhibitors has been used previously by a number of other groups. For example, dimerization of peptides or alkaloids, through a flexible linker, generated potent and specific agonists and antagonists of opioid receptors (Portoghese *et al.*, 1986; Erez *et al.*, 1982; Shimohagishi *et al.*, 1982). Analogously, Maraganore and co-workers (1990) have recently described a class of peptides (hirulogs) that act as heterobifunctional bivalent inhibitors of thrombin, and Lee and co-workers (1989) have demonstrated the increase in affinity for Gal/GalNAc-binding lectin that may be acheived with multidentate ligands.

The distribution of SA binding loci on the surface of influenza A virus is not known. Thus, in order to identify potential bivalent inhibitors of influenza virus we have synthesized three classes of bivalent sialosides containing spacers of different lengths. By testing the affinity of these compounds for influenza virus we have identified inhibitors of hemagglutination that appear to bind to influenza A virus by two point attachment to the virus surface. We discuss these results and draw some conclusions regarding the ordering of HA on the surface of the virion (Glick and Knowles, 1991).

EXPERIMENTAL PROCEDURES[2]

Synthesis of Bivalent Sialosides—The materials and methods for the synthesis of all of the compounds tested, along with the relevant structural characterization data, are contained in the Miniprint.

Materials—Covered microtiter plates containing 96 V-shaped wells were obtained from Flow Laboratories Inc. (Mclean, VA). All hemagglutination inhibition experiments were performed with recombinant influenza A virus strain X-31 (Kilbourne, 1969). Phosphate-buffered saline (PBS) refers to NaCl (80 g), KCl (2 g), Na_2HPO_4 (11.5 g), and KH_2PO_4 (2 g) dissolved in H_2O (10 liters). Chicken erythrocytes were obtained from SPAFAS Inc. (Norwich, CT). NMR samples of BHA were prepared as described (Sauter *et al.*, 1989).

Preparation of Red Blood Cells—Red blood cells from 22-week-old chickens, in Elsever's solution (6 ml), were placed in a centrifuge tube and diluted with PBS to a total volume of 50 ml. The suspension was centrifuged for 5 min at 2000 rpm (500 × *g*) in a Sorvall GLC-2 centrifuge. The supernatant was removed by decantation and the pellet resuspended in an equal quantity of fresh PBS buffer. This operation was repeated five times. The final pellet was resuspended in PBS buffer to a total volume of 50 ml.

Determination of the Viral Hemagglutination Titer—Virus test solutions were prepared in microtiter plates as serial dilutions in PBS (100 μl) of the original virus stock solution (see "Materials"). Red blood cell suspension (100 μl of the 0.5% solution prepared above) was then added to each well at 4 °C. The plates were read after approximately 2 h. The lowest concentration of virus that causes the blood cells to agglutinate is defined as one hemagglutinating unit. A solution containing virus at a concentration equivalent to four hemagglutinating units was used for all hemagglutination inhibition assays.

Hemagglutination Inhibition Assay—Each test compound was prepared as a 10 mM solution in PBS and adjusted to pH 7 with 1 M NaOH. Serial dilutions of the inhibitor were made in three lanes of a microtiter plate to give sample volumes of 50 μl. A solution of virus (50 μl, 4 hemagglutinating units) was added to each of the wells in two of the three lanes. PBS (50 μl) was added to each well in the third row, as a control lane. The plate was allowed to stand at 4 °C for 30 min. Red blood cell suspension (100 μl of the 0.5% solution prepared above) was added to each well in all three lanes. End points were determined after 2 h. The minimum concentration of inhibitor required to inhibit red cell agglutination gives a value for the K_i of the inhibitor with influenza A virus.

Quasi-elastic Light-scattering Analysis (QLS)—Light-scattering analysis was performed using a Brookhaven light scattering system (Brookhaven Instruments, Holtsville, NY). The system consisted of a B12005M goniometer with a photomultiplier positioned at 45 ° to the incident light beam. The light source consisted of a 2-watt argon laser at 488 nm (Lexel, Fremont, CA). The goniometer assembly was kept at 4 °C with an external water bath. Samples (1 ml) were filtered into glass disposable culture tubes (12 × 75 mm, VWR Scientific, San Francisco, CA) and placed in the goniometer with *cis, trans*-decahydronaphthalene surrounding the sample tube as an index matching fluid. The photon data were collected using a B12030 autocorrelator with 136 channels. A personal computer was used for system control and data storage. Particle size distributions were calculated from QLS autocorrelation function data using the method of constrained regularization (CONTIN) as described (Stock and Ray, 1985), and adapted by Brookhaven Instruments to run on a personal computer (Provencher, 1985).

RESULTS

Inhibitor Design and Synthesis—Since the distance between SA-binding sites on the surface of the influenza virus is unknown it seemed prudent to prepare families of related molecules having linkers of different length and of different conformational flexibility. Following synthetically convergent methodology, three families of compounds of varying linker span length were generated (see Miniprint). Molecules were synthesized containing ether linkers made from ethylene glycol, peptidic linkers derived from glycine, or urea-based linkers containing piperazine units (Fig. 1). Span lengths were measured after constructing the molecules in an extended conformation using the MACROMODEL molecular modeling software package (Mohamadi *et al.*, 1990) and then minimizing the energy of the structure with the AMBER united atom force field (Weiner *et al.*, 1984; Weiner and Kollman, 1981). These lengths are listed in Table I.

^1H NMR Assay with BHA from Influenza Virus X-31—From the crystal structure of the complex of α(2,6)-sialyllactose and BHA, it is clear that the *N*-acetyl group of the sialic acid residue lies close to tryptophan-153 (Weis *et al.*, 1988). Upon binding to the protein, the *N*-acetyl methyl group of the ligand experiences a small change in its magnetic environment. Since the ligand is in rapid exchange between bound

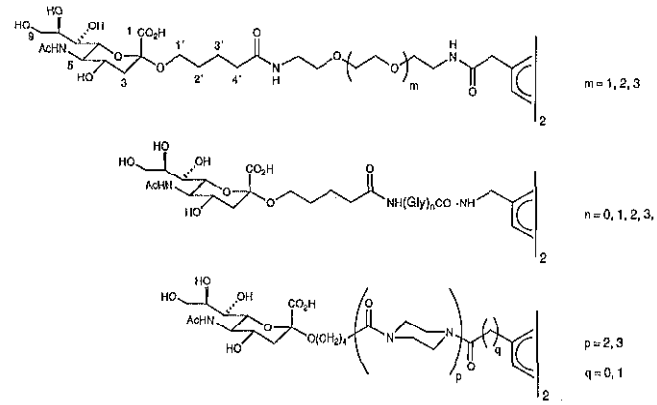

FIG. 1. **Three series of bivalent sialosides.** Key: **P(3,3)** m = 1; **P(4,4)** m = 2; **P(5,5)** m = 3; **G(3,3)** n = 3; **G(4,4)** n = 4; **U(3,3)** p = 3, q = 0; **U(3,3)'** p = 3, q = 1.

[2] The materials and methods for the synthesis of all of the compounds tested, along with the relevant structural characterization data, are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

Spans of bivalent sialosides

Compound	Span[a]	Compound	Span
	Å		Å
G(0,0)	27	**U(2,2)**	45
G(1,1)	34	**U(3,3)**	58
G(2,2)	41	**U(3,3)′**	60
G(3,3)	49	**P(3,3)**	49
G(4,4)	57	**P(4,4)**	57
		P(5,5)	65

[a] The span of a given bivalent sialoside is defined as the distance between the sialoside *N*-acetyl carbonyl carbon atoms when the molecule exists in an extended conformation. These distances were determined using MACROMODEL version 2.0 (Mohamadi *et al.* 1990).

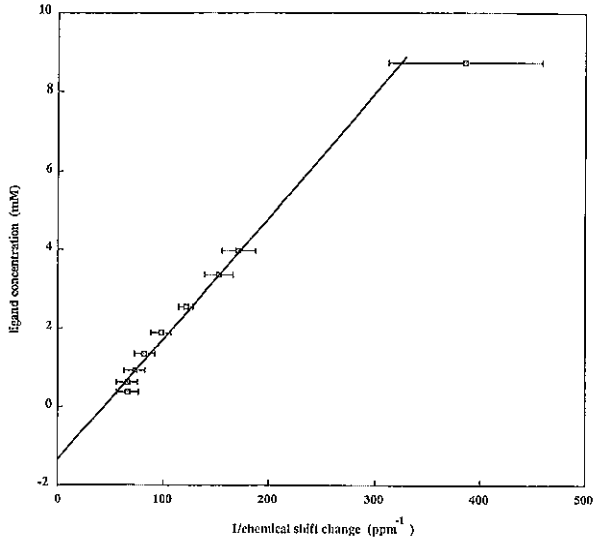

FIG. 2. **Titration of G(4,4) with BHA.** The reciprocal of the change in chemical shift of the *N*-acetyl resonance of **G(4,4)** is plotted against the concentration of **G(4,4)**. From Equation 1a in Sauter *et al.* (1989), the negative of the *y* intercept in this plot gives the K_d for the equilibrium.

and unbound states, this shielding is observed as a change in the average chemical shift of the methyl resonance. The ^{1}H NMR spectrum of the bound ligand also displays significant line broadening relative to that of the free ligand. The chemical shift change and the change in line width can each be used to obtain the ligand dissociation constant (Sauter *et al.*, 1989).

Members of each series of bivalent sialosides were assayed for binding affinity to BHA by the NMR titration method (Sauter *et al.*, 1989). Portions of a solution of BHA plus ligand were added sequentially to a solution of BHA. After each addition the NMR spectrum was recorded. The dissociation constant, K_d, was then obtained from a plot of the reciprocal of the change in chemical shift (or line width) *versus* the concentration of the inhibitor (Fig. 2). As can be seen in Table II, values of K_d for all the bivalent compounds are similar to that for the prototypical monovalent ligand, Neu5Acα2Me. All ligands are in fast exchange on the NMR time scale, and only one signal for the *N*-acetyl methyl group is observed.

Hemagglutination Inhibition Assay with Intact Influenza Virus X-31—The binding of ligands to whole virus was assessed by measuring the ability of the synthetic ligands to inhibit the agglutination of chicken erythrocytes. The results of the inhibition studies with influenza virus X-31 are given in Table II. Each compound in the G-series and the P-series and one of those in the U-series (**U(3,3)′**) inhibits agglutination more effectively than the monovalent ligand, Neu5Acα2Me.

TABLE II

Binding of bivalent sialosides to BHA and to influenza virus

Compound	K_d[a]	K_i[b]
	mM	mM
Neu5Acα2Me	2.8 ± 0.3	2.5
G(0,0)		1.25
G(1,1)		1.25
G(2,2)		1.25
G(3,3)		0.6
G(4,4)	1.5 ± 0.2	0.0030
P(3,3)		0.6
P(4,4)	2.3 ± 0.5	0.15
P(5,5)	2.2 ± 0.1	1.2
U(2,2)		>0.15
U(3,3)		>0.15
U(3,3)′	1.6 ± 0.6	1.2

[a] Binding to BHA was measured by the method of Sauter *et al.* (1989).

[b] K_i is the minimum concentration of inhibitor required to prevent hemagglutination by influenza virus A. All reported values are relative to Neu5Acα2Me that has a K_i of 2.5 mM and are accurate to a factor of two.

Quasi-elastic Light-scattering Analysis—QLS was used to determine the mean particle size in influenza A virus suspensions, in the presence and absence of the bivalent inhibitor **G(4,4)**. The neuraminidase inhibitor, 2,3-dehydro-2-desoxy-Neu5Ac, was added to all of the samples to prevent neuraminidase cleavage of sialic acid residues from the bivalent inhibitor. In a separate experiment it was shown that the addition of 2,3-dehydro-2-desoxy-Neu5Ac to virus suspensions did not affect the observed mean particle size. Solutions of influenza A virus containing 2,3-dehydro-2-desoxy-Neu5Ac (100 μM) and **G(4,4)** at concentrations of 2, 0.5, and 0.125 mM were compared with control solutions without any **G(4,4)**, over a period of 2 h. Three different concentrations of virus were examined, corresponding to 6, 60, and 120 times the concentration used in hemagglutination inhibition experiments. At these three different concentrations of virus, the formation of large viral aggregates was observed *only* when the concentration of **G(4,4)** was 2 mM. At the two lower concentrations of **G(4,4)** examined no difference in mean particle size between the control and test samples was observed. A typical set of results is shown in Fig. 3.

DISCUSSION

We have synthesized three families of bivalent sialosides to investigate the cooperative binding that occurs between influenza virus and target cells that bear oligosaccharides terminating in sialic acid residues.

The binding of the bidentate sialosides to BHA was investigated using ^{1}H NMR to follow the interaction of protein with ligand (Sauter *et al.*, 1989). It was found that all the bivalent compounds studied bind to BHA with similar affinity, and that the interaction is no tighter than that between BHA and the archetypal monovalent α-sialoside, Neu5Acα2Me (Table II). Thus none of the compounds examined appears to bind simultaneously to two SA-binding sites within a single HA trimer.

Next, we studied the interaction of the bivalent sialosides with whole influenza virus. This interaction can be assessed from the ability of these ligands to inhibit the agglutination of red blood cells by virus (WHO, 1963; Rogers *et al.*, 1983; Barrett and Inglis, 1985). The minimum concentration of ligand required for the inhibition of erythrocyte agglutination

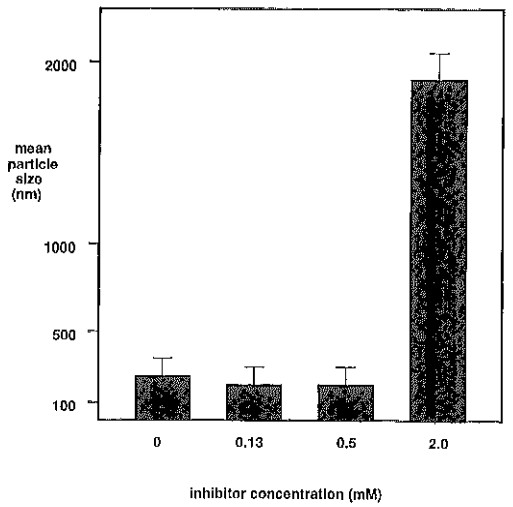

FIG. 3. Aggregation of influenza virus particles by **G(4,4)** as determined by quasi-elastic light-scattering analysis.

provides a measure of ligand binding to one of the partners involved. Although this minimum concentration does not represent a thermodynamic K_d, the *relative* inhibitory potency of different ligands correlates well with binding affinities determined by other methods (see, for example, Sauter *et al.*, 1989; Pritchett *et al.*, 1987; Kihlberg *et al.*, 1989). The inhibitory concentrations so obtained may thus be related to dissociation constants by comparing all the ligands to a single compound the K_d of which has already been determined.

Each of the compounds in the G-, P-, and U-series, when assayed for their ability to inhibit erythrocyte agglutination, binds more tightly than Neu5Acα2Me. Importantly, **G(4,4)** and **P(4,4)** show a marked increase in inhibitory potency relative to the other bivalent sialosides in their families (Table II). This increase in binding potency is not seen for monovalent analogues of **G(4,4)** and **P(4,4)** (see Table I in Supplementary Material), and the difference in binding affinity suggests that the recognition of the bivalent compounds derives from two-point attachment to the viral surface. Comparison of the binding data from NMR titration and from hemagglutination inhibition experiments further indicates that **G(4,4)** and **P(4,4)** bind much more tightly to whole virus than to isolated BHA. The data thus suggest that **G(4,4)** and **P(4,4)** bridge SA binding loci on different HA trimers on the viral surface.

Given the relationship between the span of the bivalent sialosides and their ability to bind to whole influenza virus (Tables I and II), can we draw any conclusions about the nature of the virus surface from knowledge of the solution structure of the ligands? Studies of oligoglycine chains by Raman and infrared spectroscopy have suggested that these oligomers prefer an extended conformation in aqueous solution (Smith *et al.*, 1969; Dwiveldi and Gupta, 1971). Computer-aided molecular modeling of **G(4,4)** in a fully extended conformation places the carbonyl carbons of the two sialoside *N*-acetyl groups at a distance of about 55 Å. Data on the conformational preference of polyethylene glycol chains suggest that these molecules may exist as random coils in aqueous solution (Devanand and Selser, 1990) although some spectroscopic studies have provided evidence for hydrogen-bonded structures that display a high percentage of gauche conformations along the C-C bonds (Nishide *et al.*, 1986; Matsuura and Fukuhara, 1985). The finding that **G(4,4)** and **P(4,4)** are the best inhibitors in their respective series suggests that in solution both of these compounds adopt the same effective length. Our computer modeling studies suggest that this length cannot be more than 55 Å. The best ligand in the G-series, **G(4,4)**, binds some 100 times more tightly to influenza virus than does Neu5Acα2Me, whereas the most potent inhibitor in the P-series, **P(4,4)**, binds about 20-fold better than Neu5Acα2Me, when the binding affinities of these compounds are compared on a "per sialoside" basis. It seems likely that the tighter binding of **G(4,4)** relative to **P(4,4)** is due to the smaller loss in conformational entropy in the glycine linker chain upon bidentate binding. Although **G(4,4)** contains the same number of atoms in the linker as **P(4,4)**, it is more rigid than **P(4,4)**, having the planar constraint of peptide bonds that eliminates free rotation about one third of the bonds within each of the spacer arms. The weaker binding of the bidentate inhibitors with shorter linkers is presumably a consequence of their being of inadequate length to span the distance between SA binding sites on adjacent HA trimers. Our results suggest that the distance between two adjacent SA-binding loci on the viral surface is less than or equal to 55 Å.

The mobility of HA in the membrane of influenza virus has been studied by Junankar and Cherry (1986) who concluded that HA has very little rotational mobility below 20 °C. Since our original hemagglutination inhibition assays were conducted at 4 °C, which is below the viral membrane transition temperature, these experiments were repeated at 25 °C, at which temperature the membrane should be more fluid and allow greater mobility of its integral protein components. However, the inhibitory potencies of the bidentate species from both the P-series and the G-series do not change when measurements are made at 25 °C. This result is consistent with the idea that the surface protein components of influenza A virus form a regular array on the viral surface, and that binding to cells does not involve the recruitment of HA trimers by two-dimensional diffusion in the plane of the membrane. Although investigations of influenza A virions by electron microscopy have failed to define the distribution of hemagglutinin and neuraminidase on the viral membrane, studies on influenza C show that in this case the HA trimers are closely packed and form a regular hexagonal lattice (Hewat *et al.*, 1984).

In addition to HA, the surface of influenza A virus displays the enzyme neuraminidase which also possesses an SA-binding site, and which could in principle participate in the recognition and binding of multivalent sialosides. However, as was first shown by Paulson and co-workers (Pritchett *et al.*, 1987; Rogers *et al.*, 1983), neuraminidase appears not to participate in hemagglutination or its inhibition, since addition to the assay solution of a specific neuraminidase inhibitor (2,3-dehydro-2-desoxy-Neu5Ac) (Meindl *et al.*, 1974) at levels more than 100-fold higher than its K_i value, does not alter the relative or the absolute inhibitory potency of any of the bivalent ligands investigated. Furthermore, no degradation of **P(4,4)** by viral neuraminidase was observed at 4 °C when the integrity of the bivalent sialoside was monitored by HPLC as a function of time. This result was confirmed using a fluorescence assay with 2′(4-methylumbelliferyl)NeuAc (Myers *et al.*, 1980), which showed that the aglycone is not released by neuraminidase under the conditions used in the hemagglutination inhibition assay.

It remains formally possible that the inhibition of hemagglutination by bivalent ligands that is evident from the results in Table II derives from the cross-linking and sequestration of virus by these compounds. To examine this possibility we performed light scattering experiments with influenza A virus and different concentrations of **G(4,4)** (Fig. 3). At three

different concentrations of virus we observed the formation of viral aggregates only at the highest concentration of inhibitor used (2 mM). These results suggest that at concentrations of inhibitor of 0.5 mM or less, inhibition of hemagglutination is not a result of viral aggregation. Moreover, the fall in inhibitory potency on going from **P(4,4)** to **P(5,5)** is inconsistent with viral aggregation being the explanation for the observed inhibition. This fall is, indeed, most readily accounted for by the expected drop in potency for entropic reasons when the linking chain becomes unnecessarily long. *We therefore conclude that the observed binding of molecules of the P- and G-series is HA-specific, and is localized to individual virions.*

None of the molecules in the U-series displays the same kind of cooperative binding to influenza virus as the best candidates from the G- and P-series (*i.e.* **G(4,4)** and **P(4,4)**), despite being of similar length (Tables I, II). Examination of molecular models indicates that the molecules in the U-family are considerably more rigid than compounds in either the G-series or the P-series, though computational molecular modeling indicates that an extended conformation is not energetically unfavorable in this series. These indications notwithstanding, it is possible that the conformational constraints imposed by the linker arms in the U-family are enough to preclude the simultaneous docking of two sialic acids to HA binding sites, by forcing a misorientation of the SA residues. Thus the ligand **U(3,3)′** may not exist in an extended geometry in free solution, and the molecule may be too inflexible to permit the conformational adjustments needed for two-point binding to the viral surface.

By taking advantage of the chelate effect in the use of bivalent inhibitors, the best increase in binding to influenza virus that we have achieved is about 100-fold. The naturally occurring ligand equine α_2-macroglobulin inhibits hemagglutination by influenza virus with a K_i of 10^{-8} M per SA residue. Several groups have prepared polymeric materials bearing SA residues that inhibit hemagglutination by influenza virus with a similar potency ($K_i = 10^{-7}$ M) (Spaltenstein and Whitesides, 1991; Matrosovich *et al.*, 1990). The inhibitory potency of such polysialosides, however, does not correlate with the number of sialosides available for binding, and there appears to be an optimum density of SA residues that produces maximum binding affinity. This finding most likely reflects the spatial distribution of SA binding loci on the virus surface, though insufficient structural information regarding these polymers is available to determine the interresidue distances involved. These observations suggest that extensions of the work described in this paper may lead to useful increases in binding affinity for influenza A virus.

In summary, we have prepared three sets of bivalent sialosides to evaluate the importance of binding cooperativity in the recognition of sialosides by influenza A virus. None of these compounds binds to soluble BHA in a two-point fashion. Cooperativity is seen, however, on binding to intact virus, where it is evident that the ligands interact with SA binding loci on different HA trimers on the viral surface. From studying the binding of these compounds to virus as a function of the length of the linker between the SA residues, we conclude that the distance between adjacent SA binding sites on different HA trimers is less than 55 Å. Extension of these findings to the interaction betwen virus and cells suggests that intertrimer recognition of SA residues is important, and that HA molecules may only be partially occupied by SA. In related experiments we have found that **G(4,4)** strongly inhibits the agglutination of erythrocytes by the VP1 pentamer of polyoma virus.[3] In this case, the binding of the bidentate ligand is some 500-fold tighter than Neu5Acα2Me. Further experiments with defined polyvalent receptors such as those presented here will clarify the nature of viral recognition and, perhaps, offer leads to new antiviral agents.

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SUPPLEMENTARY MATERIAL TO:
LIGAND RECOGNITION BY INFLUENZA VIRUS: THE BINDING OF BIVALENT SIALOSIDES
BY
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EXPERIMENTAL PROCEDURES

Materials - Protected amino acids were purchased either from Bachem Bioscience Inc. (Philadelphia, PA) or from Chemical Dynamics Corp. (Plainfield, NJ). Sialic acid was isolated from birds' nest substance as described.[1] All other reagents were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Methylene chloride, diisopropylethylamine, and triethylamine were each distilled from calcium hydride under N_2. Dimethylformamide was dried by storing for 1 week over activated 4 Å molecular sieves and then decanting onto fresh sieves prior to use. Dowex-50W (H+ form) (8% cross-linked) was bought from Sigma Chemical Co. (St. Louis, MO). Sephadex LH-20 and Sephadex G-25 (fine) were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Silica gel (32-63 mesh) for column chromatography was obtained from ICN Biochemicals (Cleveland, OH). Glass-backed silica gel 60 plates precoated with a layer (0.25 mm) of Kieselgel 60F-254, for thin-layer chromatography, were obtained from E. Merck (Darmstadt, Germany).

Methods - Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded variously on Bruker AM 250, 300, 400, or 500 MHz spectrometers. All NMR spectra were measured at ambient probe temperature using either $CHCl_3$ (∂ = 7.26) or HDO (∂ = 4.8) as an internal reference. Mass spectra were measured by fast atom bombardment (FAB) in the Harvard mass spectrometry facility, or at the University of California, Berkeley Mass Spectrometry Center, using either a glycerol or a glycerol/thioglycerol matrix. Gel filtration was carried out at 4 °C. High performance liquid chromatography (HPLC) was performed using two Waters Associates (Milford, MA) model 510 pumps for solvent delivery, a model 680 gradient controller, and a model 494 absorbance detector. Reverse-phase HPLC was accomplished using Waters μBondapak C_{18} columns (3.9 x 300 mm for analysis, and 19 x 150 mm for preparative runs). Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials.

Methyl (4,7,8,9-tetra-O-acetyl-N-acetyl-2-chloro-2-deoxy-β-D-neuraminate (2). Methyl *N*-acetyl-β-D-neuraminate[2] (1.49 g, 4.6 mmol) was dissolved in acetyl chloride (14 mL) and stirred for 12 h at room temperature. The excess acetyl chloride was removed under reduced pressure to give a colorless oil. This residue was concentrated three times from added CH_2Cl_2 to afford the penta-acetyl sialosyl chloride (**2**) (2.1 g, 4.1 mmol, 90%) as a white foam. The ^{1}H NMR spectrum of this compound matched the literature data[2] and the compound was used without further purification.

Methyl [(pent-5'-enyl) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-nonulopyranosid]onate (3). Compound **2** (2.1 g, 4.1 mmol) was dissolved in 5-hexen-1-ol (6 mL) and solid mercury(II) cyanide (0.12 g, 0.46 mmol, 0.11 equiv) and 3 Å molecular sieves (0.5 g) were added. The reaction mixture was stirred in the dark under argon at room temperature for 3 h. The solution was diluted with CH_2Cl_2 and washed with 0.5 M $NaI_{(aq)}$, 1 M sodium thiosulfate in H_2O, then brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give a colorless oil. The residue was chromatographed on silica gel (eluting with 70% ethyl acetate in petrol) to give compound **3** (1.46 g, 70%) as a white foam (R_f 0.35, EtOAc): ^{1}H NMR ∂(400 MHz, $CDCl_3$) 1.33-1.49 (2H, m, 3'-H), 1.49-1.90 (2H, m, 2'-H), 1.88 (3H, s, NAc), 1.92 (1H, t, J 14 Hz, 3-H_{ax}), 2.00 (3H, s, OAc), 2.02 (3H, s, OAc), 2.03 (2H, t, J 5 Hz, 4'-H), 2.11 (3H, s, OAc), 2.12 (3H, s, OAc), 2.55 (1H, dd, J 14, 4 Hz, 3-H_{eq}), 3.20 (1H, dt, J 9, 8 Hz, 1'-H), 3.71-3.80 (1H, m, 1'-H), 3.78 (3H, s, CO_2CH_3), 4.02-4.11 (3H, m, 5-H, 6-H, 9-H), 4.80 (1H, dd, J 14, 3 Hz, 9-H), 4.78-4.86 (1H, m, 4-H), 4.92 (1H, dd, J 9, 1 Hz, C=C*H trans*), 4.99 (1H, dd, J 16, 1 Hz, C=C*H cis*), 5.29 (1H, d, J 8 Hz, *NH*), 5.32 (1H, d, J 8 Hz, 7-H), 5.37-5.40 (1H, m, 8-H), 5.73-5.83 (1H, m, 5'-H).

Methyl [(4'-carboxybutyl) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-nonulopyranosid]onate (1) (Scheme 1). A solution of potassium permanganate (208 mg, 1.3 mmol, 3.2 equiv) in aqueous acetic acid (2 mL, H_2O:glacial acetic acid, 5:1) was cooled to 0 °C. The olefin (**3**) (240 mg, 0.42 mmol, 1 equiv) in acetic acid (2 mL) was added to this solution in one portion and stirred at 0 °C for 2 h. The reaction mixture was diluted with EtOAc and solid sodium sulfite (320 mg, 7 mmol, 6.5 equiv) was added followed by 1 M $HCl_{(aq)}$ (2 mL). The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure to give **1** (230 mg, 93%) as a white foam that was used without further purification (R_f 0.3, 10% MeOH in CH_2Cl_2): ^{1}H NMR ∂(400 MHz, $CDCl_3$) 1.58-1.68 (2H, m, 3'-H), 1.68-1.79 (2H, m, 2'-H), 1.89 (3H, s, NAc), 1.95 (1H, t, J 13 Hz, 3-H_{ax}), 2.02 (3H, s, OAc), 2.04 (3H, s, OAc), 2.14 (3H, s, OAc), 2.15 (3H, s, OAc), 2.40 (2H, td, J 7, 3 Hz, 4'-H), 2.58 (1H, dd, J 13, 6 Hz, 3-H_{eq}), 3.23 (1H, dt, J 10, 6 Hz, 1'-H), 3.73-3.80 (1H, m, 1'-H), 3.79 (3H, s, CO_2CH_3), 4.04-4.16 (3H, m, 5-H, 6-H, 9-H), 4.31 (1H, dd, J 6, 2 Hz, 9-H), 4.79-4.88 (1H, m, 4-H), 5.18 (1H, br d, J 9 Hz, *NH*), 5.30 (1H dd, J 9, 2 Hz, 7-H), 5.38-5.40 (1H, m, 8-H).

General procedure for the preparation of the P-series (explicitly for P(4,4)): The synthetic outline is shown in Scheme II.

Tetraethylene glycol monotosylate. Sodium hydride (1.1 g, 22.4 mmol, as a 60% dispersion in mineral oil) was washed three times with hexane, and then suspended in THF (44 mL). Tetraethylene glycol (4.4 g, 22.4 mmol) was added dropwise over 30 min and the mixture was stirred for an additional 45 min. *p*-Toluenesulfonyl chloride (4.3 g, 22.4 mmol) was added to the mixture as a solid in several portions and the stirring was continued for 1 h. The reaction mixture was concentrated under reduced pressure and the residue chromatographed on silica gel (eluting with 2% MeOH in CH_2Cl_2) to give the monotosylate (4.6 g, 60%) (R_f 0.4, 4% MeOH in CH_2Cl_2): ^{1}H NMR ∂(250 MHz, $CDCl_3$) 2.41 (3H, s, CH_3), 3.50-3.70 (14H, m, CH_2O), 4.12 (2H, t, J 6 Hz, CH_2OTs), 7.30 (2H, d, J 8 Hz, *o*-arom.), 7.74 (2H, d, J 7 Hz, *m*-arom.).

2-(2-Azidoethoxy(2-ethoxyethoxy))ethanol. Tetraethylene glycol monotosylate (5.6 g, 16 mmol, 1 equiv) was dissolved in dimethylformamide (50 mL), sodium azide (10.5 g, 160 mmol, 10 equiv) added, and the suspension stirred for 2 d at room temperature. The dimethylformamide was then removed under reduced pressure and the residue dissolved in EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure to give the azide (2 g, 60%) as a colorless oil which was used without further purification (R_f 0.3, 4% MeOH in CH_2Cl_2): ^{1}H NMR ∂(300 MHz, $CDCl_3$) 2.44 (1H, m br s, *OH*), 3.40 (2H, t, J 5 Hz, CH_2N), 3.59-3.75 (14H, m, OCH_2).

2-(2-Carbobenzoxyaminoethoxy(2-ethoxyethoxy))ethanol. 2-(2-Azidoethoxy(2-ethoxyethoxy))ethanol (2 g, 8.9 mmol, 1 equiv) was dissolved in THF (35 mL) and solid triphenylphosphine (2.6 g, 9.8 mmol, 1.1 equiv) was added. After 2 min, enough water (approximately 20 mL) was added to the reaction mixture to begin precipitation of the phosphines and the suspension was stirred vigorously overnight. The THF was removed under reduced pressure and the mixture was then filtered to remove phosphines. The solution was lyophilized to afford the amine in quantitative yield as a colorless oil. This amine was dissolved in CH_2Cl_2 and cooled to 0 °C. Triethylamine (1.8 mL, 13 mmol, 1.5 equiv) was added, followed by benzyl chloroformate (1.27 mL, 8.9 mmol, 1 equiv) and the reaction was stirred overnight. The mixture was diluted with CH_2Cl_2 and the organic layer washed with 1 M $HCl_{(aq)}$ and saturated aqueous $NaHCO_3$, and then dried over $MgSO_4$. The CH_2Cl_2 was removed under reduced pressure and chromatography of the residue on silica gel (eluting with ethyl acetate) afforded the protected amine (1.7 g, 60%) as a colorless oil (R_f 0.3, EtOAc): ^{1}H NMR ∂(400 MHz, $CDCl_3$) 3.00 (1H, br s, *OH*), 3.37 (2H, t, J 4 Hz, CH_2N), 3.51 (2H, t, J 4 Hz, CH_2CH_2N), 3.55-3.57 (1H, d, J 8 Hz, *o*-arom.), 7.74 (2H, d, J 7 Hz, *m*-arom.).

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[2] Ogura, H., Furuhata, K., Itoh, M., and Shitori, Y. (1986) *Carbohydr. Res.*158, 37-51.

2-(2-Carbobenzoxyaminoethoxy(2-ethoxyethoxy))ethanol-p-toluene sulfonate. 2-(2-Carbobenzoxyaminoethoxy(2-ethoxyethoxy))ethanol (1.6 g, 4.8 mmol, 1 equiv) and triethylamine (1 mL, 7.2 mmol, 1.5 equiv) were dissolved in CH_2Cl_2 (19 mL) and cooled to 0 °C. Dimethylaminopyridine (59 mg, 0.48 mmol, 0.1 equiv) and *p*-toluenesulfonyl chloride (0.82 g, 4.8 mmol, 1 equiv) were added in one portion and the mixture was stirred for 3 h while gradually warming to room temperature. The reaction mixture was then diluted with CH_2Cl_2 and the organic layer washed with 1 M $HCl_{(aq)}$, saturated aqueous $NaHCO_3$, brine, and then dried over $MgSO_4$. The solvent was removed under reduced pressure and chromatography of the residue on silica gel (eluting with 80% EtOAc in petroleum ether) afforded the tosylate (2 g, 87%) as a colorless oil (R_f 0.4, 80%EtOAc-petroleum ether): ^1H NMR ∂(300 MHz, $CDCl_3$) 2.43 (3H, s, CH_3), 3.39 (1H, dd, J 11, 6 Hz, CH_2N), 3.50-3.70 (12H, m, CH_2O), 4.10 (2H, dd, J 6 Hz, J' 6 Hz, CH_2OTs), 5.09 (2H, s, $PhCH_2O$), 5.31 (1H, br s, *NH*), 7.28-7.38 (7H, m, arom.), 7.79 (2H, d, J 8 Hz, arom.).

2-(2-Carbobenzoxyaminoethoxy(2-ethoxyethoxy))ethyl azide. 2-(2-Carbobenzoxyaminoethoxy(2-ethoxyethoxy))ethanol-*p*-toluene sulfonate (2 g, 4.2 mmol, 1 equiv) was dissolved in dimethylformamide (16 mL) and sodium azide (2.7 g, 42 mmol, 10 equiv) was added. The solution was stirred for 2 d at room temperature. The dimethylformamide was then removed under reduced pressure and the residue dissolved in EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. Chromatography of the residue on silica gel (eluting with EtOAc) afforded the title azide (1.3 g, 91%) as a colorless oil (R_f 0.3, EtOAc): ^1H NMR ∂(500 MHz, $CDCl_3$), 3.29-3.35 (2H, t, J 6 Hz, CH_2N_3), 3.38 (2H, dd, J 10, 5 Hz, CH_2N), 3.52-3.59(2H, t, J 5 Hz, $N_3CH_2CH_2$), 3.59-3.69 (10H, m, OCH_2), 5.09 (2H, s, $PhCH_2$), 5.29 (1H, br s, *NH*), 7.27-7.40 (5H, m, arom.).

2-(2-Carbobenzoxyaminoethoxy(2-ethoxyethoxy))ethylamine (4). (1.3 g, 3.8 mmol, 1 equiv) was dissolved in THF (10 mL) and solid triphenylphosphine (1.1 g, 4.2 mmol, 1.1 equiv) was added. After 2 min, enough water (approximately 10 mL) was added to the reaction mixture to begin precipitation of the phosphines, and the suspension was stirred vigorously overnight. *The THF was then removed under reduced pressure, and the mixture filtered to remove the* phosphines. The solution was concentrated under reduced pressure and chromatography of the concentrate on Sephadex LH-20 (100 cm x 2.5 cm, eluting with MeOH) afforded the amine (0.96 g, 80%) as a colorless oil (R_f 0.2, butanol: acetic acid: water, 5:3:1)

Coupling of 4 and 1. A solution of the acid sialoside 1 (330 mg, 0.6 mmol, 1 equiv) and 1-hydroxybenzotriazole (97 mg, 0.6 mmol, 1 equiv) in CH_2Cl_2 (2.2 mL) was cooled to 0 °C. Solid dicyclohexylcarbodiimide (115 mg, 0.6 mmol, 1 equiv) was added to the mixture followed by the amine 4 (182 mg, 0.6 mmol, 1 equiv) dissolved in CH_2Cl_2 (0.8 mL). The mixture was stirred for 12 h while gradually warming to room temperature. The reaction mixture was diluted with CH_2Cl_2 *and the dicyclohexylurea, which had precipitated during the course of the reaction,* was removed by filtration. The organic layer was washed with 1 M $HCl_{(aq)}$, brine, and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue chromatographed on silica gel (eluting with a gradient of 1-5% MeOH in CH_2Cl_2) to give the sialoside (330 mg, 66%) as a white foam (R_f 0.5, 10% MeOH in CH_2Cl_2): ^1H NMR ∂(400 MHz, $CDCl_3$) 1.50-1.59 (2H, m, 3'-*H*), 1.59-1.74 (2H, m, 2'-*H*), 1.87 (3H, s, *NAc*), 1.90 (1H, t, J 12 Hz, 3-H_{ax}), 2.00 (3H, s, OAc), 2.01 (3H, s, OAc), 2.11 (3H, s, OAc), 2.12 (3H, s, OAc), 2.14 (2H, t, J 9 Hz, 4'-H), 2.53 (1H, dd, J 13, 5 Hz, 3-H_{eq}), 3.18 (1H, dd, J 12, 4 Hz, 1'-H), 3.31-3.43 (4H, m, NCH_2CH_2), 3.57 (8H, s, OCH_2), 3.70-3.80 (1H, m, 1'-H), 3.76 (3H, s, CO_2CH_3), 3.98-3.41 (3H, m, 5-H, 6-H, 9-H), 4.30 (1H, dd, J 12, 2 Hz, 9-H), 4.75-4.83 (1H, m, 4-H), 5.07 (2H, s, $PhCH_2$), 5.20 (1H, br d, J 10 Hz, *NH*), 5.29 (1H, d, J 9 Hz, 7-H), 5.34-5.45 (2H, m, 8-H, *NH*), 6.22 (1H, br s, *NH*), 7.26-7.38 (5H, m, arom.).

Preparation of 5. A suspension of the coupled glycoside (300 mg, 0.33 mmol) and 20% $Pd(OH)_2$ on charcoal (30 mg) in ethanol (10 mL) was degassed and a hydrogen atmosphere introduced to the reaction *via* a balloon. After 1 h the reaction mixture was filtered through celite to remove the catalyst and the filtrate was concentrated under reduced pressure. The residue was chromatographed on Sephadex LH-20 (100 x 2.5 cm, eluting with MeOH) to afford the amine (5) (244 mg, 84%) as a colorless oil (R_f 0.2, butanol: acetic acid: water, 5:3:1): ^1H NMR ∂(400 MHz, $CDCl_3$), 1.52-1.62 (2H, m, 3'-H), 1.62-1.79 (2H, m, 2'-H), 1.88 (3H, s, NAc), 1.95 (1H, t, J 13 Hz, 3-H_{ax}), 2.02 (3H, s, OAc), 2.03 (3H, s, OAc), 2.15 (3H, s, OAc), 2.15 (3H, s, OAc), 2.22 (2H, t, J 7 Hz, 4'-H), 2.56 (1H, dd, J 13, 5, Hz, H-3$_{eq}$), 2.94-3.00 (2H, m, CH_2NH_2), 3.22 (1H, dt, J 9, 7 Hz, 1'-H), 3.49 (2H, br s, NH_2), 3.49-3.50 (2H, m, CH_2N), 3.51-3.60 (4H, m, NCH_2CH_2), 3.60-3.70 (8H, m, OCH_2), 3.70-3.80 (1H, m, 1'-H), 3.79 (3H, s, CO_2CH_3), 4.01-4.10 (3H, m, 5-H, 6-H, 9-H), 4.81 (1H, dd, J 13, 2 Hz, 9-H), 4.79-4.88 (1H, m, 4-H), 5.20 (1H, br d, J 9 Hz, *NH*), 5.30 (1H, d, J 8 Hz, 7-H), 5.39 (1H, ddd, J 8, 6, 2 Hz, 8-H), 5.84 (1H, br s, *NH*).

Preparation of P(4,4). A solution of phenylene 1,3-diacetic acid (27 mg, 0.14 mmol, 1 equiv) in dimethylformamide (0.25 mL) was cooled to -20 °C. Diphenylphosphoryl azide (75 μL, 0.34 mmol, 2.4 equiv) was added to the reaction followed by the amine (5) (214 mg, 0.28 mmol, 2 equiv) in dimethylformamide (0.5 mL). The pH of the mixture was adjusted to 7.5 (using moist pH paper) by the dropwise addition of triethylamine (approximately 100 μL, 5 equiv). The reaction mixture was stirred for 2 d at -20 °C, 1 d at 0 °C, and 1 d at room temperature. The dimethylformamide was then removed under reduced pressure and 1 M $NaOH_{(aq)}$ (2 mL) and MeOH (2 mL) were added to the residue. This mixture was stirred for 2 h at room temperature. The reaction mixture was then acidified to pH 2.5 by the addition of Dowex-50W (H^+ form). The Dowex resin was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was chromatographed on Sephadex G-25 (100 cm x 2.5 cm, eluting with H_2O) and the fractions containing product were pooled and lyophilized to give a white foam (186 mg). *P(4,4) was further purified by preparative reverse phase HPLC (eluting with a linear gradient of* 0.1% trifluoroacetic acid (TFA) in H_2O to 60% MeOH in 0.1% $TFA_{(aq)}$ over 80 min at a flow rate of 5 mL min^{-1}) to afford the bivalent sialoside P(4,4) (45 mg, 24%) as a white foam after lyophilization: ^1H NMR ∂(500 MHz, D_2O) 1.52-1.65 (8H, m, 2'-H, 3'-H), 1.78 (2H, t, J 14 Hz, 3-H_{ax}), 2.03 (6H, s, NAc), 2.25 (2H, t, J 7 Hz, 4'-H), 2.70 (2H, dd, J 14, 5 Hz, 3-H_{eq}), 3.38 (8H, dt, J *13, 5 Hz, CH_2N), 3.50 (2H, dt, J 9, 7 Hz, 1'-H), 3.55 (2H, d, J 9 Hz, 7-H), 3.56-3.67 (26H, m,* CH_2O, 9-H, 8-H), 3.71-3.83 (6H, m, 1'-H, 5-H, 9-H), 3.83-3.89 (4H, m, 4-H, 6-H), 7.21 (1H, s, *o*-arom.), 7.25 (2H, d, J 9 Hz, *p*-arom.), 7.39 (1H, t, J 9 Hz, *m*-arom.). FAB MS (glycerol/thioglycerol) (*m/z*): 1348 (32%) $(M+Na)^+$,.1326 (36%) $(MH)^+$.

P(3,3): ^1H NMR ∂(500 MHz, D_2O) 1.52-1.63 (8H, m, 2'-H, 3'-H), 1.73 (2H, t, J 14 Hz, 3-H_{ax}), 2.01 (6H, s, NAc), 2.23 (2H, t, J 7 Hz, 4'-H), 2.68 (2H, dd, J 14, 5 Hz, 3-H_{eq}), 3.37 (8H, dt, J 13, 5 Hz, CH_2N), 3.48 (2H, dt, J 9, 7 Hz, 1'-H), 3.52-3.66 (22H, m, CH_2O, 7-H, 8-H, 9-H), 3.69-3.79 (6H, m, 1'-H, 5-H, 9-H), 3.70-3.88 (4H, m, 4-H, 6-H), 7.20 (1H, s, *o*-arom.), 7.23 (2H, d, J 9 Hz, *p*-arom.), 7.35 (1H, t, J 9 Hz, *m*-arom.). FAB MS (glycerol/thioglycerol) (*m/z*): 1260 (10%) $(M+Na)^+$,.1238 (23%) $(MH)^+$.

P(5,5): ^1H NMR ∂(500 MHz, D_2O) 1.52-1.63 (8H, m, 2'-H, 3'-H), 1.78 (2H, t, J 14 Hz, 3-H_{ax}), 2.03 (6H, s, NAc), 2.23 (2H, t, J 7 Hz, 4'-H), 2.69 (2H, dd, J 14, 5 Hz, 3-H_{eq}), 3.37 (8H, dt, J 13, 5 Hz, CH_2N), 3.51 (2H, dt, J 9, 7 Hz, 1'-H), 3.57 (2H, d, J 9 Hz, 7-H), 3.58-3.67 (36H, m, CH_2O, 9-H, 8-H), 3.71-3.89 (10H, m, 1'-H, 4-H, 5-H, 6-H, 9-H), 7.20 (1H, s, *o*-arom.), 7.23 (2H, d, J 9 Hz, *p*-arom.), 7.37 (1H, t, J 9 Hz, *m*-arom.). FAB MS (glycerol/thioglycerol) (*m/z*): 1436 (41%) $(M+Na)^+$,.1414 (53%) $(MH)^+$.

General procedure for the preparation of the G-series. The synthesis of G(4,4) is illustrated in Scheme III.

1,3-bis-[4-(Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-nonulopyranosid)onate-2-oxy)butylcarbonylaminomethyl] benzene (octa-acetyl G(0,0)). A solution of 1 (146 mg, 0.25 mmol, 2 equiv) and 1-hydroxybenzotriazole (38 mg, 0.25 mmol, 2 equiv) in CH_2Cl_2 (1 mL) was cooled to 0 °C. Dicyclohexylcarbodiimide (51 mg, 0.25 mmol, 2 equiv) was added, followed by 1,3-bis(aminomethyl)benzene (0.16 μL, 0.12 mmol, 1 equiv). After 2 h the reaction mixture was filtered to remove the dicyclohexylurea which had precipitated during the course of the reaction, and the organic layer was washed with 1 M citric acid solution, *saturated aqueous $NaHCO_3$, brine, and then dried over $MgSO_4$. Filtration and concentration of* the filtrate under reduced pressure followed by chromatography on silica gel (eluting with a gradient of 5-10% MeOH in CH_2Cl_2) afforded the title diamide (130 mg, 81%) as a white foam (R_f 0.3, 10% MeOH in CH_2Cl_2).

Preparation of G(0,0). To a solution of octa-acetyl G(0,0) (130 mg, 0.1 mmol) in methanol (2 mL) was added 1 M $NaOH_{(aq)}$ (5 mL). The mixture was stirred for 1 h at room temperature and then acidified to pH 2.5 by the addition of Dowex-50W (H^+ form). The Dowex resin was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was chromatographed on Sephadex G-25 (100 cm x 2.5 cm, eluting with H_2O) and the fractions that *contained the product were pooled and lyophilized to give G(0,0) as a white foam (81 mg,* 87%). (R_f 0.4, butanol:acetic acid water, 5:3:1): ^1H NMR ∂(500 MHz, D_2O) 1.58-1.68 (10H, m, 2'-H, 3'-H and 3-H_{ax}), 2.02 (6H, s, NAc), 2.32 (4H, t, J 7.4 Hz, 4'-H), 2.71 (2H, dd, J 12.5, 4.7 Hz, 3-H_{eq}), 3.46 (2H, m, 1'-H), 3.57 (2H, d, J, 9 Hz, 7-H), 3.62 (2H, dd, J 12, 6 Hz, 9-H), 3.64-3.71 (4H, m, 8-H, 4-H), 3.74-3.78 (2H, m, 1'-H), 3.83 (2H, d, J 6 Hz, 5-H), 3.78-3.87 (2H, m, 6-H), 4.37 (4H, s, $PhCH_2$), *7.19 (1H, s, o-arom.), 7.22 (2H, d, J 8 Hz, p-arom.), 7.37 (1H, t, J 8 Hz, m-arom.). FAB* MS (glycerol/thioglycerol) (*m/z*): 919 (18%) (MH^+).

1,3-bis[N-(N-tert-butoxycarbonylglycyl)aminomethyl]benzene. A solution of 1,3-bis(aminomethyl)benzene (0.95 mL, 7 mmol, 1 equiv) in CH_2Cl_2 and dimethylformamide (25 mL, 1:1) was cooled to 0 °C. *N,N*-Diisopropylethylamine was added (2.6 mL, 15 mmol, 2.1 equiv) followed by *N-tert*-butoxycarbonylglycine *N*-hydroxysuccinimide ester (2.6 g, 15 mmol, 2.1 equiv). The mixture was stirred overnight while gradually warming to room temperature. The* solvent was then removed under reduced pressure and the residue was diluted with CH_2Cl_2. The organic layer was washed with 1 M citric acid solution, saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and then concentrated under reduced pressure. Chromatography of the residue on silica gel (eluting with EtOAc) afforded the title compound (3.0 g, 94%) as a white foam (R_f 0.3, EtOAc): ^1H NMR ∂(400 MHz, $CDCl_3$) 1.39 (18H, s, *tert*-Bu), 3.78 (4H, d, J 4 Hz, NCH_2), 4.32 (4H, J 4 Hz, NCH_2Ph), 5.63 (2H, br s, *NH*), 6.90 (2H, br s, *NH*), 7.08 (1H, s, *o*-arom.), 7.15 (2H, d, J 8 Hz, *p*-arom.), 7.21 (1H, t, J 8 Hz, *m*-arom.).

1,3-bis(glycylaminomethyl)benzene trifluoroacetate salt. A solution of 1,3-bis[*N*-(*N-tert*-butoxycarbonylglycyl)aminomethyl]benzene (3.0 g, 6.6 mmol) in CH_2Cl_2 (3 mL) was cooled to 0 °C and trifluoroacetic acid (10 mL) was added. The homogeneous mixture was stirred for 2 h at 0 °C. The solvent and acid were then removed under reduced pressure and the oily yellow residue was concentrated five times from CH_2Cl_2. The residue was then triturated with diethyl ether to afford the title salt as a white solid (2.6 g, 87%). (R_f 0.5, butanol:acetic acid:water, 5:3:1). This product was used without further purification: ^1H NMR ∂(400 MHz, D_2O) 3.80 (4H, s, CH_2NH_2), 4.38 (4H, s, $PhCH_2$), 7.20 (1H, s, *o*-arom.), 7.21 (2H, d, J 4 Hz, *p*-arom.), 7.35 (1H, t, J 4 Hz, *m*-arom.).

1,3-bis[N-(N-tert-butoxycarbonylglycylglycyl)aminomethyl]benzene. A solution of 1,3-bis(glycylaminomethyl)benzene trifluoroacetate salt (1.35 g, 2.8 mmol) in CH_2Cl_2 and dimethylformamide (10 mL, 1:1) was cooled to 0 °C. *N,N*-Diisopropylethylamine was added (0.98 mL, 5.6 mmol, 2.0 equiv) followed by *N-tert*-butoxycarbonylglycine *N*-hydroxysuccinimide ester (1.53 g, 5.6 mmol, 2.0 equiv). The mixture was stirred overnight while gradually warming to room temperature. The solvent was then removed under reduced pressure and the residue was diluted with CH_2Cl_2. The organic layer was washed with 1 M citric acid solution, saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. Chromatography of the residue on silica gel (eluting with 10% MeOH in CH_2Cl_2) afforded the title compound (1.2 g, 75%) as a white foam (R_f 0.3, 10% MeOH in CH_2Cl_2): ^1H NMR ∂(400 MHz, DMSO) 1.36 (18H, s, *tert*-Bu), 3.57 (4H, d, 6 Hz, NCH_2CO), 3.73 (4H, d, J 5 Hz, NCH_2CO), 4.25 (4H, d, J 6 Hz, $PhCH_2$), 7.04 (2H, br t, J 5 Hz, *NH*), 7.11 (3H, d, J 9 Hz, *o,p*-arom.), 7.23 (1H, t, J 9 Hz, *m*-arom.), 7.94 (2H, s, *NH*), 8.12 (2H, br s, *NH*), 8.29 (2H, br s, *NH*).

1,3-bis(glycylglycylaminomethyl)benzene trifluoroacetate salt (7). A solution of 1,3-bis[*N*-(*N-tert*-butoxycarbonylglycylglycyl)aminomethyl]benzene (1.2 g, 2.1 mmol) in CH_2Cl_2 (1 mL) was cooled to 0 °C and trifluoroacetic acid (10 mL) was added. The homogeneous mixture was stirred for 2 h at 0 °C. The solvent was removed under reduced pressure and the excess *trifluoroacetic acid was evaporated as an azeotrope with CH_2Cl_2. The residue was then* triturated with diethyl ether to afford 7 as a white solid (1.1 g, 95%) (R_f 0.5, butanol:acetic acid:water, 5:3:1): ^1H NMR ∂(400 MHz, D_2O) 3.31 (1H, s, *NH*), 3.86 (4H, s, NCH_2CO), 4.00 (4H, s, NCH_2CO), 4.39 (4H, s, $PhCH_2$), 7.13-7.20 (3H, m, arom.), 7.35 (1H, t, J 8 Hz, *o*-arom.).

*1,3-bis[*N*-(*N*-tert-butoxycarbonylglycylglycylglycylglycyl)aminomethyl]benzene.* A solution of 7 (1.2 g, 2.0 mmol, 1 equiv) in CH_2Cl_2 and dimethylformamide (10 mL, 1:1) was cooled to 0 °C. *N,N*-Diisopropylethylamine was added (0.72 mL, 4.0 mmol, 2.0 equiv) followed by *N-tert*-butoxycarbonylglycine *N*-hydroxysuccinimide ester (1.1 g, 4.0 mmol, 2.0 equiv). The mixture was stirred overnight while gradually warming to room temperature. The solvent was then removed under reduced pressure and the residue was diluted with CH_2Cl_2. The organic layer was washed with 1 M citric acid solution, saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and then concentrated under reduced pressure. Chromatography of the residue on silica gel (eluting with 20% MeOH in CH_2Cl_2) afforded the title compound (1.0 g, 70%) as a white foam (R_f 0.3, 20% MeOH in CH_2Cl_2).

1,3-bis[glycylglycylglycylaminomethyl]benzene trifluoroacete salt. A solution of 1,3-bis[*N*-(*N-tert*-butoxycarbonylglycylglycylglycyl)aminomethyl] benzene (0.8 g, 1.2 mmol) in CH_2Cl_2 (1 mL) was cooled to 0 °C and trifluoroacetic acid (10 mL) was added. The homogeneous mixture was stirred for 2 h at 0 °C. The solvent was removed under reduced pressure and the excess trifluoroacetic acid was evaporated as an azeotrope with CH_2Cl_2. The residue was then triturated with diethyl ether to afford a white solid (0.75 g, 90%), (R_f 0.5, butanol:acetic acid:water, 5:3:1): ^{1}H NMR ∂(500 MHz, D_2O) 3.90 (4H, s, NCH_2CO), 3.99 (4H, s, NCH_2CO), 4.05 (4H, s, NCH_2CO), 4.41 (4H, s, PhCH_2), 7.19-7.23 (3H, m, arom.), 7.36-7.40 (1H, m, *o*-arom.).

*Methyl [4′-(benzyl-*N*-glycylglycinate)carboxamidobutyl]-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-*D*-glycero-α-*D*-galacto-*nonulopyranosid]onate.* A solution of 1 (230 mg, 0.39 mmol), glycylglycine benzyl ester *p*-toluenesulfonate salt (146 mg, 0.39 mmol), and 1-hydroxybenzotriazole (60 mg, 0.39 mmol) in CH_2Cl_2 (1.5 mL) was cooled to 0 °C and dicyclohexylcarbodiimide (80 mg, 0.39 mmol 1 equiv) was added. After 2 min *N,N*-diisopropylethylamine (68 µL, 0.39 mmol) was added and the mixture was stirred overnight while gradually warming to room temperature. The dicyclohexylurea that had precipitated during the course of the reaction was removed by filtration and the organic layer was washed with 1 M citric acid solution, saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. Chromatography of the residue on silica gel (eluting with a gradient of MeOH (1-6%) in CH_2Cl_2) afforded the title sialoside (230 mg, 75%) as a white foam (R_f 0.5, 7% MeOH in CH_2Cl_2): ^{1}H NMR ∂(400 MHz, $CDCl_3$) 1.51-1.65 (2H, m, 3′-H), 1.65-1.78 (2H, m, 2′-H), 1.85 (3H, s, NAc), 1.92 (1H, t, J 12 Hz, 3-H_{ax}), 2.00 (6H, s, OAc), 2.11 (6H, s, OAc), 2.28 (2H, t, J 8 Hz, 4′-H), 2.52 (1H, dd, J 12, 5 Hz, 3-H_{eq}), 3.19-3.24 (1H, m, 1′-H), 3.69-3.78 (1H, m, 1′-H), 3.78 (3H, s, CO_2CH_3), 3.94 (2H, t, J 7 Hz, NCH_2CO), 3.99 (1H, dd, J 14, 8 Hz, 9-H), 4.02-4.08 (5H, m, 5-H, 6-H, 9-H, NCH_2CO), 4.30 (1H, dd, J 12, 4 Hz, 9-H), 4.77-4.83 (1H, m, 4-H), 5.09 (1H, br s, 7-H), 5.15 (2H, s, PhCH_2), 5.25 (1H, br d, J 9 Hz, NHAc), 5.38-5.41 (1H, m, 8-H), 6.65 (1H, br s, N*H*), 6.72 (1H, br s, N*H*), 7.5 (5H, br s, arom.).

*Methyl [4′-glycylglycinecarboxamidobutyl]-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-*D*-glycero-α-*D*-galacto-nonulopyranosid]onate.* A suspension of the methyl [4′-(benzyl-*N*-glycylglycinate}carboxamidobutyl]-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-*D*-glycero-α-*D*-galacto-nonulopyranosid]onate (230 mg, 0.29 mmol) and 20% Pd(OH)$_2$ on carbon (30 mg) in ethanol (3 mL) was degassed and a hydrogen atmosphere introduced to the reaction *via* a balloon. After 20 min the reaction mixture was filtered through celite to remove the catalyst and the filtrate was concentrated under reduced pressure to give a white foam. The residue (180 mg, 88%) was used without further purification (R_f 0.6, butanol: acetic acid: water, 5:3:1): ^{1}H NMR ∂(400 MHz, $CDCl_3$) 1.59-1.69 (2H, m, 3′-H), 1.69-1.85 (2H, m 2′-H), 1.90 (3H, s, NAc), 1.99 (1H, t, 14 Hz, 3-H_{ax}), 2.02 (3H, s, OAc), 2.04 (3H, s, OAc), 2.13 (3H, s, OAc), 2.18 (3H, s, OAc), 2.30-2.38 (2H, m, 4′-H), 2.54 (1H, dd, J 5 Hz, 3-H_{eq}), 3.21-3.29 (1H, m, 1′-H), 3.70 (2H, s, NCH_2CO), 3.65-3.80 (1H, m, 1′-H), 3.97-4.10 (7H, m, NCH_2CO, 5-H, 6-H, 9-H), 4.33 (1H, dd, J 11, 2 Hz, 9-H), 4.78-4.85 (1H, m, 8-H), 6.80 (1H, br t, J 7 Hz, N*H*), 7.03 (1H, br t, J 7 Hz, N*H*).

Preparation of G(4,4). A solution of 6 (165 mg, 0.23 mmol, 2 equiv) in dimethylformamide (1.5 mL) was cooled to -25 °C and diphenylphosphoryl azide (65 µL, 0.29 mmol, 2.4 equiv) was added dropwise. A solution of triethylamine (74 µL, 0.54 mmol, 4.5 equiv) and 7 (70 mg, 0.12 mmol, 1 equiv) in dimethylformamide, precooled to -5 °C (0.5 mL), was then added dropwise over 5 min. After 2 hr the reaction mixture had formed a thick gel and more dimethylformamide (0.5 mL) was added. Stirring was continued at -25 °C overnight, for 1 d at 4 °C, and 1 d at room temperature. The dimethylformamide was then removed under reduced pressure and 1 M $NaOH_{(aq)}$ (5 mL) and MeOH (5 mL) were added to the residue and stirred for 2 h at room temperature. The reaction mixture was acidified to pH 2.5 by the addition of Dowex-50W (H+ form). The Dowex resin was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was chromatographed on Sephadex G-25 (100 cm x 2.5 cm, eluting with H_2O) and the fractions containing product were pooled and lyophilized to give a white foam (186 mg). **G(4,4)** was further purified by preparative reverse phase HPLC (eluting with a linear gradient of 0.1% TFA in H_2O to 15% MeOH in 0.1% $TFA_{(aq)}$ over 70 min at a flow rate of 4 mL min^{-1}) to afford G(4,4) (47 mg, 33%) as a white foam after lyophilization: ^{1}H NMR ∂(400 MHz, D_2O) 1.56-1.67 (8H, m, 2′-H, 3′-H), 1.71 (2H, t, J 14 Hz, 3-H_{ax}), 2.02 (6H, s, NAc), 2.32 (4H, t, J 9 Hz, 4′-H), 2.70 (2H, dd, J 14, 4 Hz, 3-H_{eq}), 3.49 (2H, dt, J 11, 8 Hz, 1′-H), 3.57 (2H, d, J 10 Hz, 7-H), 3.62 (2H, dd, J 12, 8 Hz, 9-H), 3.70 (2H, dd, J 11, 4 Hz, 8-H), 3.72-3.79 (4H, m, 9-H, 1′-H), 3.81-3.87 (6H, m, 4-H, 5-H, 6-H), 3.91 (4H, s, NCH_2CO), 3.95 (4H, s, NCH_2CO), 3.97 (4H, s, NCH_2CO), 3.98 (4H, NCH_2CO), 4.40 (4H, s, PhCH_2), 7.16 (1H, s, *o*-arom.), 7.20 (2H, d, J 9 Hz, *p*-arom.), 7.37 (1H, t, J 9 Hz, *m*-arom.). FAB MS (glycerol/thioglycerol) (*m/z*): 1376 (41%) (MH$^+$).

G(1,1): ^{1}H NMR ∂(300 MHz, D_2O) 1.48-1.7 (8H, m, 2′-H, 3′-H), 1.68 (2H, t, J 13 Hz, 3-H_{ax}), 2.00 (6H, s, NAc), 2.32 (4H, t, J 8 Hz, 4′-H), 2.69 (2H, dd, J 13, 5 Hz, 3-H_{eq}), 3.42 (2H, dt, J 11, 8 Hz, 1′-H), 3.55 (2H, dd, J 10, 5 Hz, 7-H), 3.60-3.88 (14H, 1′-H, 4-H, 5-H, 6-H, 8-H, 9-H), 3.90 (4H, s, NCH_2CO), 4.39 (4H, s, PhCH_2), 7.15 (1H, s, *o*-arom.), 7.19 (2H, d, J 9 Hz, *p*-arom.), 7.36 (1H, t, J 9 Hz, *m*-arom.). FAB MS (glycerol/thioglycerol) (*m/z*): 1055 (33%) (M+Na)$^+$, 1033 (27%) (MH)$^+$.

G(2,2): ^{1}H NMR ∂(500 MHz, D_2O) 1.55-1.65 (8H, m, 2′-H, 3′-H), 1.63 (2H, t, J 13 Hz, 3-H_{ax}), 2.06 (6H, s, NAc), 2.34 (4H, t, J 8 Hz, 4′-H), 2.74 (2H, dd, J 13, 5 Hz, 3-H_{eq}), 3.44-3.50 (2H, m, 1′-H), 3.60 (2H, d, J 8, Hz, 7-H), 3.65 (2H, dd, J 12, 6 Hz, 9-H), 3.70-3.80 (6H, m, 1′-H, 8-H, 9-H), 3.80-3.90 (6H, m, 4-H, 5-H, 6-H), 3.95 (4H, s, NCH_2CO), 4.00 (4H, s, NCH_2CO), 4.44 (4H, s, PhCH_2), 7.18 (1H, s, *o*-arom.), 7.23 (2H, d, J 8 Hz, *p*-arom.), 7.40 (1H, t, J 9 Hz, *m*-arom.). FAB MS (glycerol/thioglycerol) (*m/z*): 1170 (15%) (M+Na)$^+$, 1148 (22%) (MH)$^+$.

G(3,3): ^{1}H NMR ∂(400 MHz, D_2O) 1.58-1.67 (8H, m, 2′-H, 3′-H), 1.73 (2H, t, J 13 Hz, 3-H_{ax}), 2.06 (6H, s, NAc), 2.34 (4H, t, J 8 Hz, 4′-H), 2.74 (2H, dd, J 13, 5 Hz, 3-H_{eq}), 3.50-3.53 (2H, m, 1′-H), 3.59 (2H, dd, J 8, 2 Hz, 7-H), 3.66 (2H, dd, J 12, 6 Hz, 9-H), 3.70-3.80 (6H, m, 1′-H, 8-H, 9-H), 3.84-3.90 (6H, m, 4-H, 5-H, 6-H), 3.96 (4H, s, NCH_2CO), 4.00 (4H, s, NCH_2CO), 4.01 (4H, s, NCH_2CO), 4.44 (4H, s, PhCH_2), 7.20 (1H, s, *o*-arom.), 7.23 (2H, d, J 8 Hz, *p*-arom.), 7.40 (1H, t, J 9 Hz, *m*-arom.). FAB MS (glycerol/thioglycerol) (*m/z*): 1284 (19%) (M+Na)$^+$, 1262 (15%) (MH)$^+$.

General procedure for the preparation of the U-series. The synthesis of ***U(3,3)′*** is illustrated in Scheme IV.

*N-Carbobenzoxy-*N*-formyl-piperazine.* To a solution of piperazine-1-carboxaldehyde (500 µL, 4.85 mmol, 1 equiv), 4-dimethylaminopyridine (59 mg, 0.49 mmol, 0.1 equiv) and triethylamine (2.02 mL, 14.5 mmol, 3 equiv) in CH_2Cl_2 (20 mL) at room temperature under nitrogen was added benzylchloroformate (76 µL, 5.34 mmol, 1.1 equiv) dropwise with stirring. The mixture was stirred overnight at room temperature and then quenched by the addition of saturated aqueous $NaHCO_3$. The product was extracted into CH_2Cl_2 and the combined extracts washed with saturated aqueous NH_4Cl then dried over $MgSO_4$. Removal of the solvent under reduced pressure and chromatography on silica gel (eluting with EtOAc) gave the title compound as a colorless oil (1.19 g, 99%): ^{1}H NMR ∂(300 MHz, $CDCl_3$) 3.36 (2H, br s, 3-H, 5-H), 3.50-3.55 (6H, m, 2-H, 3-H_{eq}, 5-H, 6-H), 5.15 (2H, s, PhCH_2), 7.33-7.38 (5H, m, arom.), 8.07 (1H, s, C*H*O).

N-Carbobenzoxypiperazine. A solution of *N*-carbobenzoxy-*N*-formyl-piperazine (28 mg, 113 µmol) in 1:20 c.HCl:MeOH (10 mL) was stirred overnight at room temperature. After cooling to 0 °C the reaction was quenched with saturated aqueous $NaHCO_3$ and the product extracted into CH_2Cl_2. The combined organic layers were dried over $MgSO_4$. Removal of the drying agent and evaporation of the solvent gave a colorless oil (25 mg, 113 µmol, 99%) (R_f 0.18, 10% MeOH in CH_2Cl_2): ^{1}H NMR ∂(300 MHz, $CDCl_3$); 1.75 (1H, s, N*H*), 2.82 (4H, br s, 3-H, 5-H), 3.47 (4H, t, J 5 Hz, 2-H, 6-H), 5.13 (2H, s, PhCH_2), 7.37 (5H, br s, arom.).

*N-Carbobenzoxy-*N′*-formyl-bis-piperazyl urea.* To a solution of phosgene (1.93 M in toluene, 165 µL, 0.32 mmol, 1 equiv) and triethylamine (134 µL, 0.96 mmol, 3 equiv) in CH_2Cl_2 (4 mL) at -78 °C under argon, was added a solution of piperazine-1-carboxaldehyde (33 µL, 0.32 mmol, 1 equiv) in CH_2Cl_2 (4 mL) dropwise over 50 min. The solution turned a deep yellow color. The reaction mixture was stirred at -78 °C for 20 min. A solution of 1-carbobenzoxypiperazine (70 mg, 0.32 mmol, 1 equiv) in CH_2Cl_2 (2 mL) was added dropwise over 10 min and the reaction mixture allowed to warm slowly to room temperature over 3.5 h. Saturated aqueous $NaHCO_3$ was then added and the product was extracted into CH_2Cl_2. The combined extracts were washed with saturated aqueous NH_4Cl and dried over $MgSO_4$. Removal of the solvent under reduced pressure and chromatography on silica gel (eluting with a gradient of MeOH (5-10%) in CH_2Cl_2) gave the product urea as a colorless foam (100 mg, 67 %) (R_f 0.69,10% MeOH in CH_2Cl_2): ^{1}H NMR ∂(300 MHz, $CDCl_3$) 3.11-3.32 (8H, m, 3-H, 5-H, 3′-H, 5′-H), 3.32-3.43 (2H, m, 2′-H, 6′-H), 3.45-3.68 (6H, m, 2-H, 2′-H, 6-H, 6′-H), 5.15 (2H, s, PhCH_2), 7.35 (5H, s, arom.), 8.06 (1H, s, C*H*O).

N-Carbobenzoxy-bis-piperazyl urea (8). A solution of *N-carbobenzoxy-*N′*-formyl-bis-piperazyl urea* (115 mg, 0.32 mmol) in 0.05 M methanolic HCl (4 mL) was allowed to stand at room temperature overnight. Saturated aqueous $NaHCO_3$ was then added and the product was extracted into CH_2Cl_2. The combined extracts were dried over $MgSO_4$. Removal of the solvent and chromatography on silica gel (eluting with a gradient of MeOH (10-20%) in CH_2Cl_2) gave the amine as a colorless oil (87 mg, 87%) (Rf 0.24, 10% MeOH in CH_2Cl_2): ^{1}H NMR ∂(300 MHz, $CDCl_3$) 2.28 (1H, br s, N*H*), 2.88 (4H, dd, J 5, 5 Hz, 2′-H, 6′-H), 3.12-3.32 (8H, m, 3-H, 5-H, 3′-H, 5′-H), 3.45-3.55 (4H, m, 2-H, 6-H), 5.14 (2H, s, PhCH_2), 7.35 (5H, s, arom.).

*Benzene-1,3-bis(*N*-carbobenzoxy-bis-piperazyl urea carboxamide) (9).* To a solution of *N*-carbobenzoxy-bis-piperazyl urea (8) (712 mg, 2.14 mmol, 2 equiv) and triethylamine (895 µl, 6.42 mmol, 6 equiv) in dry dimethylformamide (8 mL) under argon was added a solution of isophthaloyl dichloride (217 mg, 1.07 mmol 1 equiv) in dry dimethylformamide (4 mL), dropwise. The mixture was yellow and milky. After stirring the mixture overnight at room temperature, the solvents were removed by evaporation, and the residue was purified by chromatography on silica gel (eluting with 5% MeOH in CH_2Cl_2) to give 9 as a white solid (621 mg, 73 %): ^{1}H NMR ∂(400 MHz, $CDCl_3$) 3.15-3.55 (30H, br m, piperazine CH_2 groups including 2-H, 6-H, 2′-H, 6′-H), 3.79 (4H, br s, 2-H, 6-H, 2′-H, 6′-H), 5.15 (4H, s, CH_2Ph), 7.30-7.50 (10H, m, arom.).

Hydrogenolysis of 9. Compound 9 (621 mg, 0.782 mmol) was dissolved in MeOH:AcOH:H_2O (20:1:2, by volume) containing 20% Pd(OH)$_2$ on charcoal (130 mg) and the flask charged with an atmosphere of hydrogen. The mixture was stirred for 2 h at room temperature. After replacement of the hydrogen with air, the reaction mixture was filtered through celite and the solvent was removed under reduced pressure. The acetate salt was obtained as a white solid which was used without further purification.

Preparation of U(2,2). To a solution of 1-hydroxybenzotriazole (168 mg, 1.24 mmol, 2.5 equiv) and dicyclohexylcarbodiimide (256 mg, 1.24 mmol, 2.5 equiv) in CH_2Cl_2 under argon at room temperature was added a solution of 1 (530 mg, 0.97 mmol, 2 equiv) in dry CH_2Cl_2 (10 mL + 2 mL rinse) followed by *N*-ethylmorpholine (473 µL, 3.72 mmol, 6 equiv). The mixture was stirred for 15 min at room temperature, and a solution of deprotected 9 (300 mg, 460 µmol, 1 equiv) in CH_2Cl_2 (6 mL) was then added dropwise. The reaction mixture was stirred overnight at room temperature. The reaction was then quenched by the addition of methanol (2 mL), followed by 1 M $HCl_{(aq)}$. The product was extracted into CH_2Cl_2 and the combined organic layers washed with 1 M $HCl_{(aq)}$ and brine, then dried over $MgSO_4$. Chromatography on silica gel (eluting with 10% MeOH in CH_2Cl_2) gave a white solid. This product was dissolved in MeOH and cooled to 0 °C. 1 M $NaOH_{(aq)}$ (600 µL) was added dropwise over 10 min. The mixture was then warmed to room temperature over 2.5 h. The reaction was quenched by the addition of Dowex-50W (H+ form) and the resin removed by filtration after 15 min. Removal of the solvent by evaporation gave a white residue that was passed through a column of Sephadex G-25 (100 cm x 2.5 cm, eluting with H_2O). The fractions that contained the required product were combined and lyophilized. The residue was purified by reverse phase HPLC (eluting with 30% MeOH in 0.1% $TFA_{(aq)}$) to give U(2,2) (12 mg, 2% from 9): ^{1}H NMR ∂(500 MHz, D_2O) 1.59 (8H, br s, 2′-H, 3′-H), 1.70 (2H, t, J 12 Hz, 3-H_{ax}), 2.05 (6H, s, NAc), 2.40-2.59 (4H, m, 4′-H), 2.66 (2H, dd, J 13, 5 Hz, H-3$_{eq}$), 3.67-3.87 (48H, m, NCH_2, 4-H, 5-H, 6-H, 7-H, 8-H, 9-H), 3.47 (1H, s, *o*-arom.), 7.65-7.74 (4H, m, arom.). FAB MS (nitrobenzylalcohol) (*m/z*): 1332 (50%) (M+Na$^+$), 1310 (100%) (MH$^+$).

N-Carbobenzoxy-N'-formyl-tris-piperazyl-bis-urea. To a solution of triphosgene (373 mg, 1.32 mmol, 0.3 equiv) in CH_2Cl_2 (150 mL) at -78 °C under argon was added a cold (-78 °C) solution of triethylamine (3 mL, 2 mmol, 6 equiv) and piperazine-1-carboxaldehyde (386 mL, 3.76 mmol, 1 equiv) in CH_2Cl_2 (15 mL) under argon, over 2 h. The reaction was stirred for a further 30 min at -78 °C. A solution of 8 (1.25 g, 3.76 mmol, 1 equiv) in CH_2Cl_2 (50 mL) at -78 °C under argon was added rapidly over 30 min. The mixture was stirred for a further 1 h at -78 °C then allowed to warm slowly to 4 °C and stirred for a further 14 h. The reaction was then quenched with saturated aqueous $NaHCO_3$ and the product extracted into CH_2Cl_2. The combined extracts were washed with saturated aqueous NH_4Cl then brine and dried over $MgSO_4$. The product was purified by chromatography on silica gel (eluting with 5% MeOH in CH_2Cl_2) to give a white solid (1.14 g, 64%) (R_f 0.4, 10% MeOH in CH_2Cl_2): ^1H NMR ∂(400 MHz, $CDCl_3$) 3.23-3.31 (16H, m, piperazine CH_2), 3.39-3.42 (2H, m, *H*CNCHO), 3.51-3.53 (4H, m, *H*CNCbz), 3.53-3.58 (2H, m, *H*CNCHO), 5.15 (2H, s, CH_2Ph), 7.34-7.37 (5H, m, arom.), 8.08 (1H, s, *CH*O).

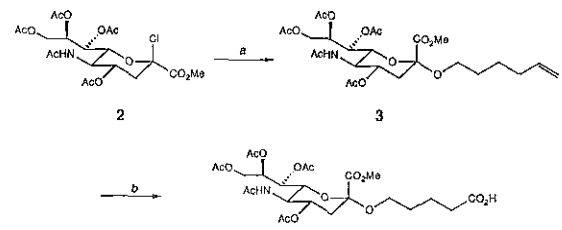
N-Carbobenzoxy-tris-piperazyl-bis-urea (10). A suspension of *N*-carbobenzoxy-*N'-formyl-tris-piperazyl-bis-urea (1.01 g, 2.3 mmol) in 0.05 M methanolic HCl (70 mL) was stirred at* room temperature for 24 h. The solid was gradually replaced by a flocculent white precipitate. Solid $NaHCO_3$ (8.4 g) was added at 0-4 °C and the solvent was removed by evaporation. Chromatography of the residue on silica gel (applied to the column in MeOH, eluting with a gradient of MeOH (30%-40%) in CH_2Cl_2) gave a white solid (900 mg, 2.03 mmol, 95 %) (R_f 0.06,10% MeOH in CH_2Cl_2): ^1H NMR ∂(400MHz, D_2O) 3.19-3.27 (4H, m, *H*CNH), 3.27-3.37 (12H, m, piperazine CH_2), 3.42-3.62 (8H, m, piperazine CH_2), 5.17 (2H, s, CH_2Ph), 7.35-7.47 (5H, m, arom.); FAB MS (glycerol) (*m/z*) 457 $(M+Na)^+$, 445 $(MH)^+$, 356 $(MH\text{-}C_7H_6)^+$.

Coupling of 10 and 1. To a solution of 10 (500 mg, 1.13 mmol, 1 equiv), 1 (800 mg, 1.35 mmol, 1.2 equiv) and benzotriazol-1-yl-*N*-oxytris(dimethylamino) phosphonium hexafluorophosphate (530 mg, 1.2 mmol, 1.06 equiv) in acetonitrile (50 mL) under nitrogen at room temperature was added triethylamine (350 µL, 2.5 mmol, 2.2 equiv). The reaction was shielded from light and stirred at room temperature for 13 h. Saturated aqueous $NaHCO_3$ was added and the product extracted exhaustively into CH_2Cl_2. The combined organic layers were washed with brine and dried over $MgSO_4$. Chromatography on silica gel (eluting with 5% MeOH in CH_2Cl_2) gave a white foam (1.02 g, 0.99 mmol, 88%) (R_f 0.39, 10% MeOH in CH_2Cl_2): ^1H NMR ∂(400 MHz, $CDCl_3$) 1.45-1.70 (4H, m, 2'-H, 3'-H), 1.79 (3H, s, NAc), 1.92 (3H, s, OAc), 1.93 (1H, t, J 13 Hz, 3-H_{ax}), 1.95 (3H, s, OAc), 2.03 (3H, s, OAc), 2.03 (3H, s, OAc), 2.26 (2H, t, J 9 Hz, 4'-H), 2.55 (1H, dd, J 13, 6 Hz, 3-H_{eq}), 3.15-3.30 (16H, br m, NCH_2) 3.43-3.50 (6H, m, NCH_2), 3.42 (2H, br s, 1'-H), 3.80 (3H, s, CO_2CH_3), 4.01-4.14 (3H, m, 5-H, 6-H, 9-H), 4.21 (1H, dd, J 12, 3 Hz, 9-H), 4.68-4.76 (1H, m, 4-H), 5.05 (2H, s, CH_2Ph), 5.24 (1H, br d, J 9 Hz, 7-H), 5.25-5.35 (1H, m, 8-H), 5.82 (1H, d, J 10 Hz, N*H*), 7.29-7.39 (5H, m, arom.)

Preparation of 11. A solution of *N*-carbobenzoxy protected 11 (1 g, 0.98 mmol, 1 equiv) in MeOH (50 mL) containing 20% $Pd(OH)_2$ on carbon (40 mg) was stirred at room temperature *under an atmosphere of hydrogen for 2 h. Replacement of the hydrogen by air, followed by* filtration of the solution through a celite plug and removal of the solvent under reduced pressure, gave a white solid (920 mg) which was used without further purification.

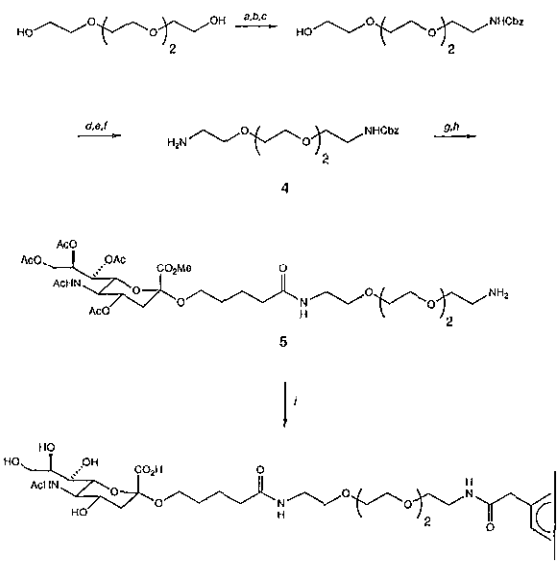
Preparation of U(3,3). A solution of the amine 11 (267 mg, 300 µmol, 2 equiv) and triethylamine (126 µL, 910 µmol, 6 equiv) in dry dimethylformamide (3 mL) under argon was cooled to 0 °C, and a solution of isophthaloyldichloride (31 mg, 150 µmol, 1 equiv.) in dimethylformamide (2 mL) was added dropwise with stirring. The mixture was allowed to warm slowly to room temperature, and then stirred overnight. The solvents were removed under reduced pressure, and the residue was chromatographed on silica gel (eluting with 10% MeOH in CH_2Cl_2) to give protected U(3,3) as a white solid. This solid was dissolved in MeOH (2 mL) and 1 M NaOH (0.5 mL) was added dropwise. After stirring for 2 h at room temperature Dowex-50W (H^+ form) (0.5 g) was added, and the stirring continued for 10 min. The resin was removed by filtration, and the solvents then evaporated under reduced pressure. Purification of the residue by size exclusion chromatography on Sephadex G-25 (100x2.5 cm, eluting with H_2O) followed by reverse phase HPLC, gave the product as a white solid (22 mg, 14 µmol, 5%): ^1H NMR ∂(500 MHz, D_2O) 1.60-1.69 (8H, m, 2'-H, 3'-H), 1.78 (2H, t, J 11 Hz, 3-H_{ax}), 2.05 (6H, s, NAc), 2.45-3.00 (4H, m, 4'-H), 2.71 (2H, dd, J 11, 4 Hz, 3-H_{eq}), 3.27-3.32 (55H, m, NCH_2, 4-H, 5-H, 6-H, 7-H, 8-H, 9-H), 7.49 (1H, s, *o*-arom.), 7.66-7.68 (4H, m, arom.). FAB MS (nitrobenzylalcohol) (*m/z*): 1578 (20%) $(M^+\text{-}H+2Na)$, 1556 (58%) $(M+Na^+)$, 1534 (26%) (MH^+).

*Preparation of U(3,3)´. To a solution of 11 (400 mg, 0.43 mmol 1 equiv), benzotriazol-*1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (203 mg, 0.46 mmol, 1 equiv) and phenylene 1,3-diacetic acid (43 mg, 0.22 mmol, 0.5 equiv) in acetonitrile (5 mL) shielded from light was added *N,N*-diisopropylethylamine (160 µL, 0.92 mmol, 2 equiv). The reaction was stirred overnight at room temperature. The solvent was replaced with CH_2Cl_2 and the mixture was washed with saturated aqueous $NaHCO_3$ and brine, then dried over $MgSO_4$. Chromatography on silica gel (eluting with a gradient MeOH (8-10%) in CH_2Cl_2) gave a white solid (73 mg) (R_f 0.71, 20% MeOH in CH_2Cl_2). This compound was dissolved in 2:1 MeOH:1 M $NaOH_{(aq)}$ and stirred at room temperature for 2 h. The mixture was neutralised by the addition of Dowex-50W (H^+ form), the resin was removed by filtration, and the solvents evaporated to give a white solid. A solution of this product in H_2O was passed down a G-25 size exclusion gel column (100 cm x 2.5 cm, H_2O) at 4 °C. The fractions that contained the required product were pooled and lyophilized, and the residue was purified by reverse phase HPLC (eluting with 40% MeOH in 60% 0.1 % $TFA_{(aq)}$), to give a white solid (21.6 mg, 14 µmol, 3% overall from amine 11): ^1H NMR ∂(500 MHz, D_2O), 1.65 (8H, br s, 2'-H, 3'-H), 1.74 (2H, t, J 12 Hz, 3-H_{ax}), 2.04 (6H, s, NAc), 2.46-2.52 (4H, m, 4'-H), 2.72 (2H, dd, J 12, 5 Hz, 3-H_{eq}), 3.17-3.22 (4H, br m, NCH_2), 3.27-3.92 (55H, m, NCH_2, 4-H, 5-H, 6-H, 7-H, 8-H, 9-H), 7.14 (1H, s, *o*-arom.), 1.22 (2H, *d*, 7 Hz, *m*-arom.), 7.23 (1H, t, 7 Hz, *p*-arom.). FAB MS (glycerol) (*m/z*): 1561 (64%) (MH^+).



Scheme I: Synthesis of Sialoside 1

[a] 6-Hexen-1-ol, $Hg(CN)_2$, 3Å sieves (70%). [b] $KMnO_4$, $AcOH_{(aq)}$ (93%).

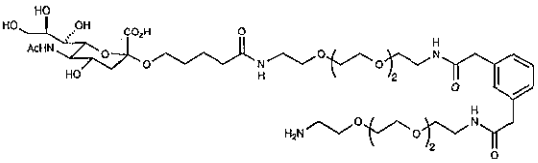
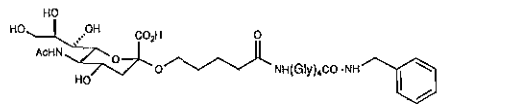


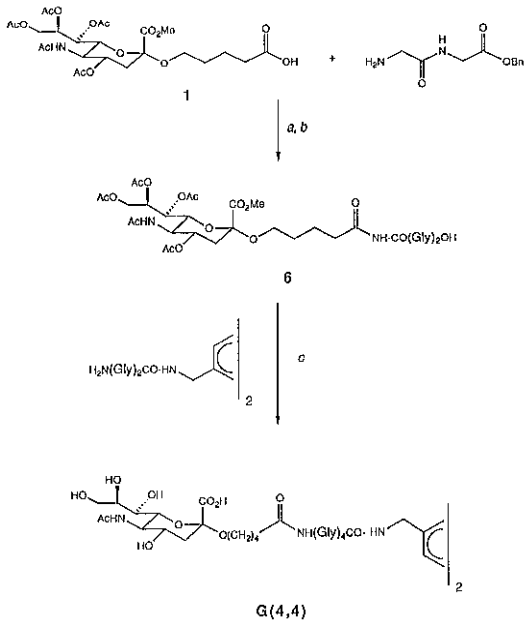
Scheme II: Synthesis of P(4,4)

[a] NaH, *p*-toluenesulfonyl chloride, THF (60%). [b] NaN_3, DMF (60%). [c] PPh_3, THF-H_2O; Et_3N, benzyl chloroformate (60%). [d] Et_3N, *p*-toluenesulfonyl chloride, DMF (87%). [e] NaN_3, DMF (91%). [f] PPh_3, THF-H_2O (80%). [g] 1, 1-hyroxybenzotriazole, dicyclohexylcarbodiimide, CH_2Cl_2 (66%). [h] $Pd(OH)_2$, H_2, EtOH (84%). [i] Phenylene 1,3-diacetic acid, diphenyphosphoryl azide, DMF; $NaOH_{(aq)}$, MeOH (24%).

TABLE I

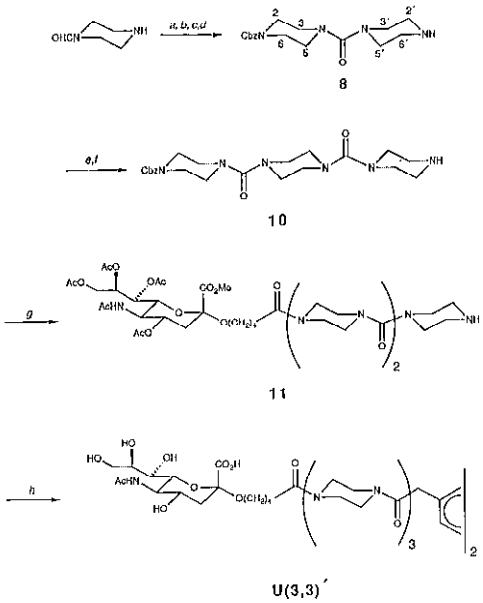
Binding of monovalent sialosides to influenza virus

Compound	K_I
	mM
	0.6
	0.6



Scheme III: Synthesis of G(4,4)

[a] 1-Hydroxybenzotriazole, dicyclohexylcarbodiimide, $^{i}Pr_2EtN$ (75%). [b] $Pd(OH)_2$, H_2, EtOH (88%). [c] Diphenylphosphoryl azide, Et_3N, DMF; $NaOH_{(aq)}$, MeOH (33%).



Scheme IV: Synthesis of U(3,3)´.

[a] Benzylchloroformate, Et_3N, 4-dimethylaminopyridine, CH_2Cl_2 (99%). [b] 12 M $HCl_{(aq)}$: MeOH (1:20) (99%). [c] Piperazine-1-carboxaldehyde, phosgene, Et_3N (67%). [d] 12 M $HCl_{(aq)}$:MeOH (1:20), (87%). [e] Triphosgene, Et_3N, 1-piperazine carboxaldehyde, CH_2Cl_2 (64%). [f] 12 M $HCl_{(aq)}$:MeOH (1:20) (95%). [g] 1, benzotriazol-1-oxytris(dimethylamino)phosphonium hexafluorophosphate, Et_3N, CH_3CN; $Pd(OH)_2$, H_2, MeOH (88%). [h] Phenylene 1,3-diacetic acid, benzotriazol-1-yl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate, $^{i}Pr_2EtN$, CH_3CN; $NaOH_{(aq)}$, MeOH (3%).