Polyglycine II Nanosheets: Supramolecular Antivirals?

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Tetraantennary peptides [glycine$_n$-NHCH$_2$]$_4$C can form stable noncovalent structures by self-assembly through intermolecular hydrogen bonding. The oligopeptide chains assemble as polyglycine II to yield submicron-sized, flat, one-molecule-thick sheets. Attachment of α-N-acetylneuraminic acid (Neu5Acα) to the terminal glycine residues gives rise to water-soluble assembled glycopeptides that are able to bind influenza virus multivalently and inhibit adhesion of the virus to cells 10^3-fold more effectively than a monomeric glycoside of Neu5Acα. Another antiviral strategy based on virus-promoted assembly of the glycopeptides was also demonstrated. Consequently, the self-assembly principle offers new perspectives on the design of multivalent antivirals.

KEYWORDS:

antiviral agents · glycopeptides · nanostructures · self-assembly · sialic acids

Introduction

Increasing interest in the self-assembly of small molecules into ordered supramolecular structures has arisen from the design of new materials and molecular devices.[1–4] Well-defined supramolecular architectures can be obtained if self-assembly is governed by hydrogen bonding. This self-assembly results in one-, two-, or three-dimensional structures—bands, sheets, nanotubes, and so on.[5–8] Such assemblies, though readily formed in organic solvents, are rather unstable in aqueous media, where there is competition for hydrogen bonding. As a rule, additional van der Waals or ionic interactions are needed to achieve stability of noncovalent structures in aqueous solutions. For example, the classic amphiphiles (that is, lipids and surfactants) are able to form durable supramolecular assemblies (micelles, liposomes, etc.) in water.[9]

Our aim was to design noncovalent polymers that are stable in aqueous solution and also display a carbohydrate moiety with specific biological activity. These supramolecular assemblies were expected to possess a dramatically higher activity when compared to a single carbohydrate ligand because of their multipoint binding to a complementary protein, a phenomenon that is well known for multivalent carbohydrate conjugates.[10–12] Additional criteria for the self-assembling material in this work are chemical simplicity and the absence of hydrophobicity, thus avoiding any nonspecific interaction with cell membranes.

Results and Discussion

Synthesis and self-assembly

The term *tecton*[13] is used to describe the monomeric unit used here, while the assembled structures are referred to as *tectomers*.

Tetraantennary peptides and glycopeptides capable of self-assembly are shown in Scheme 1. We previously described the syntheses of similar nonassembling compounds.[14] The compounds were synthesized by using conventional peptide chemistry with tetra(aminomethyl)methane as the starting material. Glycine residues were inserted individually or in a block as hydroxysuccinimide-activated *tert*-butoxycarbonyl (Boc) derivatives. The structures of the synthesized peptides were confirmed by NMR spectroscopy and mass spectrometry.

Self-assembly of the peptides [Gly$_n$-NHCH$_2$]$_4$C in aqueous solution occurs when $n \geq 7$; however, the properties of the three homologues with $n = 7$, 8, and 9 differ considerably. The free base [Gly$_7$-NHCH$_2$]$_4$C exists as a water-insoluble tectomer, whereas light scattering results indicate that the hydrochloride [HCl · Gly$_7$-NHCH$_2$]$_4$C does not self-assemble, presumably because of

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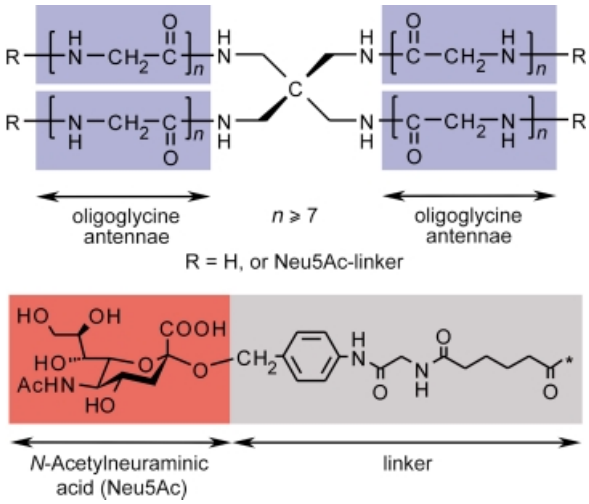

Scheme 1. *Structures of the tetraantennary peptides and glycopeptides capable of self-assembly.*

repulsion between the positively charged terminal NH_3^+ groups. When $n = 8$, the tendency towards self-assembly is so strong that even the hydrochloride $[HCl \cdot Gly_8\text{-}NHCH_2]_4C$ forms a water-soluble tectomer. When $n = 9$, the assembled hydrochloride is almost insoluble in water.

N-acetylneuraminic acid (Neu5Ac), known as a receptor for influenza viruses A and B,[15] was chosen as the biologically active ligand. The carbohydrate ligand was coupled to tetraantennary peptides by acylation of the amino group of the terminal Gly residue with Neu5Acα-linker-COONp (Np = 4-nitrophenyl) by two routes (see the Experimental Section and Figure 1). In method A, Neu5Ac residues were coupled with a preformed $[Gly_7\text{-}NHCH_2]_4C$ tectomer; the obtained product was not stochiometric (75 – 90% acylation depending on the Neu5Acα-linker-COONp excess and the reaction time) and thus its formula is given in quote marks, "[Neu5Acα-linker-Gly_7-$NHCH_2]_4C$", so that it can be discriminated from the glycopeptide obtained by method B. In this latter method, acylation of $[Gly_7\text{-}NHCH_2]_4C$ was performed in concentrated aqueous lithium bromide, an agent that destroys H bonds and impedes assembly. The Neu5Ac residues were thus attached to the monomeric peptide molecules. Method B allows complete substitution by Neu5Ac even if the starting peptide tectomer, for example, $[Gly_9\text{-}NHCH_2]_4C$, is insoluble in water. The glycopeptides synthesized by method B are incapable of self-assembly into extended flat tectomers, probably because of spatial hindrance by bulky Neu5Ac groups. In this case, small assemblies coexist with the monomeric form (see below).

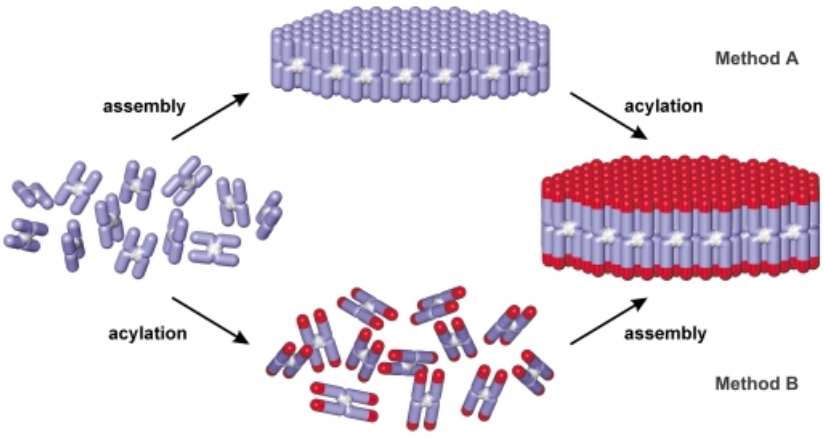

Figure 1. *Two routes for glycopeptide synthesis. Method A: Self-assembly of oligoglycine tectons into a [2 + 2] conformation (see Figure 5) gives rise to extended flat sheets that are one molecule thick. Carbohydrate groups were then coupled to a preformed peptide tectomer. Method B: Acylation of the peptide in the presence of LiBr gives rise to a glycopeptide that forms assemblies after removal of the LiBr.*

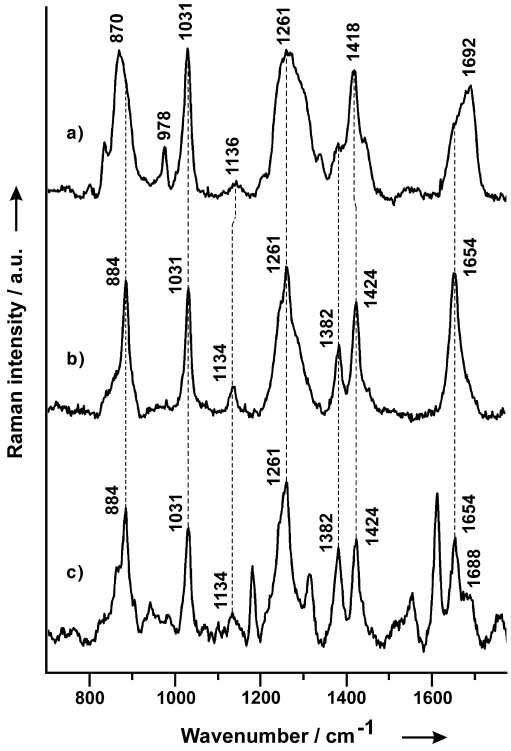

Figure 2. *Raman spectra of (a) monomeric $[HCl \cdot Gly_7\text{-}NHCH_2]_4C$ in solution, (b) assembled $[Gly_7\text{-}NHCH_2]_4C$ in the solid phase, and (c) assembled glycopeptide "[Neu5Acα-linker-Gly_7-$NHCH_2]_4C$" in solution. The spectrum of the peptide tectomer (b) contains bands at 884, 1382, 1424, 1654 cm^{-1}, identical to the bands of crystalline polyglycine II.[16] The assembled glycopeptide in solution (c) reveals the same profile with additional bands that arise from the amide groups of the Neu5Ac moiety and the linker. Spectrum (a) is interpreted as the superposition of the spectrum of the unordered polypeptide with elements of the polyglycine I and polyglycine II spectra (these results are also confirmed by Fourier-transform infrared spectroscopy).*

The obtained glycosylated tectomers are water soluble. The existence of submicron-sized aggregates in glycopeptide solutions was evident from laser light scattering; for "[Neu5Acα-linker-Gly_7-$NHCH_2]_4C$" the maximum of the size distribution is observed at 200 nm. Gel-permeation chromatography evaluation of the molecular weight of "[Neu5Acα-linker-Gly_7-$NHCH_2]_4C$" and [Neu5Acα-linker-Gly_n-$NHCH_2]_4C$ ($n = 7 – 9$) tectomers gave rise to a value greater than 2000 kD. The tectomers are

stable in the presence of salts at physiological concentrations, sodium dodecyl sulphate at pH 2.0 – 9.0, and on addition of MeOH or MeCN up to 70% v/v. Reversible disintegration of the tectomers was observed in concentrated solutions of lithium bromide or upon heating to 60 °C.

Structure

Raman spectroscopy, atomic force microscopy (AFM), electron microscopy (EM), and molecular modeling were applied to define the spatial organization of the tectomers.

The so-called polyglycine II structure was identified in all tectomers. The Raman spectra of both the peptide and the glycopeptide tectomers (Figure 2) have a band pattern with a position, form, and relative intensity consistent with the crystalline structure of polyglycine II.[16] This structure is distinct from the canonical α-helix and β-sheet structures. Previously, polyglycine II was found in the crystalline glycine polymer,[17–19] bolaamphiphils,[20] and nylons,[21] in which all the NH and CO groups of the 3_1 helices ($\phi = -76.9°$, $\psi = 145.3°$) form intermolecular hydrogen bonds with the six surrounding chains, although no intrachain H bonds are formed. Contrary to all cited examples, in which polyglycine II was only observed in a solid phase, the tectomers described here are stable in aqueous solution.

The Raman spectra only testify to the type of polyglycine II helix packing and to the mutual disposition of the chains into the hexagonal array, they do not show the supramolecular organization of the tectomers. Therefore, the shape and size of the structures were investigated by AFM. These studies showed the tectomers as thin, flat sheets (Figure 3). The planar size of the "[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C" aggregates is in the range

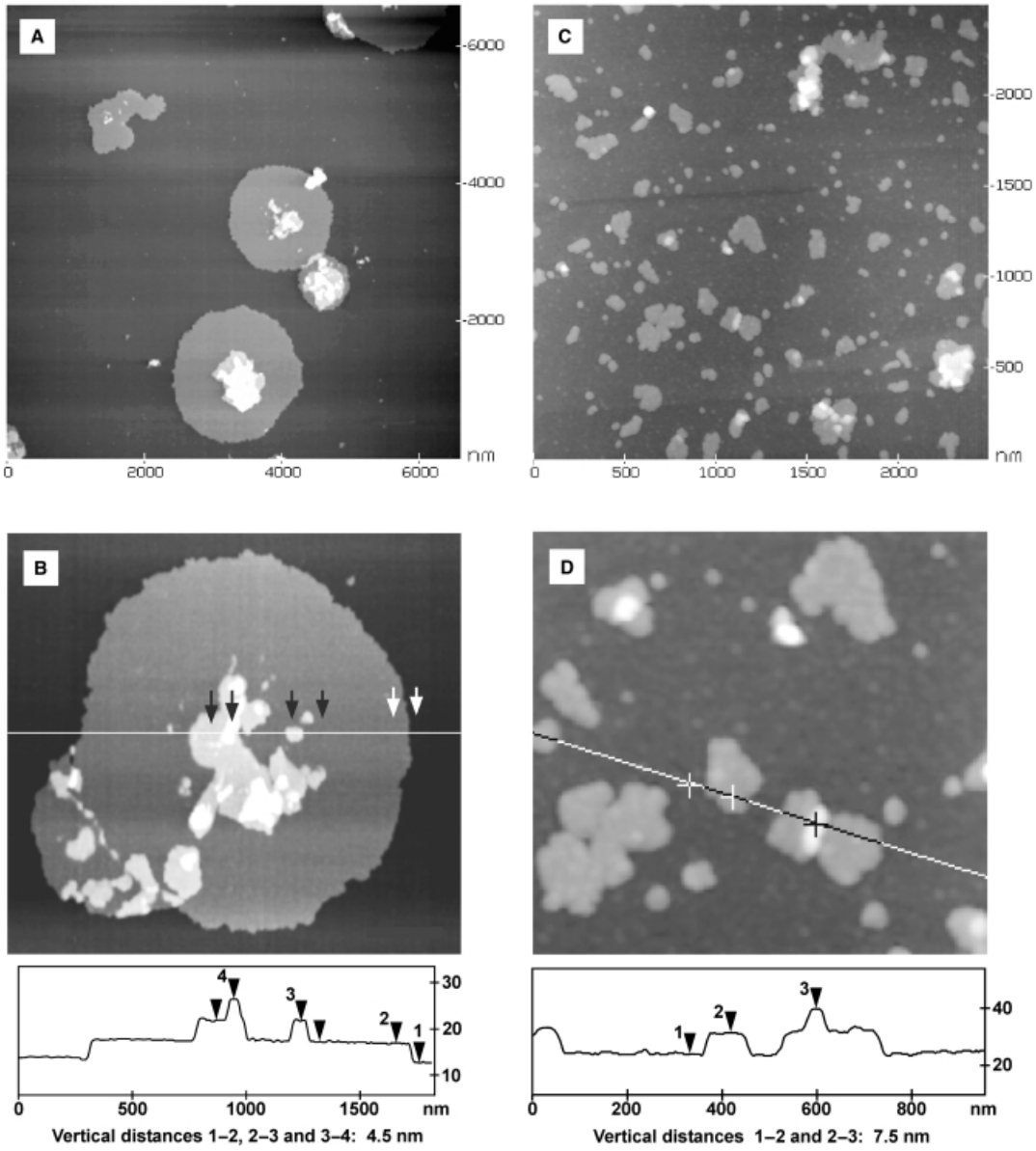

Figure 3. *A, B) AFM images (mica) of the water-insoluble tectomer [Gly$_7$-NHCH$_2$]$_4$C; the tectomer monolayer thickness is 4.5 nm. C, D) AFM images (pyrographite) of the water-soluble tectomer "[Neu5Acα-linker-Gly$_7$ -NHCH$_2$]$_4$C"; the monolayer thickness is 7.5 nm.*

30 – 500 nm, with the maximum of the distribution at 50 nm. Glycopeptides synthesized in the presence of LiBr form small (30 – 50 nm) associates (Figure 4) that coexist with a minor monomeric form (GPC and EM data). It is noteworthy that the

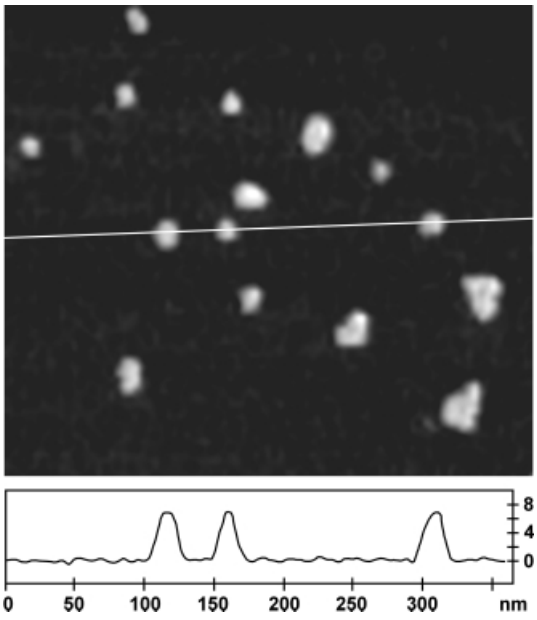

Figure 4. *AFM image (pyrographite) of the water-soluble small associates of [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C synthesized in the presence of LiBr.*

morphology of the tectomers is unaffected by the supporting matrix (such as mica or pyrographite), which confirms that the tectomers are indeed formed in solution. Additional confirmation that aggregation takes place in solution is provided by light scattering results, which show that submicrometer-sized particles are present in solution. AFM data give an experimentally determined thickness of the [Gly$_7$-NHCH$_2$]$_4$C peptide tectomer of 45 ± 5 Å, whereas the extended glycopeptide tectomer "[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C" obtained by method A is 75 ± 5Å thick. The thickness of the small [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C tectomer is also about 75 Å.

In principle, several different packings of the tetraantennary tecton appear possible, all of which result in flat monolayers. The most probable structures are outlined in Figure 5. The size of the tecton was calculated for three conformations and compared with the AFM data. The [2 ⊥ 2] tecton (Figure 5 A) was incapable of packing within the hexagonal array of the polyglycine II structure because of the thickened node [-Gly-NHCH$_2$]$_4$C, while the other two conformers packed into a lattice with an interchain distance of 5 Å, which is normal for polyglycine II.[16] The calculations for the nonglycosylated molecule [Gly$_7$-NHCH$_2$]$_4$C yield thicknesses of 50 Å and 25 Å for the [2 + 2] and [4 + 0] structures, respectively. The first value agrees best with the experimental value of 45 ± 5 Å. Similar calculations for the [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C molecule give thicknesses of 95 Å and 70 Å for the [2 + 2] and [4 + 0] conformations, respectively. Although the experimental value of 75 ± 5 Å fits the [4 + 0] variant, the [2 + 2] structure seems more realistic. This

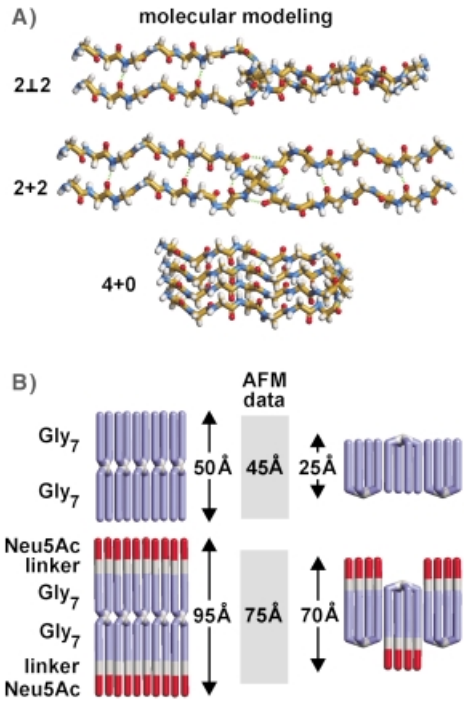

Figure 5. *The conceivable types of packing for [Gly$_7$-NHCH$_2$]$_4$C peptide and the corresponding glycopeptide. A) [2 ⊥ 2] conformation, in which the antennae pairs are located in perpendicular planes; [2 + 2], [Gly$_7$-NHCH$_2$]$_4$C conformation with four antennae in the same plane; [4 + 0] conformation, bush-type variant. Geometry optimization was performed with MM2 software[31] and images were processed on a DS ViewerPro 5.0 instrument (Accelrys Inc.). B) The calculated and experimental measurements of [Gly$_7$-NHCH$_2$]$_4$C and [Neu5Ac-linker-Gly$_7$-NHCH$_2$]$_4$C for the conformations [2 + 2] and [4 + 0].*

apparent discrepancy may be explained by underestimation of the layer thickness determined by AFM (in contact mode) as a result of compression of the sample at measurement or during drying.[22] The close agreement of the experimental and calculated [2 + 2] values for [Gly$_7$-NHCH$_2$]$_4$C is explained by a higher rigidity of the nonglycosylated tectomer compared to the glycosylated one. Thus, the geometry of tetraantennary tectons in the tectomer resembles the letter "H". Although we here analyze the data only in relation to [Gly$_7$-NHCH$_2$]$_4$C and the corresponding glycopeptide, the Gly$_8$ and Gly$_9$ homologues were demonstrated to have analogous spectral and structural features, which points to an analogous pattern of assembly.

The data collected by Raman spectroscopy, AFM, EM, and molecular mechanics calculations indicate that the tectomer may be considered as a two-dimensional crystal (Figure 1) that is rigid as a result of a highly cooperative system of hydrogen bonds. The unusual stability of the tectomer in aqueous media can be explained by the participation of all CO and NH glycine groups in H bonding in a highly cooperative manner, and the inaccessibility to water molecules of hydrogen bonds inside the two-dimensional crystal. Moreover, each of the Gly$_n$ antennae in a hexagonal array is covalently bound to the three others through the core [NHCH$_2$]$_4$C fragment. This tetraantennary design of the tecton appears crucial for tectomer stability.

Tectomers as a novel antiviral strategy

The anti-microbial-adhesion activity of a small molecule can be enhanced by linking of the molecule to a true polymer.[10–12] The same would be expected for attachment of a biologically active ligand to a tectomer. Adhesion of the influenza virus to cells is mediated by its surface protein hemagglutinin, which binds to the Neu5Acα-containing oligosaccharides of the host cell.[15] The Neu5Acα-containing tectomers can therefore act as inhibitors of the binding. Table 1 outlines the relationship between the structures of Neu5Ac glycopeptides and their antiviral activity. The nonassembling glycopeptides of general formula [Neu5Acα-linker-Gly$_n$-NHCH$_2$]$_4$C ($n = 1-6$) did not show a substantial difference in activity compared with monovalent Neu5AcαOBn. However, elongation of the oligoglycine fragment up to Gly$_{7-9}$ enhanced the activity by at least three orders of magnitude. AFM, EM, GPC, and light scattering data showed that the active compounds are high-molecular-weight aggregates.

It should be pointed out that the length of the glycopeptide molecules is not sufficient to realize multipoint binding with a viral surface.[14] Thus, the contribution of the monomeric form to the antiviral activity of the associating glycopeptides could be neglected. Taken together, the inhibition assay (Table 1) and EM/AFM (Figure 6 A, 6 B) data show that enhanced antiviral activity of the associating glycopeptides is achieved by tight multipoint binding of tectomers with virus particles. We also believe that the adsorption of the large glycopeptide sheets onto the viral surface shields the virus and blocks its ability to bind sialic receptors.

Table 1. Relative activity of the glycopeptides in the influenza virus receptor-binding inhibition assay.[a]

Inhibitor	Relative activity
Neu5AcαOBn (reference compound)	1
PAA-Neu5Acα[b]	4000
nonassembling glycopeptides	
[Neu5Acα-linker-Gly$_n$-NHCH$_2$]$_4$C ($n = 1-6$)	1 – 3
assembled glycopeptides (tectomers)	
"[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C"	1000
[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C	1000
[Neu5Acα-linker-Gly$_8$-NHCH$_2$]$_4$C	1400
[Neu5Acα-linker-Gly$_9$-NHCH$_2$]$_4$C	3300
"[Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C"	inactive
[Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C	inactive

[a] Virus strain A/H3N2/NIB/44/90M was used; Bn = benzyl; linker = $-OCH_2(p\text{-}C_6H_4)NHCOCH_2NH\text{-}CO(CH_2)_4CO-$; see the Experimental Section for a detailed description of the assay. [b] Polyacrylamide conjugate of Neu5Ac$\alpha OCH_2(p\text{-}C_6H_4)NHCOCH_2NH_2$ (20 mol %), an example of a multivalent inhibitor based on a true polymer.

The specificity of virus – tectomer binding was demonstrated by the fact that "[Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C", the tectomer that bears unnatural Neu5Acβ groups, was not active in the antiviral assay. The same conclusion was reached from EM data: the Neu5Acα tectomer formed complexes with virus particles,

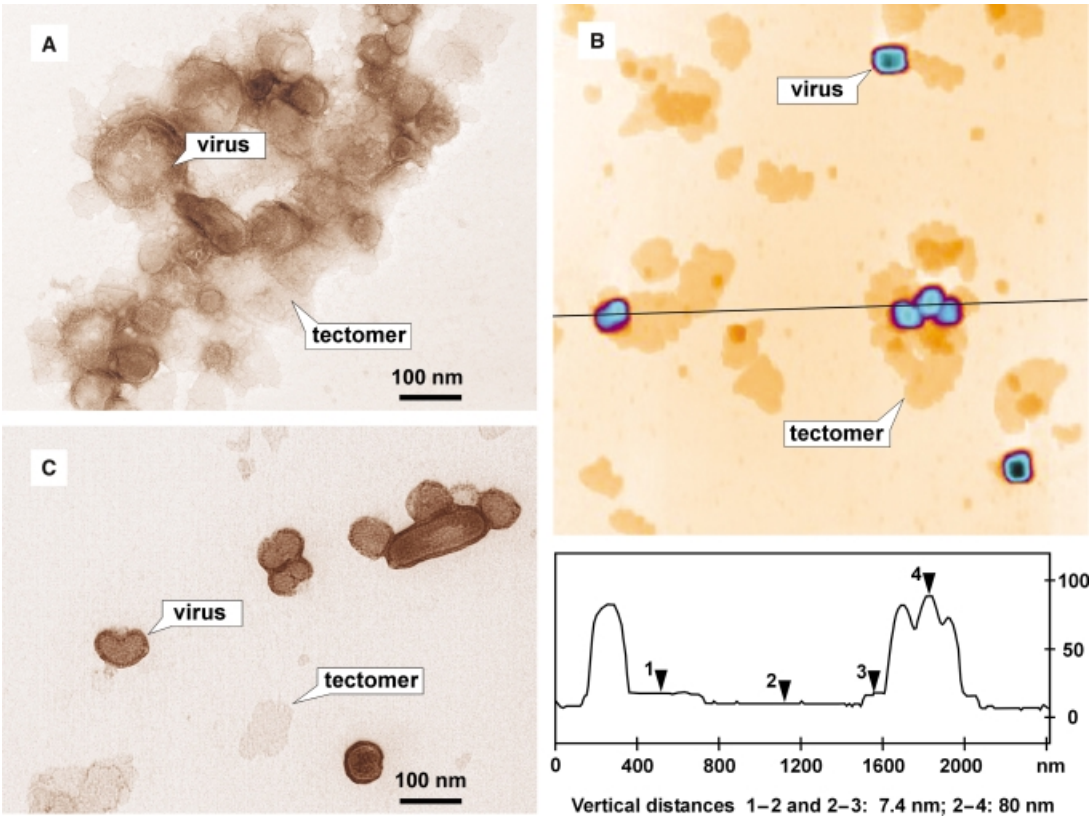

Figure 6. Images of the influenza virus in the presence of tectomers. A) EM micrograph and (B) AFM image (pyrographite) of the virus with "[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C" tectomer; the tectomer particles are in close proximity to the virions. C) EM micrograph of the virus and "[Neu5Acβ-linker-Gly$_7$ -NHCH$_2$]$_4$C" with unnatural Neu5Acβ residues; the tectomer particles are distanced from the virions.

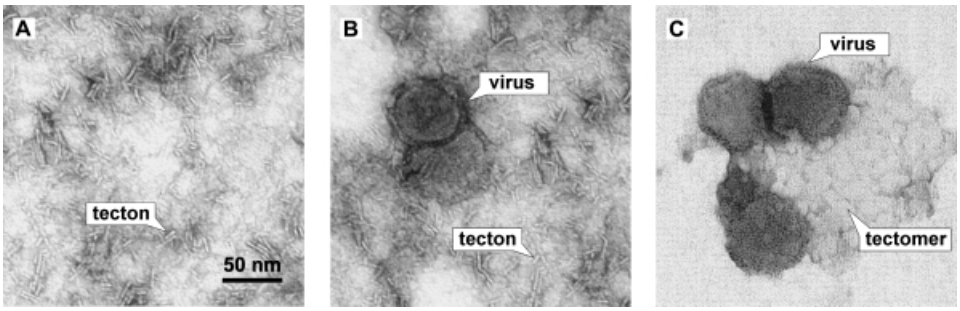

Figure 7. EM micrographs demonstrating the interaction of influenza virus with glycopeptides synthesized in the presence of LiBr. A) [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C exists as small tectomers and monomeric tectons; a similar picture was observed for the unnatural Neu5Acβ analogue. B) Addition of the virus does not affect assembly of [Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C (compare with (A)). C) The virus promoted assembly of [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C. The glycopeptide is fully assembled into a tectomer; the monomeric form has disappeared. The presence of a neuraminidase inhibitor (4-amino-4-deoxy-Neu5Ac2en, 3 μM) does not change the picture, which suggests that the virus-promoted assembly is not the result of viral neuraminidase splitting off any extra Neu5Ac residues that constrain the assembly.

while the structurally analogous Neu5Acβ tectomer (investigated by light scattering, GPC, AFM) exhibited no binding (Figure 6C).

The antiviral activities of the Neu5Acα tectomers approach those of polyacrylamide derivatives of Neu5Acα.[23, 24] Although a number of polyvalent sialosides have previously demonstrated higher antiviral activity,[12] the tectomers have clear advantages as potential therapeutics since they are, as aggregates of small molecules, capable of surmounting the problem of inconsistency and the disadvantageous pharmacokinetics of true polymers.

While the present study focused on the activity of preformed glycopeptide tectomers, a more promising approach is the specific generation of extended assemblies that only form in the presence of a virus. Examples of surface-promoted assembly were described for molecules possessing two different binding sites, one for homotypic binding and another for interaction with a carbohydrate-covered surface.[25–27] From the perspective of antiviral therapy, the assembly of the tectomer directly on the virus particle has clear advantages compared to the administration of the preformed tectomer. We propose that such virus-promoted assembly was realized in our in vitro studies (Figure 7), in which the EM data show that nonassembled tectons form tectomers upon contact with influenza virus.

In this study, we have presented the application of glycopeptide tectomers as antivirals, but both the mode of self-assembly and the architecture of the tectomers itself may be of interest for other applications. The rigidity of the peptide tectomers distinguishes these structures from liquid-crystalline assemblies or lipid self-assembled monolayers. Tectomers capable of forming perfectly flat and rigid layers of a predetermined thickness appear promising for the design of new nanomaterials and as a platform for nanodevices.

Experimental Section

Synthesis:

[BocGly$_7$-NHCH$_2$]$_4$C: Et$_3$N (0.3 mL) followed by BocGlyGlyONs (725 mg, 2.2 mmol) were added to a suspension of [HCl·Gly$_5$-NHCH$_2$]$_4$C[14] (710 mg, 0.5 mmol) in dimethyl sulfoxide (DMSO; 30 mL). The mixture was stirred for 24 h at 20 °C, evaporated, and DMSO was removed under a vacuum. The obtained white residue was suspended in MeOH and the precipitate was washed with MeOH and dried in a vacuum. Yield: 990 mg (93%). ^{1}H NMR (500 MHz, [D$_6$]DMSO, 30 °C): δ = 8.545, 8.118, 8.092, 8.085, 8.081, 8.018, 7.814 and 6.970 (t, 1H; COCH$_2$N*H*), 3.840 (d, 2H; CH$_2$CO), 3.751 (m, 8H; 4CH$_2$CO), 3.723 and 3.581 (d, 2H; CH$_2$CO), 2.688 (br s, 2H; CCH$_2$), 1.378 (s, 9H; OCMe$_3$) ppm. MS (MSVK instrument, ^{252}Cf plasma desorption TOF, "Electron" company, Sumy, Ukraine): *m/z*: 2152 [*M* + Na]$^+$. Elemental analysis calcd (%) for C$_{81}$H$_{132}$N$_{32}$O$_{36}$ (2130.2): C 45.67, H 6.25, N 21.04; found: C 45.08, H 6.20, N 20.78.

[HCl·Gly$_7$-NHCH$_2$]$_4$C: [BocGly$_7$-NHCH$_2$]$_4$ (852 mg, 0.4 mmol) was dissolved in CF$_3$COOH (3 mL) and kept for 2 h at 20 °C. The mixture was diluted with toluene (10 mL), evaporated, and dried in a vacuum. The residue was dissolved in HCl (1 M, 2.4 mL) and evaporated to dryness. The crystalline residue was washed with MeOH (4 mL) and dried in a vacuum. Yield: 720 mg (96%). TLC: R_f = 0.67 (eluent: 2 M HCl). ^{1}H NMR (500 MHz, [D$_2$]H$_2$O, 30 °C): δ = 4.074, 4.063, 4.021, 4.015 (× 2), 3.958 and 3.919 (s, 2H; CH$_2$CO), 2.922 (br s, 2H; CCH$_2$) ppm. Elemental analysis calcd (%) for C$_{61}$H$_{104}$Cl$_4$N$_{32}$O$_{28}$ (1875.5): C 39.07, H 5.59, Cl 7.56, N 23.90; found: C 38.54, H 5.91, Cl 7.80, N 23.88. Analytical HPLC (Column Nucleosil C18 – 100 2.0 × 75 mm, MeCN/H$_2$O/TFA 1:1:0.1, 1 mL min^{-1}, UV detection at 210 and 246 nm): a single peak with a retention time of 10.31 min was detected.

[BocGly$_8$-NHCH$_2$]$_4$C: A solution of saturated aqueous LiCl (1.5 mL) followed by BocGlyONs (145 mg, 0.533 mmol) was added to a solution of [HCl·Gly$_7$-NHCH$_2$]$_4$C (50 mg, 26.7 μmol) in water (0.3 mL). After addition of Li$_2$CO$_3$ (24 mg, 0.32 mmol) the mixture was stirred for 24 h at 20 °C, then diluted with water (30 mL) and NaHCO$_3$ (1 M, 2 mL) and kept at 5 °C for 20 h. The white precipitate was filtered off, washed with water and MeOH (1 mL) and dried in a vacuum. Yield: 52 mg (82%) Boc-derivative. Elemental analysis calcd (%) for C$_{89}$H$_{144}$N$_{36}$O$_{40}$ (2358.4): C 45.33, H 6.15, N 21.38; found: C 44.92, H 6.16, N 21.21.

[HCl·Gly$_8$-NHCH$_2$]$_4$C: [BocGly$_8$-NHCH$_2$]$_4$C (40 mg, 17 μmol) was dissolved in CF$_3$COOH (1 mL) and kept at 20 °C for 2 h. The mixture was diluted with toluene (5 mL) and evaporated to dryness. The residue was evaporated twice with HCl (0.05 M, 1.4 mL) and freeze-dried. Yield: 34 mg (94%). TLC (eluent: 2 M HCl): R_f = 0.63 (monomer) and R_f = 0 (assembled). ^{1}H NMR (500 MHz, [D$_2$]H$_2$O, 30 °C): δ = 4.087, 4.074, and 4.037 (s, 2H; CH$_2$CO), 4.026 (s, 6H; 3 CH$_2$CO), 3.969 and 3.931 (s, 2H; CH$_2$CO), 2.936 (s, 2H; CCH$_2$) ppm.

[BocGly$_9$-NHCH$_2$]$_4$C and [HCl·Gly$_9$-NHCH$_2$]$_4$C: Obtained by a similar method to that described above for [HCl·Gly$_8$-NHCH$_2$]$_4$C. Yields: 92% for [Boc-Gly$_9$-NHCH$_2$]$_4$C and 90% for [HCl·Gly$_9$-NHCH$_2$]$_4$C. [Boc-Gly$_9$-

$NHCH_2]_4C$ elemental analysis calcd (%) for $C_{97}H_{156}N_{40}O_{44}$ (2586.6): C 45.04, H 6.08, N 21.66; found: C 44.54, H 5.99, N 21.47.

Neu5Acα-linker-COONp: A solution of di(4-nitrophenyl)adipate (330 mg, 0.85 mmol) in dimethylformamide (DMF; 2 mL) was added to a solution of Neu5Acα2-OCH$_2$(p-C$_6$H$_4$)NHCOCH$_2$NH$_2$[28] (80 mg, 0.17 mmol) in DMSO (0.5 mL). The mixture was stirred for 24 h at 20 °C then diluted with water (15 mL) containing AcOH (0.1 mL) and filtered. The filtrate was evaporated. Exclusion chromatography of the residue on Sephadex LH-20 in MeCN/H_2O (1:1, 0.2% AcOH) and freeze-drying yielded Neu5Acα-linker-COONp (92 mg; 75%). TLC (2-propanol/EtOAc/water 4:3:2): $R_f = 0.62$. ^{1}H NMR (500 MHz, [D$_4$]MeOH, 30 °C): $\delta = 8.467$ and 7.561 (d, $J = 8.8$ Hz, 2H; Ar), 7.707 and 7.474 (d, $J = 8.3$ Hz, 2H; Ar), 4.973 and 4.653 (d, $J = 11$ Hz, 1H; OCH_2Ar), 4.196 (s, 2H; COCH_2NH), 4.083 (ddd, 1H; H-8 Neu5Ac), 4.047 (dd, $J_8 = 2$ Hz, 1H; H-9b Neu5Ac), 3.978 (ddd, 1H; H-5 Neu5Ac), 3.924 (ddd, 1H; H-4 Neu5Ac), 3.840 (dd, $J_{9b} = 12$, $J_8 = 6$ Hz, 1H; H-9a Neu5Ac), 3.821 (dd, $J_5 = 10$ Hz, 1H; H-6 Neu5Ac), 3.743 (dd, $J_6 = 1.5$, $J_8 = 9$ Hz, 1H; H-7 Neu5Ac), 2.976 (dd, $J_4 = 4.5$, $J_{3ax} = 13$ Hz, 1H; H-3$_{eq}$ Neu5Ac), 2.874 and 2.565 (t, $J = 6.8$ Hz, 2H; CH$_2$CO), 2.205 (s, 3H; NCOCH$_3$), 1.980 (m, 4H; CH_2CH_2CH$_2$CO), 1.968 (dd, 1H; H-3$_{ax}$ Neu5Ac) ppm.

Glycopeptides:

Method A:

"[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C": Neu5Acα-linker-COONp (87 mg, 120 μmol) was added to a solution of [HCl·Gly$_7$-NHCH$_2$]$_4$ (38 mg, 20 μmol) in water (1.5 mL). NaHCO$_3$ (1 M) was added until pH 8 was reached (opalescence appears) and the mixture was stirred for 48 h at 20 °C. Exclusion chromatography on Sephadex LH-20 in aqueous NH_3 (0.1 M) and freeze-drying yielded the glycopeptide tectomer (59 mg; 71%). Carbohydrate analysis of glycopeptide: calcd: 0.97 μmol Neu5Ac/mg; found: 0.84 (corresponds to about 75% acylation). The ^{1}H NMR spectrum is similar to that of [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C.

"[Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C" was prepared from Neu5Acβ-linker-COONp and [HCl·Gly$_7$-NHCH$_2$]$_4$ by method A. Yield: 75%. Carbohydrate analysis of glycopeptide: calcd: 0.97 μmol Neu5Ac/mg; found: 0.86 (corresponds to about 80% acylation). The ^{1}H NMR spectrum is similar to that of [Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C.

Method B:

[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C: Saturated aqueous LiBr (0.4 mL) and Neu5Acα-linker-COONp (31 mg, 43 μmol) were added consecutively to a solution of [HCl·Gly$_7$-NHCH$_2$]$_4$C (10 mg, 5.3 μmol) in water (0.3 mL). Li_2CO_3 (6 mg, 80 μmol) was added and the mixture was stirred for 48 h at 20 °C then diluted with water (0.7 mL). Exclusion chromatography on Sephadex LH-20 in aqueous NH_3 (0.1 M) and freeze-drying yielded the glycopeptide tectomer (19 mg; 87%). Carbohydrate analysis of glycopeptide: calcd: 0.97 μmol Neu5Ac/mg; found 0.98; the glycopeptides synthesized by method B have stoichiometric Neu5Ac content. ^{1}H NMR (500 MHz, [D$_2$]H$_2$O, 30 °C): $\delta = 7.455$ (m, 4H; Ar), 4.770 and 4.550 (d, $J = 11$ Hz, 1H; ArCH_2), 4.085 and 4.078 (s, 2H; CH$_2$CO), 4.020 (s, 8H; 4 CH$_2$CO), 3.980 and 3.972 (s, 2H; CH$_2$CO), 2.929 (s, 2H; CCH$_2$), 2.401 (m, 4H; 2 COCH_2CH$_2$), 1.687 (m, 4H; COCH$_2$CH_2); Neu5Acα-unit: $\delta = 3.887$ (dd, $J_{9b} = 12$, $J_8 = 2.3$ Hz, 1H; H-9a), 3.862 (dd, 1H; H-5), 3.820 (ddd, 1H; H-8), 3.769 (dd, $J_7 = 1.5$, $J_5 = 10.2$ Hz, 1H; H-6), 3.739 (ddd, $J_5 = 9.8$ Hz, 1H; H-4), 3.676 (dd, $J_8 = 6$ Hz, 1H; H-9b), 3.638 (dd, $J_8 = 9$ Hz, 1H; H-7), 2.819 (dd, $J_{3ax} = 12.5$, $J_4 = 4.6$ Hz, 1H; H-3eq), 2.079 (s, 3H; NAc), 1.722 (dd, $J_4 = 12$ Hz, 1H; H-3ax) ppm.

[Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C was prepared from Neu5Acβ-linker-COONp and [HCl·Gly$_7$-NHCH$_2$]$_4$ by method B. Yield: 89%. Carbohydrate analysis of glycopeptide: calcd: 0.97 μmol Neu5Ac/mg; found: 0.96. ^{1}H NMR (500 MHz, [D$_2$]H$_2$O, 30 °C): $\delta = 7.487$ (m, 4H; Ar), 4.622 and 4.307 (d, $J = 10.4$ Hz, 1H; ArCH_2), 4.086 and 4.076 (s, 2H; CH$_2$CO), 4.017 (s, 8H; 4 CH$_2$CO), 3.981 and 3.970 (s, 2H; CH$_2$CO), 2.928 (s, 2H; CCH$_2$), 2.404 (m, 4H; 2 COCH_2CH$_2$), 1.686 (m, 4H; COCH$_2$CH_2) ppm; Neu5Acα unit: $\delta = 3.956$ (dd, 1H; H-5), 3.898 (dd, $J_{9b} = 12$, $J_8 = 2.7$ Hz, 1H; H-9a), 3.727 (dd, $J_8 = 5.5$ Hz, 1H; H-9b), 3.619 (dd, $J_8 = 9.5$ Hz, 1H; H-7), 2.435 (dd, $J_{3ax} = 12.6$, $J_4 = 5$ Hz, 1H; H-3eq), 2.085 (s, 3H; NAc), 1.729 (dd, $J_4 = 12$ Hz, 1H; H-3ax).

[Neu5Acα-linker-Gly$_8$-NHCH$_2$]$_4$C and [Neu5Acα-linker-Gly$_9$-NHCH$_2$]$_4$C were prepared by method B. Yields: 90% and 87%, respectively. Carbohydrate analysis of glycopeptides: [Neu5Acα-linker-Gly$_8$-NHCH$_2$]$_4$C: calcd: 0.92 μmol Neu5Ac/mg; found: 0.92; [Neu5Acα-linker-Gly$_9$-NHCH$_2$]$_4$C: calcd: 0.87 μmol Neu5Ac/mg; found: 0.88.

Analysis:

Raman spectroscopy: Raman spectra were recorded with a Ramanor HG-2S (Jobin Yvon) spectrometer with an Anaspec 300S monochromator; the 514.5 nm line of an argon laser (Spectra Physics, model 164 – 03) was used for excitation. The irradiation power in the sample chamber did not exceed 300 mW in Raman experiments. All spectra were recorded upon scanning in 2-cm^{-1} steps with an integration time of 3 s. Data was accumulated from 3 – 5 independent scans and averaged.

Atomic force microscopy: AFM images of the water-insoluble tectomer [Gly$_7$-NHCH$_2$]$_4$C were obtained by adding $NaHCO_3$ (four equiv) to a solution of the corresponding tectomer hydrochloride (1 mg mL^{-1}). Images of a number of AFM samples prepared from the solution at 10-min intervals were then recorded. Extended flat sheets were observed 1 – 2 h after [Gly$_7$-NHCH$_2$]$_4$C solution preparation (at the beginning of the appearance of opalescence). Before this time, no associates were detected; 5 – 6 h later, the deposit had formed. In the case of the water-soluble tectomers "[Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C" and [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C, a 1-mg mL^{-1} solution was used. To obtain images of tectomer – virus complexes, a solution of glycopeptide (1 mg mL^{-1}) and a suspension of A/H3N2/NIB/44/90M influenza virus (0.25 mg of protein per mL) purified by centrifugation in 30% sucrose were mixed in a 1:1 (v/v) ratio and incubated for 30 min at room temperature. The samples were placed on a fresh mica or graphite surface, kept for 5 min, and then unabsorbed material was removed by an argon stream. To decrease the relative humidity in the scanning zone, a hot nitrogen stream was blown over the sample placed on the microscope working table. The samples were imaged with a NanoScope II instrument (Digital Instruments, Santa Barbara, CA) at a constant force in vertical mode, with commercial silicon nitride cantilevers (0.06 and 0.53 N m^{-1} force constants, Park Scientific Instruments, USA). The scanning rate was 3 – 7 Hz and the scanning angles were 180° and 270°. The images were filtered with a flatten filter from the NanoScope II software. Ruby mica was purchased from TED PELLA Co. Highly ordered pyrolytic graphite was produced by the Moscow Institute of Graphite (Russia).

Electron microscopy: Samples of the glycopeptide solution in water (0.1 mg mL^{-1}), a suspension of purified A/H3N2/NIB/44/90M influenza virus (0.25 mg of protein per mL), or a 1:1 (v/v) mixture of the two substances, were incubated for 30 min at room temperature and used to make EM preparations. The samples were placed onto freshly prepared parlodium films, dried, negatively stained with uranyl acetate (1% aqueous solution), and deposited on EM grids. The preparations were examined with a JEOL EM 100CX microscope (JEOL, Japan).

Light scattering experiments: Light scattering of aqueous solutions of the peptides and glycopeptides (1 mg mL^{-1}) was studied with an automatic submicron particle analyzer "Coulter N4MD" (He – Ne laser, λ: 632.8 nm, detection angle:62.5°). Estimation of the tectomer sizes

was performed by using the firmware software and assuming a spherical shape.

HPLC: Gel-permeation HPLC of glycopeptides was performed on a TSK-4000 7.5 × 300 mm column calibrated with a set of globular proteins. Mobile phase: 0.2 M NaCl, 1 mL min^{-1}; UV detection at 250 nm.

Carbohydrate (Neu5Ac) analysis: The glycopeptides were analyzed by HPLC[29] with Neu5ProβBn as internal standard (ODS VYDAC 300A, 4.6 × 250 mm column, mobile phase: 2.5% *n*BuOH).

Influenza virus receptor-binding inhibition assay:[30] A suspension of A/H3N2/NIB/44/90M influenza virus in phosphate buffered saline containing 1:50 – 1:200 hemagglutination titre was incubated in the wells of a fetuin-precoated polystyrene 96-well microplate (0.1 mL/well) for 2 h at 4 °C. The plate was washed with the same buffer with 0.01% Tween 20 added (PBST buffer). A PBST-diluted mixture (0.1 mL) of horseradish-labeled fetuin (final concentration 2×10^{-8} M with respect to fetuin) and 4-amino-4-deoxy-Neu5Ac2en (3×10^{-6} M) was added to the wells. After incubation for 2 h at 4 °C the plate was washed and the peroxidase activity was measured (*o*-phenylenediamine – H_2O_2 reagent, absorbance at 492 nm). The data obtained were used to calculate the dissociation constant of the virus – inhibitor complex. The relative activities of the glycopeptides as inhibitors were calculated as the ratio (Neu5AcαBn dissociation constant)/(glycopeptide dissociation constant). The dissociation constant for Neu5AcαBn with the A/H3N2/NIB/44/90M influenza virus strain is 100 μM.

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