Inhibition of Herpes Simplex Virus-1 Entry into Human Cells by Non-Saccharide Glycosaminoglycan Mimetics

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Materials and Methods

Chemicals, reagents, analytical chemistry, and protein

Unfractionated heparin (UFH), and chondroitin sulfate A (CSA) were from Sigma-Aldrich. Fondaparinux was purchased from VCU Health Systems pharmacy and used as such. Sucrose octasulfate (SOS) was from Santa Cruz Biotechnology (Santa Cruz, CA). NMR of these agents, especially fondaparinux and SOS, confirmed their purity of greater than 95%. Recombinant gD (amino acids 21 through 339) was obtained from ViroStat (Portland, ME). Stock solutions of gD was prepared in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% PEG8000. Sulfated NSGMs, synthesized earlier as reported in several papers, were used as stock solutions in dd H_2O (10–30 mM). Specifically, the synthesis of **5** and **6** is described in reference S1; that of **7** is described in reference S2; **9** in references S3 and S4; and NSGMs **10** – **13** in reference S5. Synthesis, purity and characterization of NSGM **8** is described below. The sulfated NSGMs were characterized using NMR and MS techniques. The purity of NSGMs was evaluated by LC-MS/MS and found to be >95%.

Cells and viruses

HeLa and HFF-1 (human foreskin fibroblasts; ATCC SCRC-1041) cells were grown in Dulbecco's modified Eagle medium (DMEM; Corning cellgro) supplemented with 10% and 15% fetal bovine serum (FBS; Atlanta Biologicals Inc.), respectively, and penicillin/streptomycin. VK2/E6E7 (human vaginal epithelial cells; ATCC CRL-2616) cells were grown in Keratinocyte-Serum Free media (Life Technologies) supplemented with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, $CaCl_2$ (final concentration 0.4 mM) and penicillin/streptomycin. Cells were grown at 37 °C and 5% CO_2, and trypsinized and passaged after reaching near-confluence. This study used the reporter based β-galactosidase-expressing

HSV-1 KOS (gL86) strain. Viral stocks were propagated at a low multiplicity of infection (MOI) in a complementing 79B4 Vero cell line, their titers were determined by plaque assay on Vero cells and they were stored at -80 °C. Viruses were diluted in Opti-MEM reduced serum media (Life Technologies) to 5 MOI for viral entry assays.

Affinity Studies

Fluorescence experiments were performed using a QM4 spectrofluorometer (Photon Technology International, Birmingham, NJ) in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG8000 at 37° C. The affinity of gD for **1**–**13** was measured using the change in the intrinsic tryptophan fluorescence (λ_{EX} = 280 nm, λ_{EM} =333 nm) as a function of ligand concentrations [L], as described earlier.[S6] Titrations were performed by adding aliquots of aqueous stock solutions of **1**–**13** to 200 μL of gD (175 nM) and monitoring the emission intensity. The excitation and emission slits were set to 1.0 mm. The observed change in fluorescence (ΔF) relative to initial fluorescence (F_O) was fitted using a standard binding equation to obtain the dissociation constant (K_D) and the maximal change in fluorescence (ΔF_{MAX}) at saturation. Each measurement was performed at least in triplicate. Negative control experiments included monitoring gD fluorescence upon addition of buffer aliquots parallel to experiments with the library of ligands.

Molecular Modeling

The 3D coordinates of gD were retrieved from the PDB (ID: 1JMA) and prepared for molecular modeling studies using the protein preparation tool in Tripos Sybyl-X v2.2 (www.tripos.com/sybyl). Hydrogen atoms were added and minimized. To identify potential GAG binding site on gD, the entire sequence was analyzed for the presence of consensus sequence pattern[S7] and the surface electrostatics were calculated using APBS tool in PyMOL (PYMOL

Molecular Graphics System, v1.5.0.4, Schrödinger, New York, NY). For molecular modeling experiments, we considered two sites (sub-site 1 and sub-site 2). All residues within 32 Å centered on Arg130 and Lys245 were defined as part of the GAG binding site 1 and 2. Molecules from both groups, sulfated saccharides (**1** and **2**) and NSGMs (flavonoid homodimers **5-6**, benzofuran homodimers **7-8**, glycosides or pseudo-glycosides **9-13)** were docked onto gD using GOLD v5.2, as described earlier.[S8 – S11] For each molecule a triplicate run comprising, 300 genetic algorithm run, 100,000 iterations were employed. Each docked pose was scored using GOLDSCORE and all docked poses were analyzed for consistency of binding with gD.

Viral Entry Assay

Individual cell types were seeded in a 96-well plate at an approximate density of 50,000 cells/well after overnight growth at 37 °C and 5% CO_2. The following day, cells were washed with 1× phosphate buffer saline (PBS) and replaced with Opti-MEM. Then cells were pre-treated with varying levels of **9** (0.195 to 100 μM) and incubated for 1 hr at 37° C and 5% CO_2. After 1 hr of incubation, the cells were washed with PBS and then challenged with HSV-1 KOS (gL86) strain in Opti-MEM at MOI of 5. After 6 hrs of challenge at 37° C and 5% CO_2, the cells were washed with PBS to remove extracellular viral particles followed by addition of 50 μL of ONPG (3 mg/ml ortho-nitrophenyl-β-galactoside, 0.6% NP40 in PBS). The plate was incubated at 37° C and 5% CO_2 for 1 hr and then read at 405 nm on the Multiskan FC Microplate Photometer (ThermoFisher). As a positive control, a column of wells was left untreated with **9** and infected. As a negative control, a column of wells was left untreated and uninfected. GraphPad Prism 7.0 was used to generate graphs, analyze results, and determine IC_{50} values. Each experiment was performed a minimum of 3 times and each data point was replicated 8 times.

Cytotoxicity Assay

Cellular toxicity of **9** was assayed using lactate dehydrogenase (LDH) cytotoxicity assay kit (Pierce, Thermo Scientific) as per the manufacturers' instructions. LDH is a cytosolic enzyme that is only released upon damage of the plasma membrane. All three cell types were plated at optimal cell density (15,000 cells/well) in triplicate and grown overnight in complete media followed by dosage-dependent treatment with and without **9**. 10× lysis buffer was used for maximum LDH activity controls, while 10 µL sterile water was used for the spontaneous LDH release controls. Following incubation at different time points, 50 µL supernatant was combined with 50 µL reaction mixture and the plate was incubated at room temperature for 30 minutes. The LDH activity was determined by measuring the absorbance at 492 nm and 620 nm on Multiskan FC Microplate Photometer and subtracting the 620 nm values from the 492 nm values to remove background. Each experiment was performed a minimum of three times.

Plaque Reduction Assay.

Vero cells were grown to confluence overnight in a 12 well plate and incubated with **9** (10 µM or 100 µM) for 1 h at 37□C in 5% CO_2, control wells were mock treated. Following treatment, cells were incubated with HSV-1 KOS (0.1 MOI) for 2 h with gentle shaking every 30 minutes. Virus inoculum was removed and cell monolayers were washed 3 times with PBS. Following infection, cells were incubated for 72 h in 1.5 ml overlay media containing DMEM supplemented with 5% heat-inactivated FBS, 1.2% Avicel RC-591 (DuPont, Wilmington, DE), and with either 10 µM or 100 µM **9**, 5µg Acyclovir (Sigma, St. Louis, MO), or mock treatment. After 72 h, cells were washed 3x in PBS and fixed for 5 minutes in methanol. Cell monolayers were then stained with Giemsa (Sigma, St. Louis, MO) and plaques were counted under 10x magnification. Viral inhibition (%) was calculated as follows: [1- (# plaques test / average #

plaques control)] x 100%, where “test” indicates the average number of plaques counted for cells treated with **9**, acyclovir, or both and “control” indicates the number of plaques counted for untreated, virus-infected cells.

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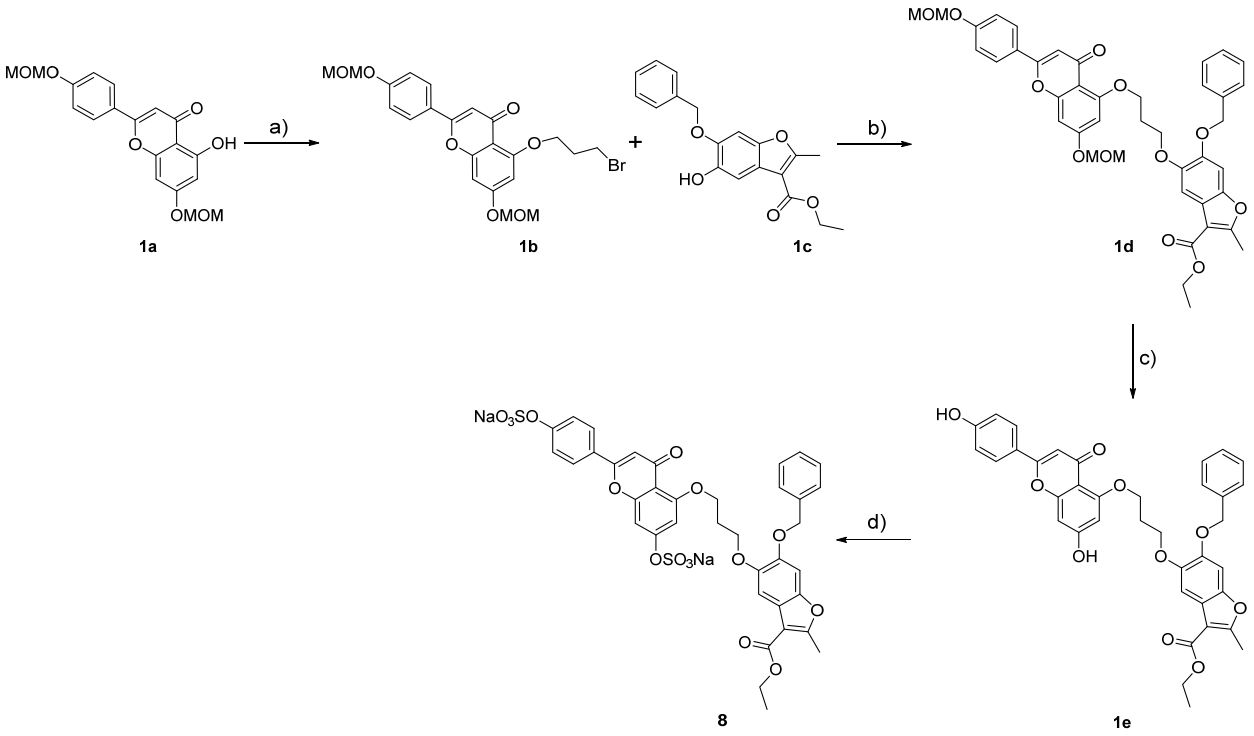
Synthesis and Characterization of NSGM 8.



Scheme S1. Synthesis of NSGM **8**. a) K_2CO_3 (5 eq.), 1,3-dibromo propane (4 eq.), DMF, rt/18 h, 60%, b) K_2CO_3 (2 eq.) rt/18 h, DMF,50%, c) TMS-Br (12 eq.) 0 ºC, 12 h, 70%, d) SO_3:Me_3N (12 eq.), Et_3N (20 eq.), CH_3CN, microwave, 90 ºC, 2 h,70%.

Synthesis of 1b

The synthesis of **1a** is described in S.R1. To a solution of **1a** (1.06 mM) in DMF was added potassium carbonate (5 eq.) and 1,3-dibromo propane (4 eq.). The reaction was stirred under an atmosphere of nitrogen for 18 hours, then quenched with 2N HCl and extracted with ethyl acetate. The organic layer was dried on an. Na_2SO_4 and then purified on silica gel (20–30% EtOAc in hexanes) to obtain **1b** in 60 % yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, *J* = 8.8 Hz, 2H), 7.09 – 7.07 (m, 2H), 6.96 – 6.93 (m, 1H), 6.78 – 6.76 (m, 1H), 6.46 – 6.45 (m, 1H), 5.22 (s, 2H), 5.18 (s, 2H), 4.16 (t, *J* = 6.1 Hz, 2H), 3.76 (t, *J* = 6.1 Hz, 2H), 3.46 (s, 3H), 3.43 (s, 3H), 2.37 – 2.31 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.70, 160.22, 159.76, 128.48, 116.69, 98.83, 95.93, 94.54, 94.27, 66.75, 56.62, 56.30, 31.86, 30.51.

Synthesis of 1d

The synthesis of **1c** is described in S.R2. To a solution of **1c** (0.313 mM) in DMF was added potassium carbonate (2 eq.) and **1b** (1 eq.), stirred under an atmosphere of nitrogen for 24 hours, then quenched with 2N HCl, extracted with ethyl acetate and dried using an. Na_2SO_4. The ethyl acetate extract was evaporated to dryness, purified on silica gel (25 – 35% EtOAc in hexanes) to obtain **1d** in 50% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 – 7.79 (m, 2H), 7.42 (s, 1H) 7.37 – 7.30 (m, 2H), 7.28 – 7.16 (m, 4H) 7.10 – 7.05 (m, 2H), 6.90 – 6.89 (m, 1H), 6.72 (s, 1H), 6.44 (s, 1H), 5.17 (m, 4H), 5.03 (s, 2H), 4.37 – 4.23 (m, 6H), 3.44 – 3.41 (m, 6H), 2.60 (s, 3H), 2.39 – 2.34 (m, 2H), 1.38 – 1.32 (m, 3H).

Synthesis 1e

To a solution **1d** in dichloromethane, TMS-Br (12 eq.) was added. The reaction mixture was stirred at -30ºC for 1 hour and then at 0 ºC for 12 – 18 hours. The reaction was monitored with MS until completion, purified on silica gel (5% MeOH in DCM) and evaporated to dryness to obtain **1e** in 70% yield.

^{1}H NMR (400 MHz, DMSO-d_6): δ 10.58 – 10.53 (br, 1H), 7.72 (d, J = 8.5 Hz, 2H), 7.37 – 7.17 (m, 7H) 6.81 (d, J = 8.5 Hz, 2H), 6.43 (s, 1H), 6.34 (s, 1H), 6.30 (s, 1H), 5.05 (s, 2H), 4.28 – 4.18 (m, 4H), 4.15 – 4.06 (m, 2H), 2.56 (s, 3H), 2.20 – 2.10 (m, 2H), 1.25 (t, J = 6.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.21, 161.99, 146.91, 146.68, 137.02, 128.33, 127.70, 127.44, 121.26, 118.17, 115.80, 107.40, 105.46, 98.09, 70.46, 66.09, 66.23, 59.96, 28.88, 14.10, 14.04.

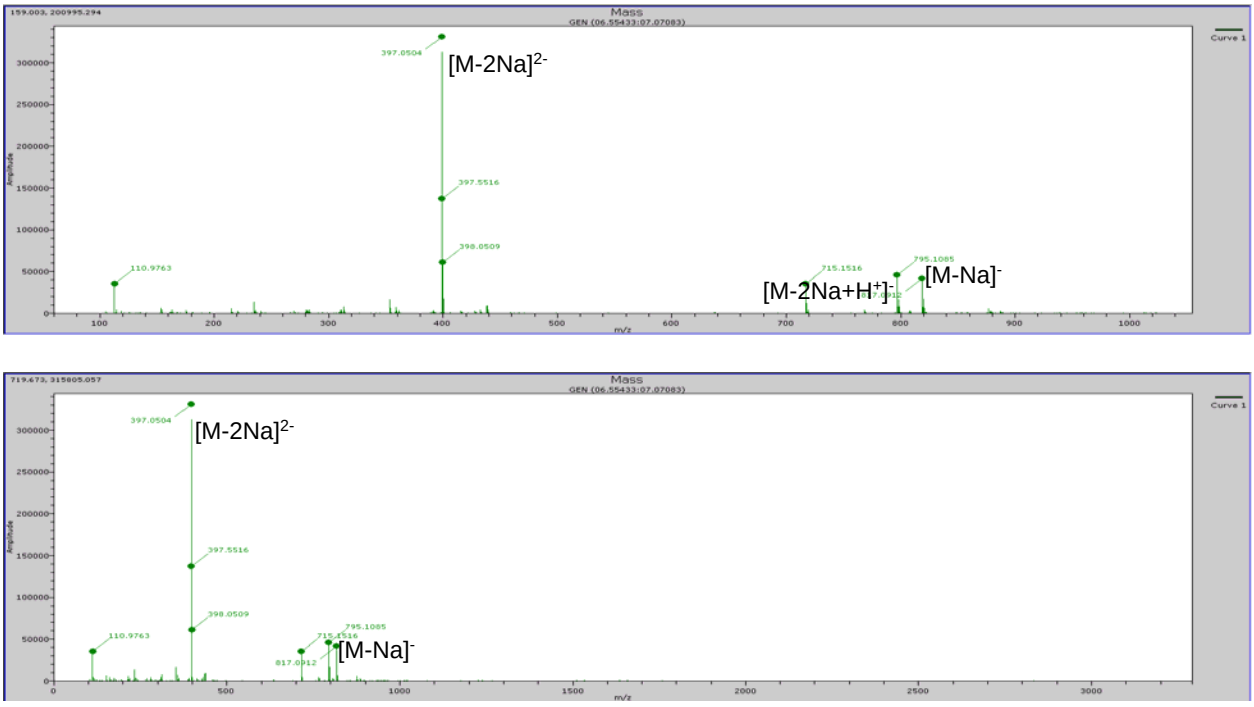
Synthesis of 8

To a solution of **1f** (0.08 mM) in anhydrous CH_3CN (~3 mL), Et_3N (20 eq) and SO_3/Me_3N complex (12 eq) were added. The reaction vessel was sealed and microwaved (CEM Discover, Cary, NC) for 2 hours at 90 ºC. The reaction mixture was cooled and concentrated *in vacuo* at temperature <30°C. The reaction mixture was then purified on Combiflash RF system (25 % MeOH in DCM) obtain 8 in 70% yield. The fractions were pooled together, concentrated *in vacuo*, and re-loaded onto a SP Sephadex C-25

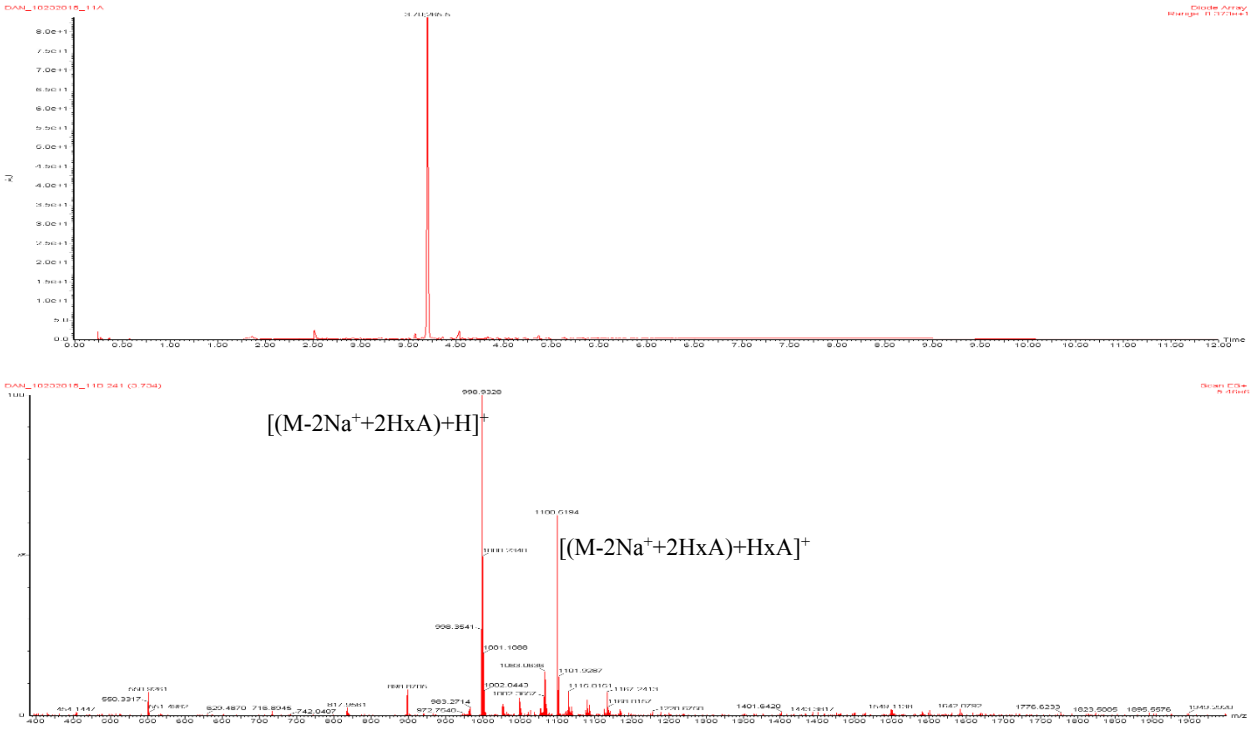
column for sodium exchange. Desired fractions containing 8 were pooled, concentrated *in vacuo*, and lyophilized to obtain a fluffy white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 7.93 (d, J = 8.4 Hz, 2H), 7.48 – 7.43 (m, 2H), 7.42 – 7.28 (m, 7H), 7.14 (d, J = 2.0 Hz, 1H), 6.72 (d, J = 2.0 Hz, 1H), 6.60 (s, 1H), 5.15 (s, 2H), 4.36 – 4.27 (m, 4), 4.21 (t, J = 5.8 Hz, 2H), 2.65 (s, 3H), 2.29 – 2.21 (m, 2H), 1.34 (t, J = 7.06 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.68, 161.98, 158.97, 158.14, 156.35, 147.61, 146.95, 146.67, 136.97, 128.38, 127.74, 127.52, 126.96, 125.03, 120.12, 118.11, 109.57, 107.21, 99.65, 70.48, 66.00, 65.43, 59.97, 14.10, 14.02. HRMS calculated for $C_{37}H_{30}Na_2O_{16}S_2$ $[(M\text{-}Na^+)]^-$, m/z 817.0873, m/z 817.0912; calculated for $C_{37}H_{30}Na_2O_{16}S_2$ $[(M\text{-}2Na^+)]^{2-}$, m/z 397.0488, found m/z 397.0504; calculated for $C_{37}H_{30}Na_2O_{16}S_2$ $[(M\text{-}2Na^+ + H^+)]^-$, m/z 795.1054, found 795.1085.

HRMS Profile for NSGM 8

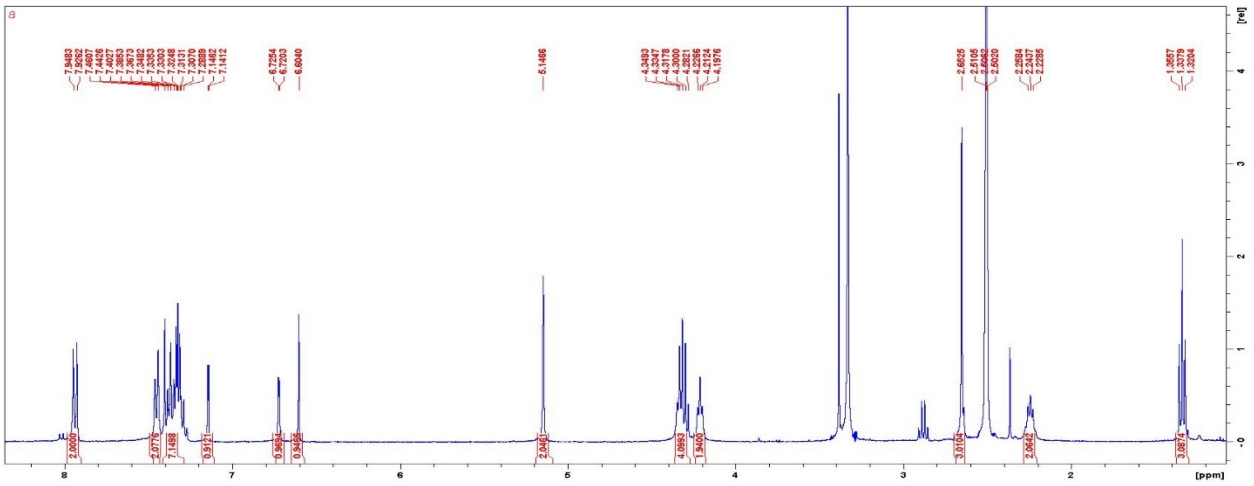


UHPLC-MS Profile for NSGM 8



ESI-MS calculated for $C_{37}H_{30}Na_2O_{16}S_2$ $[(M-2Na^+ +2HxA)+HxA]^+$, m/z 1100.4818, found m/z 1100.52; calculated for $[(M-2Na^+ +2HxA)+H]^+$, m/z 999.3614, found 998.93.

NMR Spectra for NSGM 8



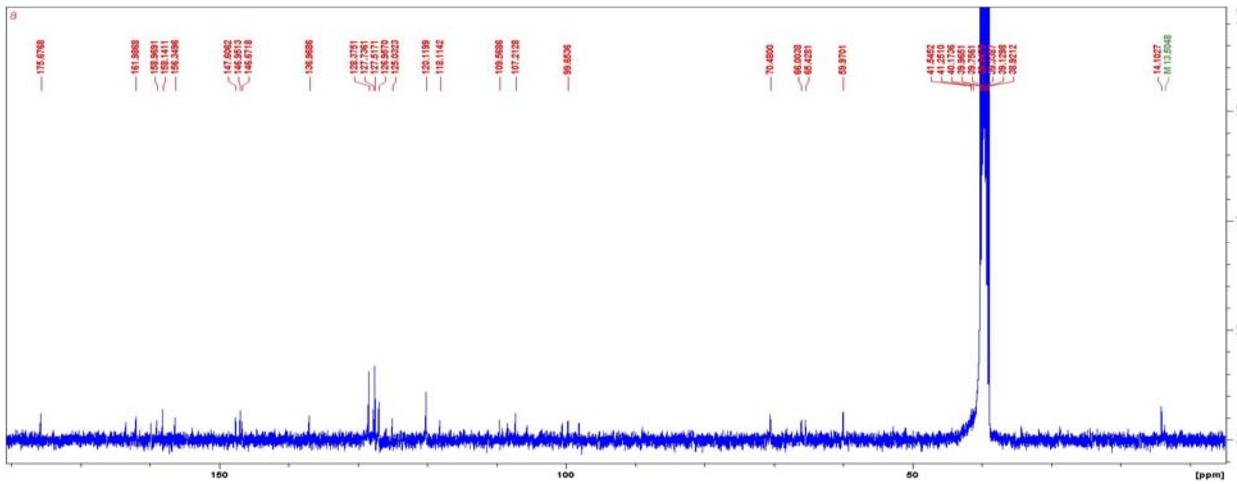

[rel]
1.5
1.0
0.5
-0.0
150
100
50
[ppm]

TABLE S1. GOLDSCORES of **1**, **2** and **5** – **13** following docking onto gD in sub-site 1 and sub-site 2.[a]

NSGM	Sub-site 1	Sub-site 2
1	100.21	93.51
2	84.19	82.62
5	120.00	113.39
6	116.11	105.00
7	89.99	87.18
8	76.48	84.74
9 (X = OH)	137.37	123.77
9 (X = OSO_3Na)	155.63	121.52
10	107.31	97.03
11	110.16	105.05
12	128.61	120.34
13	131.35	92.89

[a]The two sites are shown in Figure 3A. Sub-site 1 includes Lys1, Arg35, Arg130, Arg134, Arg229 and sub-site 2 includes Arg64, Arg67, His183, Arg184, Lys186, Arg190, Lys237, Arg245. For modeling all residues within 32 Å centered on Arg130 and Lys245 were defined sites 1 and 2.

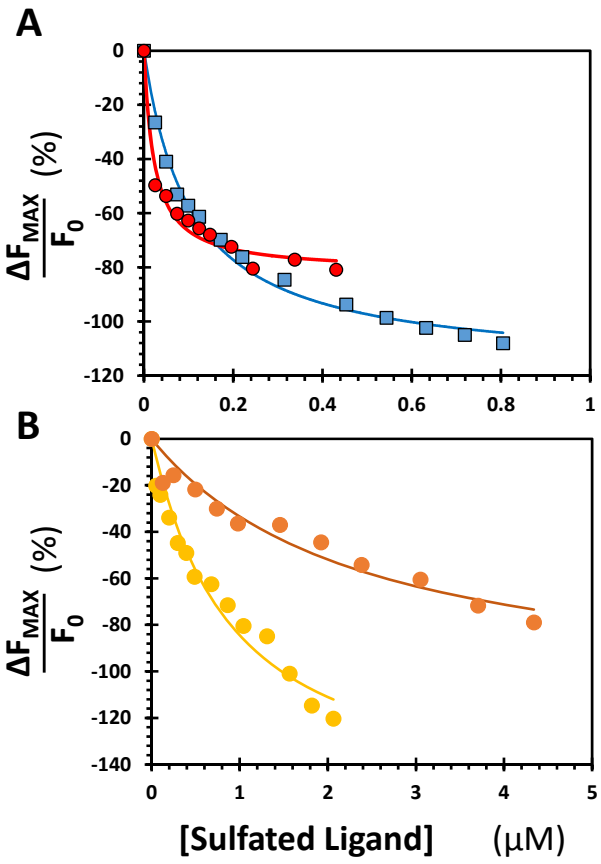


Figure S1. Spectrofluorometric titrations of gD binding to NSGMs **5** and **6** (A) and **7** and **8** (B) at pH 7.4 and 37 °C monitored by intrinsic fluorescence. Solid lines represent nonlinear regression to obtain K_D.

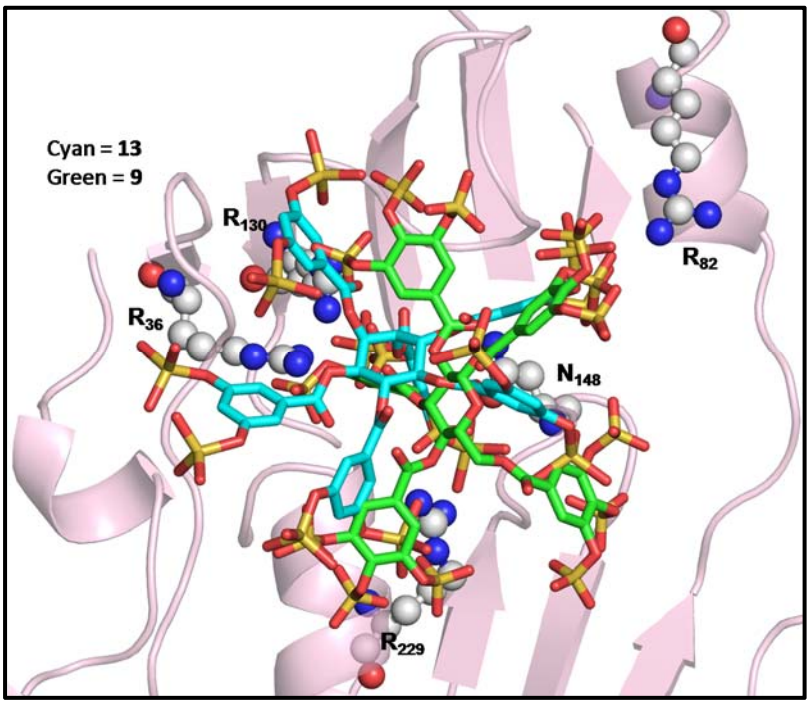


Figure S2. Comparison of most preferred binding poses of NSGMs 9 (green sticks) and 13 (cyan sticks) onto sub-site 1 of gD. Multiple sulfate groups are recognized by arginines 82, 229, 36 and 130 and asparagine 148. gD is shown in ribbon diagram.

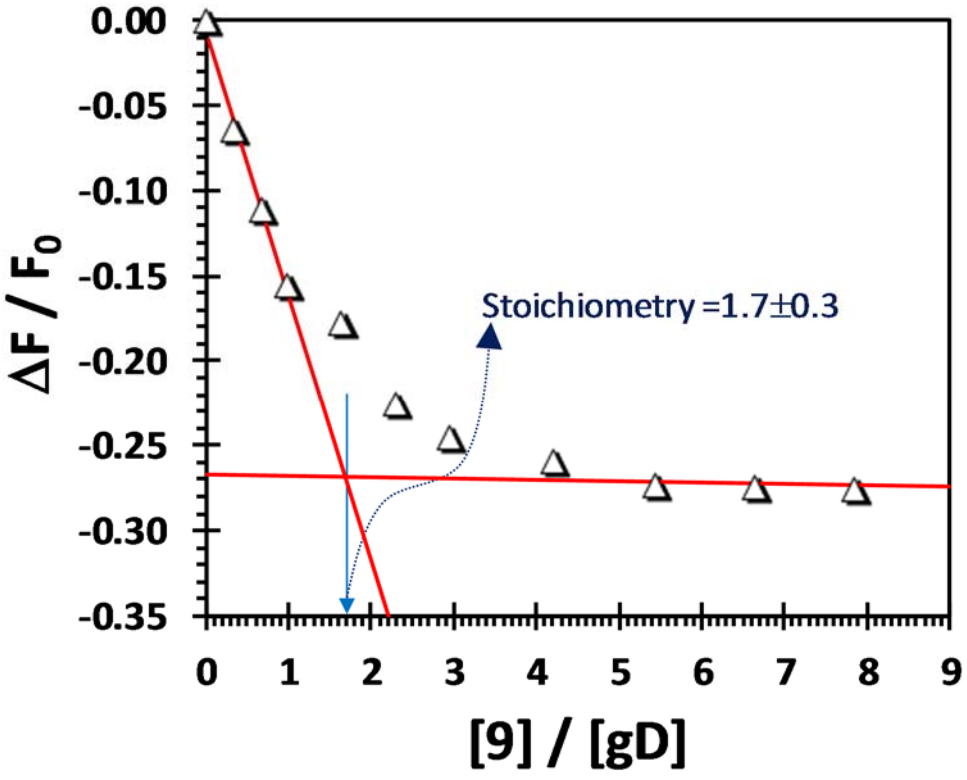


Figure S3. Spectrofluorometric titration of gD binding to NSGM **9** at pH 7.4 and 37 °C monitored by intrinsic fluorescence under stoichiometric conditions ([gD] >> K_D). Solid lines represent linear regressions in the low and high ligand ranges to calculate the stoichiometry of binding. The average molecular weight (M_R = 2178) of **9** was used in calculations, in the manner implemented for affinity measurement.

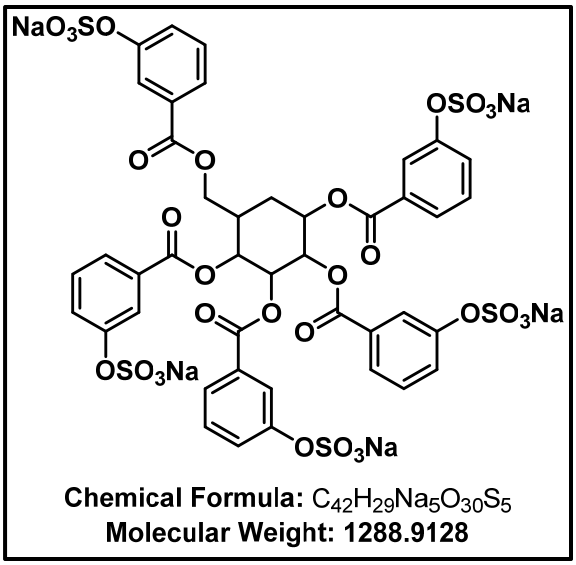


Figure S4. Structure of an NSGM analog designed on basis of common interactions deduced for **13** and **9**.

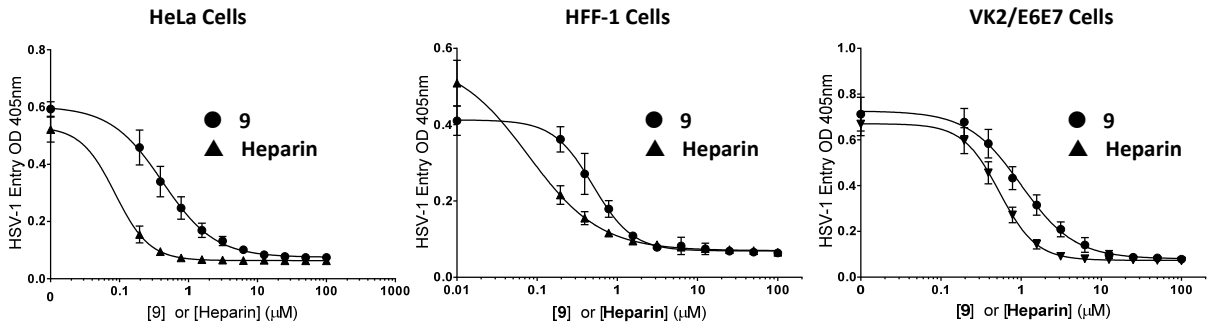


Figure S5. Comparison of anti-HSV-1 entry activity between **9** and unfractionated heparin. Cells were pre-treated with **9** or heparin for 1 hr at 37 °C and 5% CO_2, washed and challenged with HSV-1 KOS (gL86) for 6 hr. Viral entry was measured by the expression of β-galactosidase activity using colorimetry.

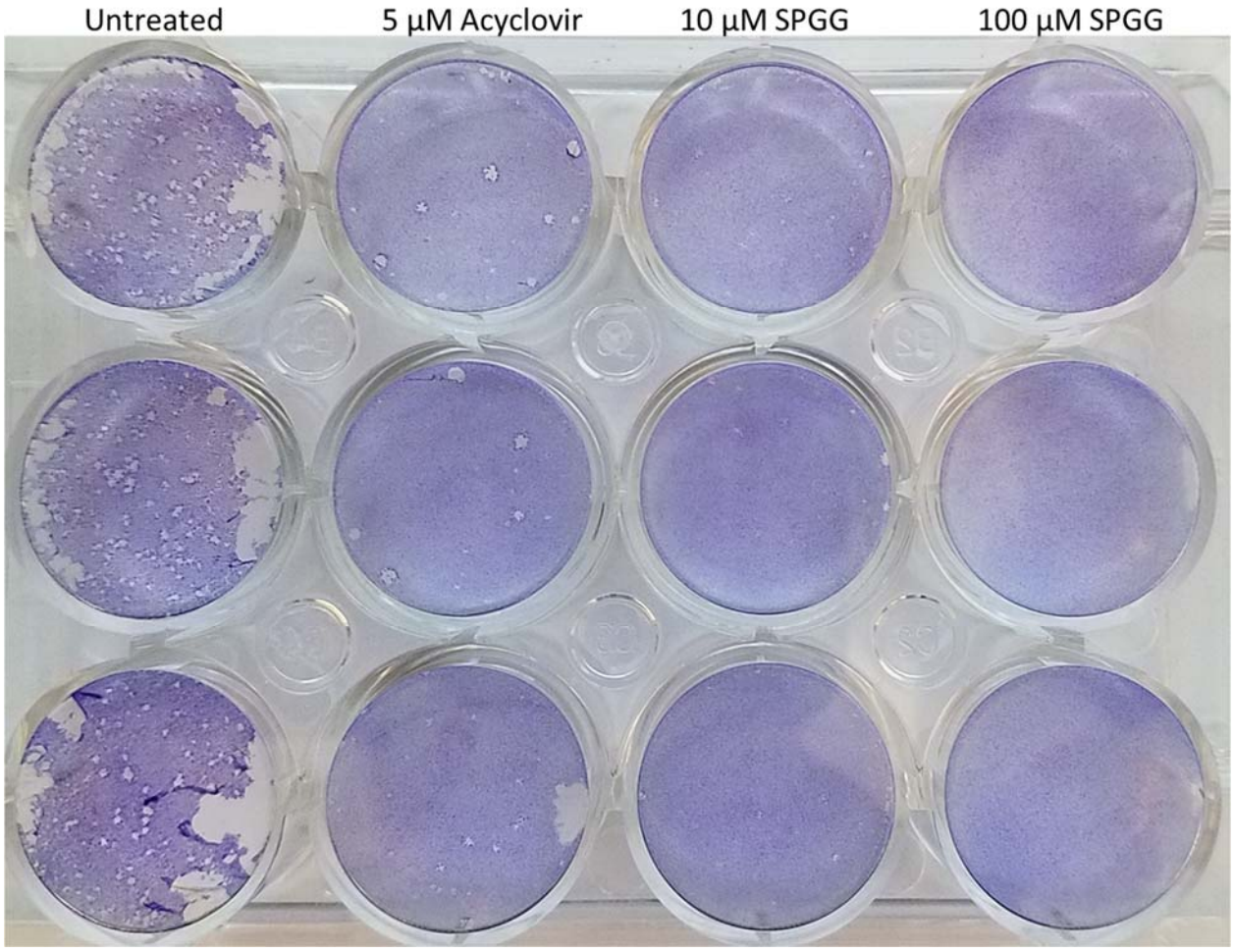


Figure S6. Plaque reduction by **9**. Confluent vero cells were incubated with **9** (0, 10 or 100 µM) for 1 h at 37°C in 5% CO_2 and then incubated with HSV-1 KOS (0.1 MOI) for 2 h. Following infection and washing, cells were incubated for 72 h and plaques were counted under 10x magnification.