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Tethered Neuraminidase Inhibitors which Bind Influenza Virus: A First Step Towards a New Diagnostic Method for Influenza

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Synthesis of 5-acetamido-7-(6'-biotinylaminohexyl)carbamoyloxy-4-guanidino-2,3,4,5-tetradeoxy-D-glycero-D-galacto-non-2-enopyranosonic acid (Compound **4a**)

(a) Compound **6** was prepared following the method of Andrews et al.[9] and isolated as a white foam.

IR ($CHCl_3$): 3328, 2734, 2099, 1799, 1732, 1682 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): δ = 1.20-1.40 (m, 4H), 1.40-1.60 (m, 13H), 2.07 (s, 3H), 3.01-3.42 (m, 5H), 3.84 (s, 3H), 4.50-4.85 (m, 4H), 4.93 (m, 2H), 5.03 (m, 1H), 5.41 (m, 1H), 5.95 (d, 1H), 6.48 (d, 1H).
MS (FAB): 599 $(M+1)^+$

(b) A mixture of compound **6** (500 mg, 0.836mmol), acetic acid (105 mg) and Palladium on charcoal (10%) (100 mg) was added to a mixture of methanol (30 mL) and toluene (18 mL) and the whole was agitated under an atmosphere of hydrogen for 1 hr. The reaction mixture was filtered through a pad of Celite, the filter cake was washed with methanol (50 mL) and the filtrate and washings were combined and evaporated to dryness under reduced pressure. The residue was subjected to flash column chromatography over silica-gel using ethyl acetate/isopropanol/water (5:2:1) as eluent to afford the 4-amino derivative (285 mg, 59.6%) as a white foam. The 4-amino compound (125 mg, 0.219mmol) was dissolved in methanol (15 mL) and treated with N,N'-bis-tert-butoxycarbonyl-1H-pyrazole-1-carboxamidine (310 mg, 0.996 mmol). The solution was stirred under argon at 30-35^oC for 5 days and then the solvent was removed under reduced pressure. The residue was subjected to flash column chromatography [SiO_2, hexane/ethyl acetate (1:8) and then ethyl acetate] to give the protected 4-guanidino compound **7** (149 mg, 83.4%) as a white foam.

IR ($CHCl_3$): 3313, 2978, 1805, 1727, 1643, 1610, 1558 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): δ = 1.11-1.82 (m, 35H), 1.91 (s, 3H), 2.98-3.21 (m, 4H), 4.13 (dd, 1H), 4.32 (dd, 1H), 4.50-4.80 (m, 3H), 4.91-5.22 (m, 3H), 5.45 (dd, 1H), 5.89 (d, 1H), 6.69 (br d, 1H), 7.63 (d, 1H), 8.48 (d, 1H).
MS (FAB): 815 $(M+1)^+$

(c) Compound **7** (176 mg, 0.216 mmol) was stirred in trifluoroacetic acid (TFA, 5 mL) under argon at room temperature for 1 hr and then the TFA was removed under reduced pressure to afford compound **8** as the bis TFA salt. The crude compound **8** was then treated with a solution of the N-hydroxy-succinimide ester of biotin (110 mg, 0.324 mmol) in water/acetone (1:2, 22.5 mL) containing sodium bicarbonate (27 mg, 0.32 mmol) and sodium carbonate (34 mg, 0.32 mmol) at room temperature for 3 hr. The mixture was evaporated to dryness under reduced pressure and the residue was stirred under argon with a mixture of methanol (25 mL) and water (25 mL) containing triethylamine (4 ml). After 16 hr the reaction mixture was evaporated to dryness under reduced pressure and the residue was subjected to column chromatography on silica-gel using firstly ethyl acetate/isopropanol/water (5:2:1) as eluent and then isopropanol/water (3:1). The fractions with R_f value of 0.25 in the final solvent system were collected, combined and evaporated to dryness. The residue from the combined fractions was

dissolved in water and treated with Dowex 50x8 (H+) resin (0.5 g) for 0.5 hr at room temperature. The resin was washed successively with water (50 mL), methanol (20 mL), water (10 mL) and then eluted with 2M NH_4OH. The eluate was evaporated to dryness and freeze-dried to afford compound **4a** (60 mg, 39.7%) as a white foam.

IR: 3330, 2934, 1674, 1616, 1429 cm^{-1}.
^1H NMR (300 MHz, D_2O): δ = 1.25-1.80 (m, 14H), 2.01 (s, 3H), 2.23 (dd, 2H), 2.78 (d, 1H), 2.98 (dd, 1H), 3.08-3.26 (m, 4H), 3.35 (m, 1H), 3.51 (dd, 1H), 3.68 (dd, 1H), 4.02-4.28 (m, 2H), 4.32-5.08 (m, 4H), 4.92 (dd, 1H), 5.76 (d, 1H).
MS (FAB): 701 $(M+1)^+$

Synthesis of 5-acetamido-7-{6'-[6"-(6'''-biotinylaminocaproyl-triaminocaproyl]-aminohexyl}carbamoyloxy-4-guanidino-2,3,4,5-tetradeoxy-D-glycero-D-galacto-non-2-enopyranosonic acid (Compound **4b)**

(a) To a solution of 6-[6'-(6"-tert-butoxycarbonyl-aminocaproyl)-diaminocaproyl]-aminocaproic acid (150 mg, 0.263 mmol) in methanol (5 mL) at -20°C were added successively potassium t-butoxide (30 mg, 0.267 mmol), N-methylmorpholine (15 mg, 0.148 mmol) and isobutyl chloroformate (40 mg, 0.293 mmol). The whole mixture was stirred under argon at -15 to -20°C for 20 min before being combined with a solution of compound **8** (140 mg, 0.238mmol) in a mixture of methanol (1.5 mL) and water (1.5mL) containing triethylamine (38 mg, 0.372 mmol) at 0-5°C. The resulting reaction mixture was stirred at 15-20°C for 4 h and then evaporated to dryness under reduced pressure. The residue was taken up into acetone (50 mL x 2), filtered and allowed to evaporate to give a white solid (224 mg) which was chromatographed (SiO_2, ethyl acetate/isopropanol/water (5:2:1)) to afford compound **9** (80 mg, 31.5%) as a white solid.

^1H NMR (300 MHz, $CDCl_3$): δ = 1.21-1.72 (m, 41H), 1.99 (s, 3H), 2.18 (t, 8H), 2.92-3.24 (m, 12H), 3.79 (s, 3H), 4.14 (dd, 1H), 4.51-4.78 (m, 4H), 5.19 (ddd, 1H), 5.59 (dd, 1H), 5.92 (d, 1H).
MS (FAB): 1068 $(M+1)^+$ 1067 (M^+)

(b) A solution of compound **9** (71.4 mg, 0.0667 mmol) in trifluoroacetic acid (2 mL) was stirred under argon at 20°C for 1 h and then evaporated to dryness. The residue was dissolved in water (5 mL) and evaporated to dryness under reduced pressure. The residue was dissolved in pyridine (6 mL) containing biotin-N-hydroxysuccinimide ester (36 mg, 0.105 mmol). The reaction mixture was stirred under argon at 40-50°C for 48 h and then evaporated to dryness under reduced pressure. The resulting residue was stirred in acetone (30 mL) at room temperature for 4 h and then filtered. The collected solid was washes with acetone (10 mL x 2) and air-dried to afford a white solid (72 mg). The solid was stirred under argon together with a mixture of methanol (25 mL), water (25 mL) and triethylamine (5 mL) for 16 h before being concentrated to dryness under reduced pressure. The crude product was subjected to chromatography (SiO_2, ethyl acetate/isopropanol/water (5:2:1) followed by isopropanol/water (3:1)) to afford the product **4b** (30.4 mg, 39.5%) as a white solid.

IR: 3292, 2931, 1638, 1541 cm^{-1}.
^1H NMR (300 MHz, D_2O): δ = 1.12-1.62 (m, 38H), 1.91 (s, 3H), 2.12 (m, 10H), 2.65 (d, 1H), 2.82-3.15 (m, 13H), 3.30-3.62 (m, 3H), 3.81-4.18 (m, 3H), 4.25-4.56 (m, 4H), 5.51 (d, 1H).
MS (FAB): 1153 $(M+1)^+$

Synthesis of (4-guanidino-Neu-5-Ac-2-en)-(6-aminohexyl)-7-carbamoyl-$(PEG)_{70}$-biotin conjugate (Compound **4c**)

(a) The bis-TFA salt of compound **8** (340 mg) was stirred under argon in a mixture of methanol (20 mL) and water (20 mL) containing triethylamine (10 mL) at room temperature for 6 h. The reaction mixture was evaporated to dryness and then the residue dissolved in water (1 mL) and freeze-dried to give the deprotected (6-aminohexyl)-7-carbamoyl-4-guanidino-Neu-5-Ac-2-en (compound **10**) as a white solid.

^1H NMR (300 MHz, D_2O): δ = 1.27-1.81 (m, 8H), 1.98 (s, 3H), 2.89-3.21 (m, 4H), 3.51 (dd, 1H), 3.69 (dd, 1H), 4.04 (m, 1H), 4.18 (dd, 1H), 4.46 (dd, 1H), 4.57 (dd, 1H), 4.96 (dd, 1H), 6.01 (d, 1H).
MS (FAB): 475 $(M+1)^+$, 589 $(M+1+TFA)^+$

(b) The aminohexyl compound from part (a) (20 mg, 0.025 mmol) and biotin-$(PEG)_{70}$-CO_2-N-hydroxysuccinimide ester (70 mg, 0.019 mmol, from Shearwater Cat. No. 0H2Z0F02) were dissolved in a mixture of pyridine (5 mL), DMF (1.5 mL) and DMSO (0.4 mL). The solution was stirred at room temperature for 72 h and then evaporated almost to dryness under reduced pressure. The crude product was purified by chromatography on a Sephadex G-25 column (13 x 310 mm) using water with a flow rate of 1.8 mL/8 minute and collecting fractions of 2.0 mL. Fractions were analysed by thin layer chromatography and fractions 6-8 were combined and freeze-dried to give compound **4c** (32 mg, 36%) as a white solid.

^1H NMR (300 MHz, D_2O): δ = 1.25-1.75 (m, 14H), 1.93 (s, 3H), 2.24 (m, 2H), 2.75 (d, 1H), 2.96 (dd, 1H), 3.0-3.12 (m, 4H), 3.25-3.5 (m, 8H), 3.7 (bs, approx 300H), 3.90 (m, 4H), 4.05 (m, 1H), 4.17 (bs, 2H), 4.35-4.41 (m, 1H), 5.62 (d, 1H).

Synthesis of 5-acetamido-7-{6'-[6"-(6'''-aminocaproyl-triaminocaproyl]-aminohexyl}carbamoyloxy-4-guanidino-2,3,4,5-tetradeoxy-D-glycero-D-galacto-non-2-enopyranosonic acid (Compound **5a)**

A solution of compound **9** (29.5 mg, 0.027 mmol) in trifluoroacetic acid (2 mL) was stirred under argon at 20°C for 1 h and then evaporated to dryness. The residue was dissolved in water (5 mL) and evaporated to dryness under reduced pressure and the resulting residue re-dissolved in water (2 mL) and freeze dried to give a fluffy white solid. This solid was dissolved in a mixture of methanol (10 mL), water (10 mL) and triethylamine (5 mL) and the solution stirred under argon at 20°C for 16 h. The reaction mixture was evaporated to dryness under reduced pressure, re-dissolved in water (10 mL) and evaporated to dryness again. The residue was triturated successively in acetone (3 x

20 mL) and ethanol (20 mL) then freeze-dried to afford compound **5a** (14 mg, 56%) as a white solid.

^{1}H NMR (300 MHz, D_2O): δ = 1.15-1.69 (m, 32H), 1.90 (s, 3H), 2.11-2.23 (m, 8H), 2.91-3.21 (m, 12H), 3.41-3.70 (m, 2H), 3.85-4.12 (m, 2H), 4.48-4.52 (m, 2H), 4.92 (dd, 1H), 5.58 (d, 1H).
MS (FAB): 927 $(M+1)^+$

Preparation of Compound **5b** – (Microspheres coated with Compound **5a)**

N-Hydroxysuccinimide (3.2 mg, 27.5μmol) was added to a suspension of carboxy functionalized microspheres (Bangs Laboratories Cat. No. P0000320CN; 100μL, 550 nmol of CO_2H) in activation buffer (0.05M MES/0.05% Tween 20, 80 μL). A solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.0mg, 11μmol) in the same buffer (50 μL) was added and the reaction was kept at 20^{o}C for 1.5 h. A solution of the TFA salt of compound **5a** (6 mg, 5.5μmol) in sodium bicarbonate (1M, 60μL) was added to the beads and then extra sodium bicarbonate (3 mg) was added and the reaction mixture was kept at 20^{o}C for 4 h and then at 4^{o}C overnight. The reaction was quenched with an aqueous solution of ethanolamine (0.9M, 61μL) for 0.25 h and then placed into dialysis tubing and the beads were purified by dialysis for two days in water containing 0.05% Tween. Microspheres **5b** were thus obtained as a suspension in water (0.9 mL) containing 0.14 μmol of attached 4-guanidino-Neu-5-Ac-2-en (assuming 25% coupling efficiency).