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Synthesis of Methyl α-Sialosides N-substituted with Large Alkanoyl Groups, and Investigation of Their Inhibition of Agglutination of Erythrocytes by Influenza A Virus.[1]

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Abstract: The synthesis of a series of new inhibitors of the binding of influenza virus to erythrocytes is described. The inhibitors are derivatives of sialic acid with C_4 - C_{14} alkanoic acid groups attached at the N5 position as acyl groups. These molecules were evaluated for their ability to prevent virus-induced agglutination of erythrocytes. There appears to be a correlation between increased chain length and increased inhibition of virus-induced agglutination of erythrocytes These derivatives did not, however, show increased binding to BHA, a soluble form of the lectin hemagglutinin that is responsible for the attachment of virus to cell.

Introduction

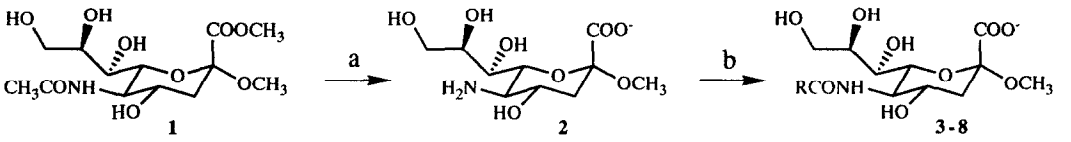
Influenza A remains a serious disease in man.[4] While the infectivity of type B and C viruses is concentrated in man, type A viruses also infect birds, horses, chickens, pigs and whales.[4] The membrane of the influenza A viruses consists of a lipid bilayer (derived from the host cell during maturation) that presents two glycoproteins on its surface: (i) hemagglutinin[5] (HA; a trimer) and (ii) neuraminidase[6] (NA, a tetramer, E.C. 3.2.1.18). In the course of an infection, viral HA initially binds to sialic acid (SA, N-acetylneuraminic acid) residues attached to glycoproteins and glycolipids on the surface of the host cell. Inhibitors of this association that bind tightly to HA would be potential inhibitors of influenza infection. Inhibitors utilizing bidentate[7] and polyvalent[8-11] derivatives of sialic acid have been reported. A number of analogs of monomeric sialic acid have also been investigated for their inhibitory potential. To date, however, the most effective monovalent inhibitors of hemagglutination are the α-glycosides of sialic acid itself.[12-16]

Results and Discussion

The binding of simple α-sialosides to HA is weak: α-methyl-sialic acid has a K_d = 2.8 mM.[13] We hypothesized that the attachment of long(er) chain alkanoyl residues to the amino group on C-5 of sialic acid might lead to tighter binding species, and therefore be better inhibitors of virus-induced agglutination of erythrocytes. An increase in binding would reflect non-specific hydrophobic interactions between the N-alkanoyl chain attached to the sialoside and lipophilic regions present on the protein surface close to the sialic acid binding site of HA.[17]

A series of N-substituted sialic acids[18] varying in the length of the alkyl group of their amide moieties was synthesized (Scheme I). The α-methyl glycoside of sialic acid **1** was treated with a methanolic solution of tetramethylammonium hydroxide[9] to afford the free amino derivative **2**. Acid residues were attached to the amino function of **2** by stirring and/or refluxing the corresponding N-hydroxysuccinimide esters with amino derivative **2** in a mixture of water and THF, while maintaining the pH of the reaction at 8.0 - 8.5 (see Experimental). The synthesis of the activated esters was straightforward. The preparation and purification of the sialic acid derivatives with chain lengths 10 carbons or longer (**6** - **8**) was complicated by their tendency to form micelles.

Scheme I



a. $(CH_3)_4NOH$, Δ ; b. RCOOSu, H_2O, THF; pH 8.5

3) R = $CH_3(CH_2)_2$-
4) R = $CH_3(CH_2)_4$-
5) R = $CH_3(CH_2)_6$-
6) R = $CH_3(CH_2)_8$-
7) R = $CH_3(CH_2)_{10}$-
8) R = $CH_3(CH_2)_{12}$-

We tested α-methyl sialic acid and compounds **3** - **8** for their potency for inhibiting the agglutination of chicken erythrocytes caused by influenza X-31 virus.[19-20] The results are summarized in Figure 1. The data indicate a significant increase in inhibitory activity for the long-chain (C_{10} and longer) α-methyl sialic acid derivatives **6** - **8**. Since the N-decanoyl derivative **6** is still relatively water soluble, it is the most easily handled of these derivatives. Longer N-alkanoyl chain lengths attached to SA lead to increased inhibitory strength in the HAI assay, but these derivatives become increasingly more difficult to prepare and isolate and are, of course, progressively less soluble in water. The N-myristoyl compound **8** was the longest alkyl chain derivative accessible using the "activated ester" method.

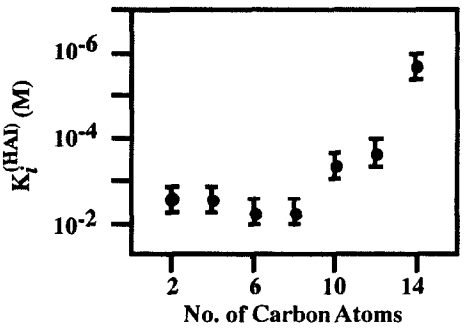


Figure 1. Correlation between the length of alkyl substitution at N5 of sialic acid and the lowest concentration of the compount that inhibits the virus-induced hemagglutination $K_i^{(HAI)}$ using a 96-well plate assay.

We do not know if the increase in inhibition of hemagglutination by derivatives **6** - **8** is caused by an additional interaction of the long chain amide residue to lipophilic regions on the HA surface, to the formation of micelles, or to some other effect. Low resolution X-ray crystallographic studies failed to show binding of these derivatives at the first hemagglutinin binding site,[5] or at other positions on the protein surface.[21] When the N-myristoyl compound **8** was incorporated into liposomes[22] no inhibition of hemagglutination took place ($K_i^{(HAI)}$> 1mM). Finally, N-octanoyl derivative **5** was unable to displace a fluorescent ligand from the active site of bromelaine-cleaved hemagglutinin (BHA) even at concentrations above 5 mM.[23] It appears that these compounds do not bind directly at the sialic acid binding site of HA.

Conclusion

The investigation of a series of different N-alkanoyl substituted sialosides showed a significant dependence of the ability of the compounds to inhibit hemagglutination on the chain length of the alkanoyl group. The origin of this inhibition is not known, and it may represent an effect unrelated to the binding of erythrocyte-surface sialic acid moieties to viral hemagglutinin. Two pieces of evidence -- the absence of binding of **5** to BHA in a competitive flourescence assay, and the absence of electron density corresponding to **8** in an initial single crystal x-ray experiment -- indicate that binding probably does not occur at the sialic acid binding site of HA. We hypothesize that inhibition may result from interaction of these amphiphilic compounds with other hydrophobic residues present on the surface of viral hemagglutinin, perhaps close to the binding site for sialic acid.

These results establish a new class of derivatives of sialic acid capable of inhibiting agglutination of erythrocytes but they make the cautionary point that inhibition of this process need not necessarily involve interaction at the sialic acid binding site of HA.

Experimental

General Methods. Reagent grade chemicals were purchased from Aldrich. Biogel P2 was purchased from BioRad. Solvents were dried and distilled before use. Analytical thin layer chromatography was performed on Merck plates (silica gel F_{254}, 0.25 mm). Compounds that were not visualized by UV were detected by spraying with a solution of 3% $Ce(SO_4)_2$ in 2*N* H_2SO_4 followed by heating to 200 °C, or by spraying with a mixture of ninhydrin (0.3 g) and acetic acid (3 mL) in ethanol (100 mL) followed by heating. Flash chromatography was performed using Merck Silica gel 60 (0.04 - 0.06 mm mesh). ^{1}H NMR chemical shifts are reported in parts per million from internal TMS (δ = 0.0) with $CDCl_3$ as solvent and the HOD peak (δ = 4.8) with D_2O as solvent. ^{13}C NMR spectra chemical shifts are reported in parts per million relative to $CDCl_3$ (δ = 77.0) with $CDCl_3$ as solvent and relative to external dioxane (δ = 66.5) with D_2O as solvent. Phosphate buffered saline (PBS) used in the HAI assay was prepared from 80 g NaCl, 2 g KCl, 11 g Na_2HPO_4 and 2 g KH_2PO_4 in 1L of distilled H_2O. This stock solution was diluted 1 part in 10 in distilled H_2O and then adjusted to pH 7.2 with 1N NaOH. Chicken erythrocytes was purchased from Spafas, Inc. (Storrs, CT 06268-1198, Tel. 203-429-1990). Influenza virus X-31 was a generous gift from Prof. D. C. Wiley and Prof. J. J. Skehel.

General procedure for the preparation of N-hydroxysuccinimide esters of alkanoic acids (C_n-OSu). To a stirred solution of N-hydroxysuccinimide (10 mmol) and triethylamine (1.5 mL) in CHCl3 (25 mL) was added the corresponding acyl chloride (10 mmol) while keeping the reaction mixture at 0°C. The mixture was allowed to warm up to RT over 1 hour followed by the addition of solid $NaHCO_3$ (200 mg). Stirring was continued for 10 minutes. The precipitate was removed by filtration and the filtrate concentrated under vacuum. The residue was purified by chromatography over silica gel (40 g) using ethyl acetate:methanol (9:1).

N-Butyroyloxysuccinimide (C_4-OSu). Yield: 92 %. ^{1}H NMR ($CDCl_3$) δ 2.78 (bs,4H), 2.53(t, 2H, αCH_2), 1.73(dt, 2H, ßCH_2), 0.99 (t, 3H, CH_3). ^{13}C NMR($CDCl_3$): δ 169.19, 168.45, 32.63, 25.48, 18.11, 13.24.

N-Hexanoyloxysuccinimide (C_6-OSu). Yield: 100 %. ^{1}H NMR ($CDCl_3$) δ 2.78 (bs, 4H), 2.55(t, 2H, αCH2), 1.69 (dt, 2H, ßCH2), 1.36 - 1.29 (m, 4H, 2xCH_2), 0.86 (t, 3H, CH_3). ^{13}C NMR($CDCl_3$): δ 169.18, 168.63, 30.79, 25.50, 24.15, 22.05, 13.72.

N-Octanoyloxysuccinimide (C_8-OSu). Yield: 95 %. ^{1}H NMR($CDCl_3$) δ 2.79 (bs, 4H), 2.55 (t, 2H, αCH2), 1.69 (m, 2H, ßCH2), 1.42 - 1.18 (m, 8H, 4xCH_2), 0.84 (t, 3H, CH3). ^{13}C NMR($CDCl_3$) δ 168.99, 168.57, 31.47, 30.91, 28.69, 25.57, 24.57, 22.45, 13.89.

N-Decanoyloxysuccinimide (C_{10}-OSu). Yield: 96 %. ^{1}H NMR($CDCl_3$) δ 2.80 (bs, 4H), 2.56 (t, 2H, αCH_2), 1.71 (m, 2H, ßCH_2), 1.40 - 1.19 (m, 12H, 6xCH2), 0.84 (t, 3H, CH_3). ^{13}C NMR($CDCl_3$) δ 169.17, 168.66, 31.77, 30.88, 29.25, 29.15, 28.73, 25.53, 24.52, 22.60, 14.05.

N-Lauroyloxysuccinimide (C_{12}-OSu). Yield: 81 %. ^{1}H NMR($CDCl_3$) δ 2.78 (bs, 4H), 2.55 (t, 2H,b αCH2), 1.69 (m, 2H, ßCH_2), 1.36 (m, 2H, CH_2), 1.31 - 1.19 (m, 14H, 7xCH_2), 0.83 (t, 3H, CH_3). ^{13}C NMR(CDCl3) δ 169.15, 168.60, 31.79, 30.82, 29.44, 29.24, 29.21, 28.98, 28.68, 25.40, 24.47, 22.57, 14.00.

N-Myristoyloxysuccinimide (C_{14}-OSu). Yield: 84 %. ^{1}H NMR($CDCl_3$) δ 2.81 (bs, 4H), 2.58 (t, 2H, αCH2), 1.72 (m, 2H, ßCH_2), 1.38 (m, 2H, CH_2), 1.30 - 1.21 (m, 18H, 9xCH_2), 0.85 (t, 3H, CH_3). ^{13}C NMR($CDCl_3$) δ 169.15, 168.64, 31.87, 30.90, 29.58, 29.50, 29.30, 29.04, 28.75, 25.54, 24.53, 22.65, 14.08.

Methyl 5-amino-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosidonic acid (2). To a solution of **1** (310 mg, 0.96 mmol) in water (6 mL) was added a solution of 25 wt% tetramethylammonium hydroxide in methanol (1 mL). The reaction mixture was heated at reflux for 24 h; the progress of the reaction was monitored by TLC using ninhydrin (CH_3CN:10%AcOH 4:1; Rf_2=0.4). The crude reaction mixture was neutralized with acetic acid and desalted by passing through a column of Biogel P2 (30 g). Lyophilization yielded 345 mg of **2** (perhaps as a hydrate).
^{1}H NMR(400 MHz, D_2O) δ 3.91 - 3.53 (m, 6H, $H_{4,6,7,8,9a,9b}$), 3.26 (s, 3H, OCH_3), 2.97 (t, 1H, J = 9.9 Hz, H_5), 2.65 (dd, 1H, J = 4.5 Hz, J = 12.4 Hz, H_{3eq}), 1.56 (dd, 1H, J = 12.3 Hz, H_{3ax}). ^{13}C NMR(D_2O) δ 174.31, 101.84, 73.61, 72.82, 69.26, 69.02, 63.50, 53.51, 52.69, 41.21.

General procedure for the preparation of the N-substituted derivatives 3-8. To a solution of amine **2** (25 mg, 0.08 mmol) in water (3 mL), adjusted to pH 8 by addition of 0.1 N NaOH, was added a solution of the corresponding C_n-OSu ester (0.1 mmol) in THF (1 mL) and the reaction mixture stirred and/or refluxed for various times as detailed under each of the compound. The reaction was monitored by TLC (CH_3CN:10%AcOH 5:1). On completion of the reaction, the entire reaction mixture was loaded on a Biogel P2 column (50 g) and eluted with distilled water. The fractions containing the product were pooled, the volume reduced to 2-3 mL and a second chromatography over Biogel P2 performed. The pooled fractions was concentrated under vacuum, the residue redissolved in methanol (1 mL) and passed over silica gel (2 g) using a mixture of acetonitrile and methanol (4:1) as eluent.

Methyl 5-N-butyroyl-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosidonic acid (3). The reaction was stirred for 24 h at RT. Yield: 70 %. ^{1}H NMR(400 MHz, D_2O) δ 3.90-3.54 (m, 7H, $H_{4,5,6,7,8,9a,9b}$), 3.33 (s, 3H, OCH3), 2.70 (dd, 1H, J = 4.5 Hz, J = 12.5 Hz, H_{3eq}), 2.26 (m, 2H, CH_2), 1.65-1.52 (m, 3H, CH_2, H_{3ax}), 0.95 (t, 3H, CH_3). ^{13}C NMR(100 MHz, D_2O) δ 73.69, 72.73, 69.40, 69.14, 63.64, 52.89, 41.36, 38.89, 20.07, 14.05, C-1 and C=O were not detected, high resolution mass spectrum (FAB) *m/z* 350.1441 [(M-H), calcd for $C_{14}H_{24}NO_9$, 350.1451].

Methyl 5-N-hexanoyl-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosidonic acid (4). The reaction mixture was stirred for 24 h at RT. Yield: 55 %. ^{1}H NMR(400 MHz, D_2O) δ 3.90-3.50 (m, 7H, $H_{4,5,6,7,8,9a,9b}$), 3.30 (s, 3H, OCH_3), 2.67 (dd, 1H, *J* = 4.68 Hz, *J* = 12.40 Hz, H_{3eq}), 2.25 (t, 2H, CH_2), 1.62-1.52 (m, 3H, CH_2, H_{3ax}), 1.29-1.20 (m, 4H, 2xCH_2), 0.84 (t, 3H, CH_3). ^{13}C NMR(100 MHz, D2O) δ 179.55, 174.53, 101.76, 73.71, 72.76, 69.48, 69.11, 63.72, 52.89, 52.64, 41.38, 36.93, 31.59, 26.15, 22.73, 14.28, high resolution mass spectrum (FAB) *m/z* 378.1755 [(M-H), calcd for $C_{16}H_{28}NO_9$, 378.1764].

Methyl 5-N-hexanoyl-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosidonic acid (5). The reaction mixture was stirred for 18 h at room temperature and then refluxed for 1h. Yield: 85%. ^{1}H NMR(400 MHz, D_2O) δ 3.87-3.50 (m, 7H, $H_{4,5,6,7,8,9a,9b}$), 3.32 (s, 3H, OCH_3), 2.69 (dd, 1H, *J* = 4.50 Hz, *J* = 12.50 Hz, H_{3eq}), 2.26 (t, 2H, CH_2), 1.65-1.50 (m, 3H, CH_2, H_{3ax}), 1.30-1.18 (m,8H,4xCH_2), 0.83 (t,3H,CH3). ^{13}C NMR (100 MHz, D_2O) δ 179.57, 174.44, 101.77, 73.72, 72.79, 69.54, 69.10, 63.80, 52.89, 52.65, 41.38, 36.96, 32.05, 29.25, 29.15, 26.46, 23.02, 14.44, high resolution mass spectrum (FAB) *m/z* 406.2071 [(M-H), calcd for $C_{18}H_{32}NO_9$, 406.2077].

Methyl 5-N-decanoyl-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosidonic acid (6). The reaction mixture was stirred for 48 h at RT. Yield: 43%. NMR showed very broad signals due to micelle formation. ^{1}H NMR (400 MHz, D2O) d 3.93-3.52 (bm, 7H, $H_{4,5,6,7,8,9a,9b}$), 3.32 (bs, 3H, OCH_3), 2.71 (bdd, 1H, H_{3eq}), 2.28 (bt, 2H, CH_2), 1.68-1.55 (bm, 3H, CH_2, H_{3ax}), 1.45-1.20 (bm, 12H, 6xCH_2), 0.84 (bt, 3H, CH_3), high resolution mass spectrum (FAB) *m/z* 434.2393 [(M-H), calcd for $C_{20}H_{36}NO_9$, 434.2390].

Methyl 5-N-lauroyl-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosidonic acid (7). The reaction mixture was stirred for 72 h at RT and then refluxed for 48 h. Yield: 21%. NMR showed very broad signals due to micelle formation. ^{1}H NMR (400 MHz, D_2O) δ 3.91-3.50 (bm, 7H, $H_{4,5,6,7,8,9a,9b}$), 3.32 (bs, 3H, OCH_3), 2.71 (bdd, 1H, H_{3eq}), 2.28 (bm, 2H, CH_2), 1.66-1.53 (bm, 3H, CH_2, H_{3ax}), 1.33-1.15 (bm, 16H, 8xCH_2), 0.83 (bt, 3H, CH_3), high resolution mass spectrum (FAB) *m/z* 462.2708 [(M-H), calcd for $C_{22}H_{40}NO_9$, 462.2703].

Methyl 5-N-myristoyl-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosidonic acid (8). The reaction mixture was stirred for 72 h at RT and then refluxed for 72 h. Yield: 39%. NMR showed very broad signals due to micelle formation. ^{1}H NMR(400 MHz, D_2O) δ 3.96-3.42 (bm, 7H, $H_{4,5,6,7,8,9a,9b}$), 3.33 (bs, 3H, OCH_3), 2.71 (bdd, 1H, H_{3eq}), 2.27 (bm, 2H, CH_2), 1.70-1.52 (bm, 3H, CH_2, H_{3ax}), 1.40-1.18 (bm, 20H, 10xCH_2), 0.85 (bt, 3H, CH_3), high resolution mass spectrum (FAB) *m/z* 490.3016 [(M-H), calcd for $C_{24}H_{44}NO_9$, 490.3016].

Hemagglutination inhibition (HAI) assay. Compounds **1** and **3 - 8** were each dissolved in PBS to make stock solutions that were 40 mM in the inhibitor. The following operations were done at 4°C. The HA titer of a stock solution of X-31 influenza virus (in PBS) was determined by serial dilution of 100 μL of the virus solution in 100 μL PBS. A suspension of chicken erythrocytes in PBS (100 μL) was added to each well, mixed and incubated for 1 h. The HA endpoint is defined as the last well before erythrocyte pellets begin to form.

The inhibitors (50 μL) were serially diluted in 50 μL of PBS using a 96-well plate set-up. To each well was added 50 μL of virus X-31. The virus and the inhibitor were incubated for 30 min after which 100 μL of 0.5% chicken erythrocytes in PBS was added and mixed. The concentration of virus X-31 in this mixture is equal to its concentration at the HA endpoint. The wells were examined for inhibition of hemagglutination after 1 hr. The highest dilution of the inhibitor that prevented hemagglutination was recorded as the $K_i^{(HAI)}$ for the inhibitor. The reproducibility of the endpoints was within one well from the reported endpoint.

References and Notes

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24. We thank N.K. Sauter and D.C. Wiley for assistance in performing the single crystal x-ray experiments.