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Synthesis and Inhibitory Properties of a Thiomethylmercuric
Sialic Acid with Application to the X-ray Structure
Determination of 9-O-Acetylsialic Acid Esterase from Influenza
C Virus

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Abstract—2-α-Thiomethylmercuryl 9-acetamido-9-deoxy-sialoside was prepared and found to inhibit the 9-O-acetylsialic acid esterase from influenza C virus in a competitive manner with a $K_i$ of $4.2 \pm 0.5$ mM. The inhibitor is being used in the X-ray determination of the crystal structure of the esterase. Copyright © 1996 Elsevier Science Ltd

Introduction

Influenza C virus causes respiratory disease in humans, distinct from the annual epidemics caused by influenza A and B viruses. A single membrane glycoprotein on influenza C virus called HEF (haemagglutinin, esterase, fusion) has three activities. It binds the virus to cellular receptors containing 9-O-acetylsialic acid, it destroys receptors on infected cells with a 9-O-acetylsialic acid esterase, (Scheme 1) and it mediates fusion of the viral and cell membranes during viral entry. The HE glycoprotein on coronaviruses, which cause 25–30% of common colds in humans, has significant sequence identity with HEF, and contains a closely related 9-O-acetylsialic acid esterase. Because these viral 9-O-acetyl esterases appear to define a new class of serine esterase, they may be good targets for synthetic viral inhibitors.

The soluble ectodomain of HEF has been crystallized from the C/Johannesburg/1/66 strain of influenza C virus. The three-dimensional structures of both the HA (haemagglutinin) and NA (neuraminidase) glycoproteins of influenza A virus have previously been determined by X-ray crystallography and have been used to design viral inhibitors. In order to determine the three-dimensional structure of HEF we have synthesized a mercury substituted sialoside inhibitor of the HEF esterase that can be used to form an isomorphous heavy atom derivative. In this note we report the synthesis of the mercury-substituted sialoside (8) and the kinetics of the inhibition of the HEF esterase with 8.

Results and Discussion

The acetamido group at the C(9)-position in 8 was chosen in order to mimic the ester functionality in the natural substrate 9-O-acetylsialic acid. For a related compound, namely the α-methyl glycoside of 9-acetamido-9-deoxy-N-acetylneuraminic acid, a $K_i$ of 2.8 mM was determined by Imhof et al. Based on this result, we expected 8 to bind similarly weakly. But, if the introduction of the heavy-metal atom does not significantly reduce the affinity, 8 should occupy the esterase site and the receptor binding site in crystals maintained in soaking solution containing 10 mM or more 8. The replacement of the 2-α-O-linkage with a thiomercuric 2-α linkage follows results obtained by Shigeta and colleagues, who used a 2-α-thiomercuric analogue of sialic acid to determine crystallographic phases for pertussis toxin, a sialoside binding protein.

The synthesis of 8 is illustrated in Scheme 2. The required starting material, sialic acid methyl ester (1), was prepared as described previously. Compound 1 was selectively tosylated at the primary hydroxy group, giving the tosylate 2 in 77% yield. The obtained
tosylate 2 was treated with sodium azide to yield the azide 3. The substitution reaction suffered from the poor thermal stability of 2, as the formation of the product was accompanied by simultaneous decomposition of the starting material. The best results were achieved, when the reaction was performed at 65 °C in DMF for 4 h. Under these conditions, the azide 3 was isolated in 46% yield after silica gel chromatography. Next, the azido functionality in 3 was reduced to an amino group with tin(II)chloride in methanol as the reducing agent. Attempts to perform the reduction through catalytic hydrogenation with a variety of catalysts (Pd/C, Lindlar, Pt) in different solvents (MeOH, water) were not successful, since in all cases partial or quantitative reduction of the α-ketoacid functionality in 3 occurred along with the desired reduction of the azido group. Using the SnCl2/MeOH-system, however, no side products were formed and the desired amine 4 was obtained in 90% yield after silica gel chromatography followed by gel filtration. Acetylation of 4 was achieved with acetic anhydride in pyridine to give the peracetate 5 as an anomeric mixture consisting of 65% α anomer and 35% β anomer.

The following steps are adaptations of procedures previously proposed by Hasegawa et al.,16, Shigeta Jr. et al.,12 and by Warner and Lee.17 Treatment of the peracetate 5 with a HCl-saturated solution of acetyl chloride yielded the β-configured sialyl chloride, which was treated with potassium thioacetate in a dichloromethane/DMF mixture to give the thioacetate 6 in 89% overall yield. Compound 6 was S- and O-deacetylated and the resulting sulfide was treated with methylmercury(II)chloride to give the mercury sialoside 7. The yield was 52% over the two steps. Basic hydrolysis with aqueous NaOH gave the target compound 8 in 97% yield.

The inhibition of 9-O-acetyl sialic acid esterase by 8 was measured using the hydrolysis of the substrate p-nitrophenyl acetate as described by Vlasak et al.18 Compound 8 was found to be a competitive inhibitor of the enzyme. The $K_i$ was determined to be 4.2 ± 0.5 mM. The inhibition constant does not greatly differ from the $K_i$ of 2.8 mM for the corresponding methyl sialoside. This result indicates that replacement of the aglycon with a thiomethylmercury group seems to be a useful strategy for preparing heavy atom derivatives of crystals of sialoside-binding proteins.12 Although not a potent inhibitor, compound 8 can be soaked into crystals of HEF at concentrations high enough to occupy the 9-O-acetyl sialoside binding sites. Further results will be reported in due course.

**General**

All reagents and solvents used were of the highest available purity. For flash chromatography silica gel 60 (230–400 mesh) from Mallinckrodt was used. 1H NMR spectra were recorded at 400 MHz using TMS (in CDCl3), or HDO (in D2O, δ=4.80) as internal reference. 13C NMR spectra were run at 100 MHz using CDCl3 (δ=77.00) or CH3CN (in D2O, δ=1.60) as internal reference. Mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded under fast-atom bombardment (FAB) conditions.

**Methyl 5-acetamido-3,5-dideoxy-9-O-((p-toluensulfonyl)-β-D-glycerol-D-galacto-nonulopyranosonate** (2). At 0 °C, p-toluensulfonyl chloride (11.3 g, 59.3 mmol) was added to a soln of N-acetyleneuraminic acid methyl ester 1(14.7 g, 45.6 mmol) in pyridine (170 mL) over 90 min. The reaction mixture was stirred at 0 °C for 12 h. The solvent was evapd and the residue purified by silica gel chromatography (eluting with 10% MeOH in CH2Cl2, followed by 33% MeOH in CH2Cl2 to give the tosylate 2 (16.7 g, 77%) and unreacted starting...
material 1 (1.4 g, 10%). 1H NMR (D2O): 7.80 (d, 2H, J = 8.4 Hz), 7.47 (d, 2H, J = 8.1 Hz), 4.29 (dd, 1H, J = 10.6, 2.4 Hz), 4.19 (dd, 1H, J = 10.6, 4.9 Hz), 4.05 (dd, 1H, J = 10.3, 10.1, 4.9 Hz), 4.00 (dd, 1H, J = 10.4, 0.9 Hz), 3.90–3.85 (m, 2H), 3.81 (s, 3H), 3.57 (dd, 1H, J = 9.2, 1.0 Hz), 2.44 (s, 3H), 2.29 (dd, 1H, J = 13.1, 4.9 Hz), 2.04 (s, 3H), 1.88 (dd, 1H, J = 13.1, 11.6 Hz). 13C NMR (D2O): δ 174.56, 172.07, 96.03, 73.25, 70.86, 68.36, 67.28, 54.23, 52.78, 39.43, 22.80, 21.57. HRMS calcd for C19H27NO13Na (M + Na+): 500.1203, found: 500.1185.

Methyl 5-acetamido-9-azido-3,5,9-trideoxy-β-D-galacto-nonulopyranosonate (3). The tosylate 2 (972 mg, 2.03 mmol) was dissolved in DMF (5 mL) and sodium azide (1.06 g, 16.3 mmol) and molecular sieves 5 Å were added. The mixture was stirred at 65 °C and the reaction progress was monitored by TLC. After 5.5 h, 20% MeOH in CH2Cl2 (5 mL) was added at rt and the ppt filtered. Concentration of the filtrate and silica gel chromatography of the residue (eluting with 20% MeOH in CH2Cl2) afforded the azide 3 (325 mg, 46%). 1H NMR (D2O): δ 4.10–4.04 (m, 1H), 4.05 (dd, 1H, J = 10.4, 1.1 Hz), 3.92 (br d, 1H, J = 10.2 Hz), 3.90–3.85 (m, 1H), 3.85 (s, 3H), 3.60 (dd, 1H, J = 13.2, 2.8 Hz), 3.55 (dd, 1H, J = 9.3, 1.0 Hz), 3.46 (dd, 1H, J = 13.2, 6.0 Hz), 2.30 (dd, 1H, J = 13.1, 4.9 Hz), 2.05 (s, 3H), 1.90 (dd, 1H, J = 13.2, 11.6 Hz). 13C NMR (D2O): δ 175.51, 172.07, 96.03, 73.25, 70.86, 68.36, 67.28, 54.23, 52.78, 39.43, 22.80, 21.57. HRMS calcd for C19H27NO13Na (M + Na+): 500.1203, found: 500.1185.

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added to a solution of 6 (172 mg, 0.313 mmol) in MeOH at rt. The solution was stirred at rt for 2.5 h and then neutralized with Dowex 50WX8 [H⁺]. The residue was dissolved in MeOH (1 mL) and methylmercury (II) chloride (80.0 mg, 0.320 mmol) was added. After 1.5 h at rt, the solution was concd and the residue chromatographed on silica gel (eluting with 20% MeOH in CH₂Cl₂) to afford the mercury-substituted methyl ester 7 (95.9 mg, 52%). ¹H NMR filtered off and the filtrate evapd. The residue was neutralized with Dowex 50WX8 [H⁺] and filtered. Evapn of the filtrate yielded the title compound 8 (65.3 mg, 97%). ¹H NMR (D₂O): δ 3.85–3.75 (m, 2H), 3.69 (dd, 1H, J=14.1, 2.6), 3.62 (dd, 1H, J=14.1, 8.5), 2.81 (dd, 1H, J=12.9, 4.7), 2.01 (br s, 6H), 1.86 (dd, 1H, J=12.9, 11.5), 0.77 (s, 3H). ¹³C NMR (D₂O): δ 176.32, 175.61, 174.99, 86.37, 75.87, 71.10, 70.34, 70.12, 69.14, 54.47, 52.29, 45.56, 0.79 (s, 3H). HRMS calcd for C₅H₂₆HgN₂O₄SC₃ (M+Na⁺): 729.0171, found: 729.0154.

5,9-Diacetamido-3,5,9-trideoxy-2-(thiomethylmercuric)-α-D-glycero-D-galacto-nonulopyranosidonic acid (8).

To a solution of the methyl ester 7 (68.7 mg, 0.115 mmol) in H₂O an aq NaOH-soln (1%) was gradually added, until the pH remained > 8. The soln was stirred at rt for 8 h, neutralized with Dowex 50WX8 [H⁺] and filtered. Evapn of the filtrate yielded the title compound 8 (65.3 mg, 97%). ¹H NMR (D₂O): δ 3.85–3.78 (m, 2H), 3.69 (dd, 1H, J=14.1, 2.6), 3.62 (dd, 1H, J=11.1, 10.1, 4.7), 3.45–3.40 (m, 2H), 3.11 (dd, 1H, J=14.1, 8.5), 2.81 (dd, 1H, J=12.9, 4.7), 2.01 (br s, 6H), 1.86 (dd, 1H, J=12.9, 11.5), 0.77 (s, 3H). ¹³C NMR (D₂O): δ 180.79, 175.74, 174.90, 86.37, 75.87, 71.10, 70.34, 70.05, 52.34, 46.30, 42.76, 22.73, 22.64, 9.38. HRMS calcd for C₅H₂₆HgN₂O₄SC₃ (M+Na⁺): 729.0154.

Kinetic measurements

The activity of 9-O-acetylsialic acid esterase was measured by the hydrolysis of p-nitrophenyl acetate. The reactions were performed in 0.5 mL of sodium phosphate buffer (pH 7.4, 100 mM) containing sodium chloride (50 mM) and the bromelain-released glyco-protein of influenza C virus. The reactions were started by adding the p-nitrophenyl acetate from 25 or 100 mM stock solutions in acetonitrile followed by sample inversion. The change in absorption was detected at 400 nm. Activity was measured at substrate concentrations of 0.125, 0.25, 0.5, 1.0, and 2.0 mM, and at inhibitor concentrations of 0.7, 1.5, and 2.5 mM. The data obtained for a given concentration of substrate was corrected for background hydrolysis of the substrate in buffer. Using these conditions, the Kᵣ of 8 was determined to be 4.2 ± 0.5 mM.

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Supplementary Material Available

Copies of ¹H NMR spectra of 2–8, and of the inhibition plots of 9-O-acetylsialic acid esterase with 8 as inhibitor (9 pp). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal and can be ordered from the ACS; see any current masthead page for ordering information.

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