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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±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

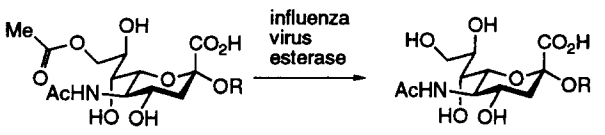
Influenza C virus causes respiratory disease in humans, distinct from the annual epidemics caused by influenza A and B viruses.[1] A single membrane glycoprotein on influenza C virus called HEF (haemagglutinin, esterase, fusion) has three activities.[2] It binds the virus to cellular receptors containing 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,[3] has significant sequence identity with HEF,[4] and contains a closely related 9-*O*-acetylsialic acid esterase.[5] Because these viral 9-*O*-acetyl esterases appear to define a new class of serine esterase,[6] they may be good targets for synthetic viral inhibitors.

The soluble ectodomain of HEF[7] has been crystallized from the C/Johannesburg/1/66 strain of influenza C virus.[8] 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[9] and have been used to design viral inhibitors.[10] 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**.

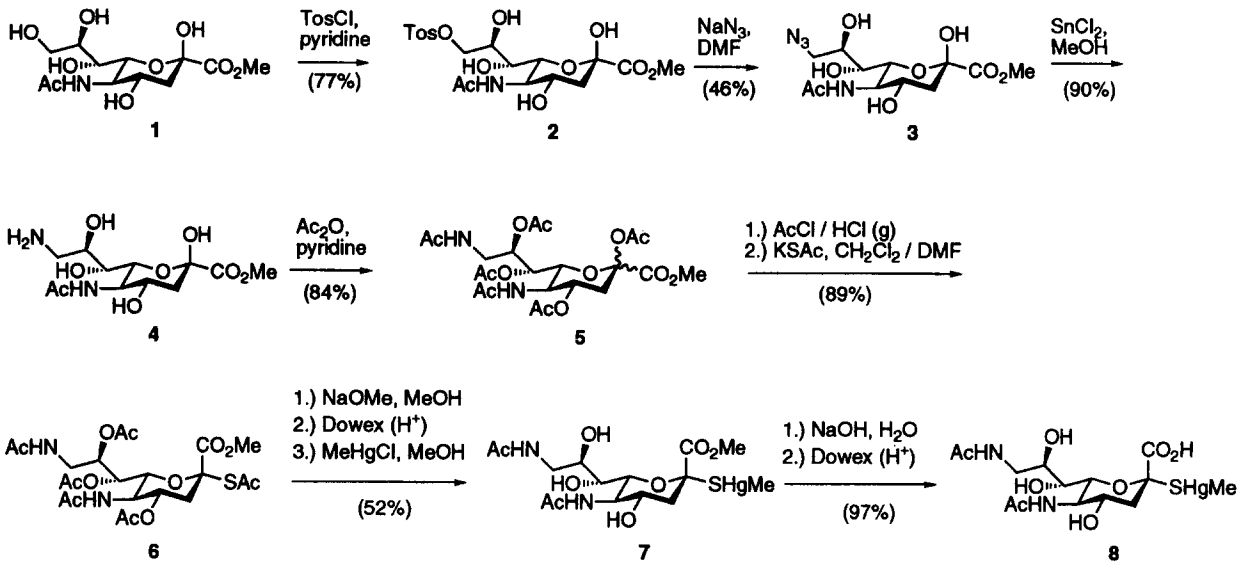
Results and Discussion

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.[11] 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.[11] 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 thiomercury 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.[12]

The synthesis of **8** is illustrated in Scheme 2. The required starting material, sialic acid methyl ester (**1**), was prepared as described previously.[13] Compound **1** was selectively tosylated at the primary hydroxy group, giving the tosylate **2** in 77% yield.[14] The obtained



Scheme 1.



Scheme 2.

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.[15] 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 $SnCl_2$/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 in situ 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*-acetylsialic 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.

Experimental

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. ^{1}H NMR spectra were recorded at 400 MHz using TMS (in $CDCl_3$), or HDO (in D_2O, δ = 4.80) as internal reference ^{13}C NMR spectra were run at 100 MHz using $CDCl_3$ (δ = 77.00) or CH_3CN (in D_2O, 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*-toluenesulfonyl)-β-D-*glycero*-D-*galacto*-nonulopyranosonate (2). At 0 °C, *p*-toluenesulfonyl chloride (11.3 g, 59.3 mmol) was added to a soln of *N*-acetylneuraminic acid methyl ester **1**[19] (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 CH_2Cl_2, followed by 33% MeOH in CH_2Cl_2 to give the tosylate **2** (16.7 g, 77%) and unreacted starting

material **1** (1.4 g, 10%). ^{1}H NMR (D_2O): 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 (ddd, 1H, J=11.3, 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). ^{13}C NMR (D_2O): δ 175.64, 172.03, 147.34, 131.25, 130.94, 128.54, 96.03, 73.25, 70.86, 68.36, 68.27, 67.28, 54.23, 52.78, 39.43, 22.80, 21.57. HRMS calcd for $C_{19}H_{27}NO_{11}SNa$ ($M+Na^+$): 500.1203, found: 500.1185.

Methyl 5-acetamido-9-azido-3,5,9-trideoxy-D-β-*glycero*-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 (4 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 CH_2Cl_2 (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 CH_2Cl_2) afforded the azide **3** (325 mg, 46%). ^{1}H NMR (D_2O): 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). ^{13}C NMR (D_2O): δ 175.51, 172.07, 96.03, 70.87, 69.67, 69.31, 67.36, 54.53, 54.20, 52.76, 39.36, 22.77. HRMS calcd for $C_{12}H_{20}N_4O_8Cs$ ($M+Cs^+$): 481.0335, found: 481.0356.

Methyl 5-acetamido-9-amino-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-nonulopyranosonate (4). To a soln of tin(II)chloride dihydrate (9.2 g, 40.8 mmol) in MeOH (50 mL) was added a soln of the azide **3** (3.55 g, 10.2 mmol) in MeOH (40 mL) at 0 °C. The mixture was stirred at rt for 22 h. The solvent was evapd and the residue suspended in 20% MeOH in CH_2Cl_2 (20 mL). After filtration, evapn of the filtrate and silica gel chromatography (eluting with a gradient starting with 20% MeOH in CH_2Cl_2 and ending with MeOH) the product fractions were concd and the residue redissolved in H_2O (5 mL). The solution was filtered through a BioGel P-2 column (3 cm × 50 cm, eluting with water) and the product containing fractions were freeze-dried to give the amine **4** (2.96 g, 90%). ^{1}H NMR (D_2O): δ 4.10–4.03 (m, 1H), 4.06 (dd, 1H, J=10.5, 1.0), 3.95–3.85 (m, 2 H), 3.83 (s, 3 H), 3.55 (dd, 1H, J=8.6, 1.0), 3.38 (dd, 1H, J=13.2, 3.2), 2.98 (dd, 1H, J=13.1, 9.4), 2.32 (dd, 1H, J=13.2, 4.9), 2.04 (s, 3H), 1.91 (dd, 1H, J=13.1, 11.6). ^{13}C NMR (D_2O): δ 174.91, 171.24, 95.29, 70.13, 70.04, 66.63, 66.42, 53.51, 51.94, 42.60, 38.69, 22.06. HRMS calcd for $C_{12}H_{22}N_2O_8Na$ ($M+Na^+$): 345.1274, found: 345.1269.

Methyl 5,9-diacetamido-2,4,7,8-tetra-*O*-acetyl-3,5,9-trideoxy-D-*glycero*-D-*galacto*-nonulopyranosonate (5). Acetic anhydride (7 mL) was added to a soln of **4** (500 mg, 1.55 mmol) in pyridine (6.2 mL) at 0 °C. After 30 min, the ice-bath was removed and the reaction mixture was stirred at rt for 12 h. The solvent was evapd and the residue was chromatographed on silica gel (eluting with CH_2Cl_2 and then with 15% MeOH in CH_2Cl_2) to afford the peracetate **5** (721 mg, 87%). ^{1}H NMR ($CDCl_3$): δ 6.43 (t, $1H_\beta$, J=6.1 Hz), 6.31 (t, $1H_\alpha$, J=6.2 Hz), 5.81 (br d, $1H_\alpha+1_\beta$, J=9.7 Hz), 5.30–5.23 (m, $1H_\beta$), 5.24 (dd, $1H_\beta$, J=7.3, 2.2 Hz), 5.18 (dd, $1H_\alpha$, J=7.8, 2.1 Hz), 5.08–4.97 (m, $2H_\alpha$), 4.84 (dd, $1H_\beta$, J=9.0, 4.7 Hz), 4.59 (dd, $1H_\alpha$, J=10.8, 2.1 Hz), 4.25–4.15 (m, $1H_\alpha+1H_\beta$), 4.12 (dd, $1H_\beta$, J=10.6, 2.2 Hz), 3.90–3.80 (m, $1H_\alpha+1H_\beta$), 3.80 (s, $3H_\beta$), 3.75 (s, $3H_\alpha$), 3.21 (ddd, $1H_\beta$, J=14.8, 5.9, 3.9 Hz), 3.00 (ddd, $1H_\alpha$, J=15.0, 5.1, 4.2 Hz), 2.59 (dd, $1H_\alpha$, J=13.2, 5.0 Hz), 2.53 (dd, $1H_\beta$, J=13.4, 5.0 Hz), 2.18 (s, $3H_\alpha$), 2.17 (s, $3H_\beta$), 2.15 (s, $3H_\beta$), 2.11 (s, $3H_\alpha$), 2.08 (s, $3H_\alpha$), 2.06 (s, $3H_\alpha$), 2.05 (s, $3H_\beta$), 2.03 (s, $3H_\beta$), 1.99 (s, $3H_\beta$), 1.98 (s, $3H_\alpha$), 1.92 (s, $3H_\alpha$), 1.92 (s, $3H_\alpha$), 1.91 (s, $3H_\beta$); ratio α anomer:β anomer = 1:2. ^{13}C NMR ($CDCl_3$): δ 171.35 (C_α), 171.07 (C_β), 170.97 (C_β), 170.86 (C_α), 170.80 (C_β), 170.75 (C_α), 170.55 (C_α), 170.48 (C_β), 170.42 (C_β), 170.08 (C_α), 168.48 (br, C_β), 168.37 (C_α), 166.62 (C_α), 97.04 (C_β), 95.72 (C_α), 73.37 (C_α), 72.71 (C_β), 72.29 (C_β), 70.29 (C_α), 68.42 (C_β), 68.26 (C_α), 67.71 (C_α), 53.26 (C_β), 52.89 (C_α), 49.04 (C_β), 38.56 (C_β), 38.42 (C_α), 36.78 (C_α), 36.18 (C_β), 23.19, 23.05 (br), 23.00, 20.94, 20.88 (br), 20.86 (br), 20.81 (br), 20.77, 20.64. HRMS calcd for $C_{22}H_{33}N_2O_{13}$ ($M+H^+$): 533.1983, found: 533.1971.

Methyl 5,9-diacetamido-4,7,8-tri-*O*-acetyl-2-*S*-acetyl-3,5,9-trideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosonate (6). AcCl was satd with HCl (700 μL) and the resulting soln was added at −20 °C to a soln of **5** (188 mg, 0.353 mmol) in AcCl. After 5 min, cooling was omitted and the reaction mixture was stirred at rt for 23 h. AcCl was removed under red. pres. and the residue was coevaporated three times with toluene (2 mL each) and redissolved in a mixture of CH_2Cl_2/DMF (4:1, 2 mL). The solution was added to a suspension of potassium thioacetate (81.0 mg, 0.706 mmol) in the same solvent at −70 °C. Cooling was continued for 15 min, after which time the mixture was stirred at rt for 12 h. The solvent was evapd and the residue purified by silica gel chromatography (eluting with 5% MeOH in CH_2Cl_2 followed by 10% MeOH in CH_2Cl_2) to give the title compound **6** (173 mg, 89%). ^{1}H NMR ($CDCl_3$): δ 6.09 (br t, 1H, J=6.1 Hz), 5.21 (br d, 1H, J=10.3 Hz), 5.15 (dd, 1H, J=8.1, 2.2 Hz), 5.05–5.00 (m, 1H), 4.93 (ddd, 1H, J=11.7, 10.6, 4.6 Hz), 4.56 (dd, 1H, J=10.8, 2.2 Hz), 4.14 (q, 1H, J=10.5 Hz), 3.97 (ddd, 1H, J=15.0, 7.5, 3.2 Hz), 3.85–3.80 (m, 1H), 3.78 (s, 3H), 2.94 (dt, 1H, J=15.0, 4.5 Hz), 2.65 (dd, 1H, J=13.0, 4.6 Hz), 2.29 (s, 3H), 2.18 (s, 3H), 2.12 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.90 (s, 3H). ^{13}C NMR ($CDCl_3$) 191.66, 171.40, 170.86, 170.39, 170.27, 170.20, 169.20, 84.56, 74.64, 70.18, 68.83, 67.99, 53.41, 49.16, 38.52, 37.50, 30.16, 23.34, 23.14, 20.99, 20.92, 20.81. HRMS calcd for $C_{22}H_{32}N_2O_{12}SCs$ ($M+Cs^+$): 681.0730, found: 681.0753.

Methyl [5,9-diacetamido-3,5,9-trideoxy-2-(thiomethylmercuric)-α-D-*glycero*-D-*galacto*-nonulopyranosid]onate (7). A soln of NaOMe in MeOH (5%, 250 μL) was

added to a soln 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 resin was filtered off and the filtrate evapd. 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 soln was concd and the residue chromatographed on silica gel (eluting with 20% MeOH in CH_2Cl_2) to afford the mercury-substituted methyl ester **7** (95.9 mg, 52%). ^{1}H NMR (D_2O): δ 3.85–3.75 (m, 2H), 3.83 (s, 3H), 3.69 (dd, 1H, J=14.2, 2.9: Hz), 3.63 (ddd, 1H, J=11.4, 10.1, 4.6: Hz), 3.54 (dd, 1H, J=10.5, 1.1: Hz), 3.40 (dd, 1H, J=9.0, 1.1: Hz), 3.13 (dd, 1H, J=14.2, 7.9: Hz), 2.82 (dd, 1H, J=13.1, 4.6: Hz), 2.01 (s, 3H), 2.00 (s, 3H), 1.96 (dd, 1H, J=13.1, 11.6: Hz), 0.79 (s, 3H). ^{13}C NMR (D_2O): δ 176.32, 175.61, 174.99, 84.51, 75.76, 70.34, 70.12, 69.14, 54.47, 52.29, 45.56, 43.36, 22.79, 22.61, 9.49. HRMS calcd for $C_{15}H_{26}HgN_2O_8SCs$ ($M+Cs^+$): 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 H_2O 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%). ^{1}H NMR (D_2O): δ 3.85–3.78 (m, 2H), 3.68 (dd, 1H, J=14.1, 2.6), 3.62 (ddd, 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). ^{13}C NMR (D_2O): δ 180.79, 175.74, 174.90, 86.37, 75.87, 71.10, 70.28, 70.05, 52.34, 46.30, 42.76, 22.73, 22.64, 9.38. HRMS calcd for $C_{14}H_{24}HgN_2O_8SNa$ ($M+Na^+$): 605.0857, found: 605.0878.

Kinetic measurements

The activity of 9-*O*-acetylsialic acid esterase was measured by the hydrolysis of *p*-nitrophenyl acetate.[18] 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 glycoprotein of influenza C virus.[7] 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, 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 K_i of **8** was determined to be 4.2 ±0.5 mM.

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Supplementary Material Available

Copies of ^{1}H 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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