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Synthesis of Sodium salt of Ortho-(difluoromethyl)phenyl-α-ketoside of N-Acetylneuraminic acid : a Mechanism-based Inhibitor of *Clostridium perfringens* Neuraminidase.

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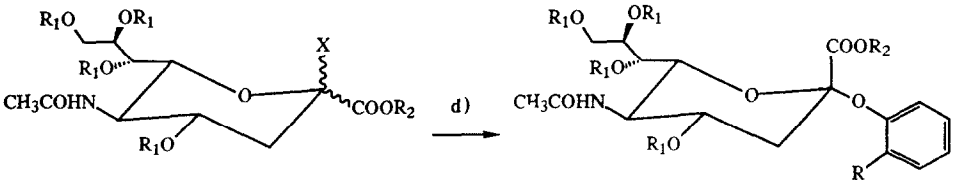
Abstract : The title compound **7** was prepared in seven steps from N-acetylneuraminic acid and proven to be an enzyme-activated irreversible inhibitor of *Clostridium perfringens* neuraminidase.

Sialidases (EC 3.2.1.18) play an important role in splitting off the α-linked-N-acetylneuraminic acid (*5-Acetamido-3,5-dideoxy*-D-glycero-D-galacto-*2-nonulopyranosonic acid*) which occupies a terminal position in glycoconjugates.[1] Due to their involvement in several viral and bacterial infections, selective inhibitors of these enzymes could be potential therapeutic agents. Thus the preparation of such compounds has been actively pursued by many groups over the past few years.[2] In designing potent inhibitors of a target enzyme, chemists have to choose mainly between two classes of compounds : transition-state analogs or mechanism-based enzyme inactivators.[3] If some compounds belonging to the first class have been reported for sialidases [4], to our knowledge, no examples of the second class have yet been mentioned in the literature. Recently Danzin *et al.* reported the preparation of some (difluoromethyl) aryl-β-D-glucosides which were shown to be enzyme-activated irreversible inhibitors of almond β-glucosidase.[5] The inhibitory effect of these molecules was explained as follows : after enzymatic cleavage of the inhibitor, the difluoromethyl-phenol thus liberated evolves into a fluoromethylene-quinone after spontaneous loss of hydrofluoric acid. Due to its high electrophilic character, this quinone is potentially able to form an irreversible covalent bond with a nucleophilic aminoacid in the active center of the enzyme. Since a similar irreversible inhibition could be envisaged for other glycosidases, we decided to prepare, as part of a program aimed at the design and synthesis of potent inhibitors of neuraminidases, the sodium salt of ortho-(difluoromethyl)phenyl-α-ketoside of N-acetylneuraminic acid **7**, and to test its eventual inhibitory activity on our target enzyme.

Synthesis. Compound **7** was synthesized as follows. First we prepared compound **2** from commercially available N-acetylneuraminic acid **1** (SIGMA-France) according to the Kuhn *et al.* procedure, [6] and transformed it into **3** using Meindl *et al.* conditions.[7] The chlorination of **3** was then carried out using similar conditions as described by Kuhn *et al.* [6] revised by Baumberger *et al.*.[8] The glycosidation of the resulting crude **4** with salicylaldehyde was accomplished by phase-transfer catalysis. The α–glycoside **5** [9, 10] thus obtained (71%) was

submitted to the action of diethylaminosulfur trifluoride (DAST) furnishing **6** (55%) which led to the final desired compound **7** after deprotection of the alcoholic functions and saponification of the ester group under the usual conditions.



a) **1** $R_1 = R_2 = H$, X = OH
b) **2** $R_1 = H$, $R_2 = CH_3$, X = OH
c) **3** $R_1 = COCH_3$, $R_2 = CH_3$, X = $OCOCH_3$
4 $R_1 = COCH_3$, $R_2 = CH_3$, X = Cl

e) **5** $R_1 = COCH_3$, $R_2 = CH_3$, R = CHO
f) g) **6** $R_1 = COCH_3$, $R_2 = CH_3$, $R = CHF_2$
7 $R_1 = H$, $R_2 = Na^+$, $R = CHF_2$

a) H^+ Dowex, MeOH. b) Ac_2O, Pyridine. c) HCl / AcCl, Et_2O. d) Salicylaldehyde, Cs_2CO_3 / H_2O, Methyltrioctyl ammonium chloride / $CHCl_3$. e) Et_2NSF_3, CH_2Cl_2. f) MeONa, MeOH. g) NaOH, H_2O.

Biochemical Studies. *Clostridium perfringens* type V was purchased from SIGMA . Compound **7** was tested for inhibitory activity against this enzyme using p-nitrophenyl-N-acetyl-α-D-neuraminate as substrate.[12] All assays were carried out in duplicate on microtiterplates. Enzyme, substrate and inhibitor were incubated for a fixed time in 50 mM NaCl 0.1 M sodium acetate buffer pH 5.4 in a final volume of 200 µl. The reaction was stopped by adding 10 µl of diethanolamine. The activity of the enzyme was measured by monitoring the increase in absorbance at 405 nm due to the release of p-nitrophenol. Optical densities were read on a plate reader (TITERTEK MCC/340).

From the kinetics of inhibition (Fig. 1) it would seem that compound **7** is either a competitive inhibitor with a Ki_{app} of 0.15 mM, or a substrate itself for *C. perfringens* neuraminidase.

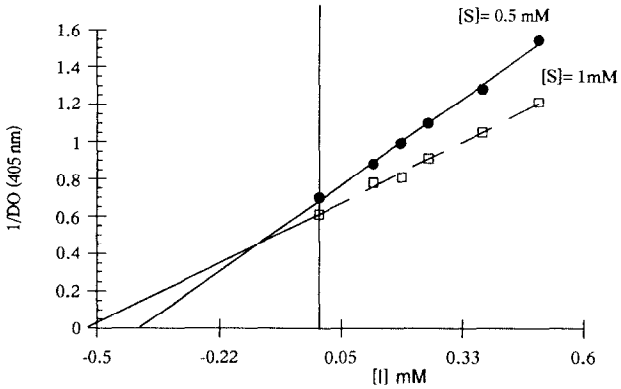


Figure 1 : **Dixon plot** [13]: substrate concentrations were 0.5 mM and 1 mM and inhibitor concentrations as shown. The reaction was initiated by adding *C. perfringens* neuraminidase (62.5 µg/mL) and allowed to proceed for 20 minutes at 37°C.

To investigate this, the capacity of **7** to inactivate the enzyme at 4°C and 37°C was examined. It was found that in the presence of inhibitor at final concentrations of 0.1 mM and 0.2 mM resulting from the 1/20 dilution step, there was little or no inhibition at 4°C whereas at 37°C appreciable inhibition was observed (Fig.2). This result suggests that an inhibitory product is derived from **7** when it is cleaved by *C. perfringens* neuraminidase.

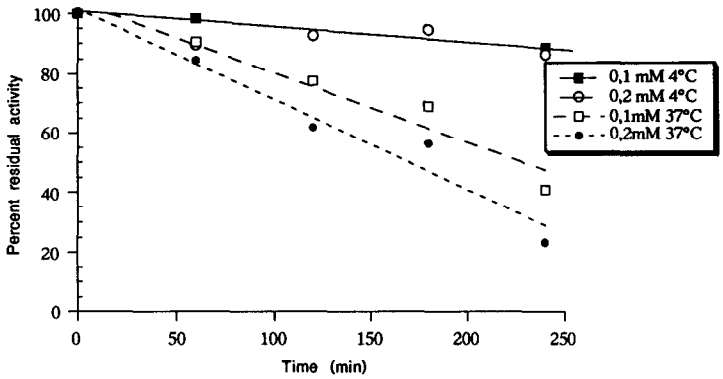


Figure 2 : **Enzymatic activity at 37°C and 4°C as a function of time.** The *C. perfringens* neuraminidase (62.5 µg/mL) and inhibitor (2 and 4 mM) were preincubated either at 37°C or at 4°C in 0.1 M acetate buffer pH 5.4 and 50 mM NaCl. At the time intervals shown, aliquots were withdrawn and diluted 1/20 to the final concentrations shown (0.1 mM to 0.2 mM). Residual activity was assessed with substrate (1.5 mM) during 40 minutes at 37°C.

To determine whether the inhibition was reversible or not, the capacity of different concentrations of **7** to inactivate the enzyme over different time intervals were insvestigated. The results (Fig. 3) show that enzyme inactivation is dependent both on the time of preincubation and the concentration of inhibitor in the medium.

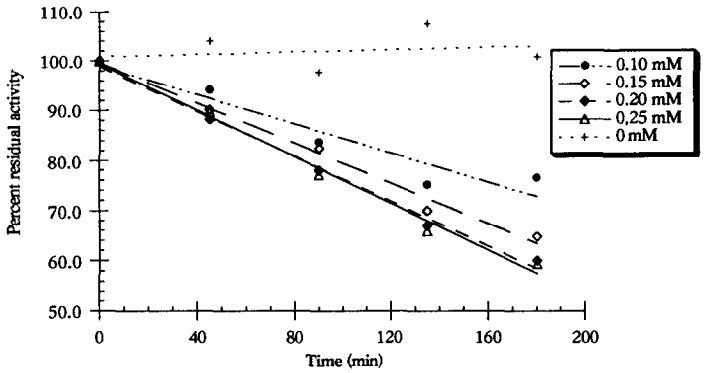


Figure 3 : **Time dependant loss of enzymatic activity at 37°C.** The experiment was carried out under the same conditions as described in figure 2 except that initial inhibitor concentrations before the dilution step were 2, 3, 4 and 5 mM.

Expressing the results as shown in Fig. 4 permitted the Ki to be calculated, 0.15 mM, which is the same value as found in Fig.1.

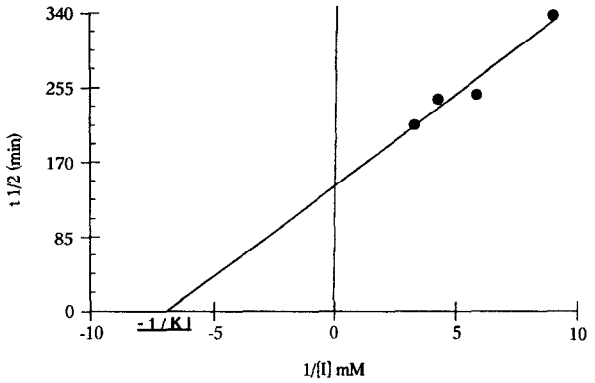


Figure 4 : **Kitz and Wilson plot.** $t_{1/2}$ calculated from figure 3 was plotted against reciprocal concentration of inhibitor.

Additional evidence for the irreversible nature of the inhibition was sought by measuring the residual activity of the enzyme which had been preincubated with inhibitor, and then dialysed for different periods of time. It is clear (Fig.5) that for an enzyme which exhibited 58% residual activity in the presence of 0.2 mM **7** at 0 time, the 64% residual activity still observed after dialysis for 46 h strongly suggests that the inhibitor has irreversibly inactivated the enzyme.

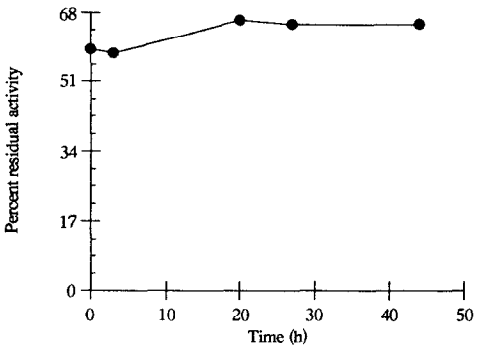


Figure 5 : **Residual activity after dialysis of enzyme preincubated with 7.** 61.5 µg/mL of neuraminidase was preincubated with 4 mM of inhibitor during 4 hours at 37°C and then was dialysed against 0.1 M acetate buffer pH 5.4 and 50 mM NaCl. Aliquots were withdrawn at different times, diluted 1/20, and tested under the same conditions as those described in figure 2.

In conclusion these results provide evidence that **7** is an enzyme activated irreversible inhibitor of *C. perfringens* neuraminidase. Its eventual inhibitory activity against *influenza* virus neuraminidase is presently being examinated.

Acknowledgments. The authors gratefully acknowledge the financial assistance of the *Région Rhône-Alpes* and of the *Ecole Interdisciplinaire du Médicament* -University of Lyon.

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9 The structures of new compounds were assigned on the basis of the appearance of their ^{1}H NMR and ^{13}C NMR (Multiplicity : DEPT) spectra and their mass spectra or elemental analysis.

Methyl(2-salicylaldehydo-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid) onate 5.

To a solution of **4** in $CHCl_3$ (80 mL) was added salicylaldehyde (1.45 mL, 5 eq.) in 0.1 M aqueous cesium carbonate solution (75 mL). Methyltrioctyl ammonium chloride (2.46 g, 2.2 eq.) was then added and the mixture was vigourously stirred for 7 h until no trace of **4** was visible by t.l.c. (eluent : ethyl acetate). The organic layer was separated and the aqueous layer extracted with $CHCl_3$ (3 x 60 mL). The combined organic layers were washed with saturated aqueous NaCl (1 x 20 mL) and dried (Na_2SO_4). After solvent evaporation *in vacuo*, the crude product was purified on silica gel (Amicon 35-70 μm; 180 g; eluent: ethyl acetate, Rf = 0.28). We thus obtained pure instable **5** (1.17 g, 71 % from **3**). **mp** 87-89°C ; **IR** ($CHCl_3$) 3420, 3050, 2960, 2880, 1740, 1680, 1600, 1480, 1370, 1250, 1025 cm^{-1} ; $[\alpha]_D^{25}$ +4.9° (c 1.00, $CHCl_3$) ; **^{1}H NMR** (200 MHz, $CDCl_3$) δ 10.35 (s, CHO), 7.81-7.11 (4H, M, arom.), 6.24 (d, $J_{NH,5}$ = 9.9 Hz, NH), 5.38-5.26 (2H, M, H-7, H-8), 4.93 (td, J_{4-3a} = J_{4-5} = 11.6 Hz, J_{4-3e} = 4.2 Hz, H-4), 4.50 (dd, J_{9-8} = 1.0 Hz, $J_{9-9'}$ = 10.8 Hz, H-9), 4.26-4.10 (3H, M, H-9', H-6, H-5), 3.56 (s, CH_3O), 2.76 (dd, J_{3e-3a} = 12.9 Hz, H-3e), 2.25 (dd, H-3a), 2.07 (3H, s, CH_3CO), 1.99 (9H, s, 3 CH_3CO), 1.87 (3H, s, CH_3CONH) ; **^{13}C NMR** (50 MHz, $CDCl_3$) δ 188.6 (d, CHO), 169.7, 169.7, 169.6, 169.0, 169.0, 166.7 (s, $\underline{C}OOCH_3$, 4 $CH_3\underline{C}OO$, $CH_3\underline{C}ONH$), 150.2 (s), 134.9 (d), 127.0 (d), 126.4 (s), 123.3 (d), 119.3 (d)(arom.) 99.4 (s, C-2), 72.6 (d, C-6), 67.8 , 67.6, 66.1 (d, C-4, C-7, C-8), 61.1 (t, C-9), 52.1 (d, C-5), 48.1 (q, OCH_3), 37.2 (t, C-3), 22.0, 20.0, 19.9, 19.8, 19.7 (q, 5 $\underline{C}H_3CO$).

Methyl(2-o-(difluoromethyl)phenyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-D-*glycero*-α-D-*galacto*-2-nonulopyranosid) onate 6.

To a solution of **5** (441 mg, 0.74 mmol) in dry CH_2Cl_2 (1.5 mL) was added dropwise, at 0°C, diethyl-

aminosulfur trifluoride (DAST) (0.4 mL, 4 eq.). The solution was then allowed to reach room temperature and stirred for 20 h. After solvent evaporation, the crude product was purified by flash chromatography (40 g, eluent : ethyl acetate, Rf = 0.32) giving pure **6** (251 mg, 55%) ; **mp** 76°C ; **IR** ($CHCl_3$) 3420, 2980, 2950, 2920, 1740, 1680, 1600, 1480, 1370, 1250, 1030 cm^{-1} ; $[\alpha]_D^{25}$ +3.6° (c 1.00, $CHCl_3$) ; **^{1}H NMR** (200 MHz, $CDCl_3$) δ 7.59-7.14 (4H,M, arom.), 6.92 (t, J_{H-F} = 55.5 Hz, CHF_2), 5.64 (d, J_{NH-5} = 10 Hz, NH), 5.43-5.34 (2H, M, H-7, H-8), 4.98 (td, J_{4-3e} = 4.5 Hz, J_{4-3a} = J_{4-5} = 12.6 Hz, H-4), 4.50 (dd, J_{9-8} = 0.7 Hz, $J_{9-9'}$ = 10.8 Hz, H-9), 4.27 (m, H-6), 4.20-4.05 (2H, M, H-5, H-9') 3.62 (3H, s, CH_3O), 2.74 (dd, J_{3e-3a} = 13.0 Hz, H-3e), 2.25 (td, H-3a), 2.15 (3H, s, CH_3COO) 2.13 (3H, s, CH_3COO), 2.05 (6H, s, 2 CH_3COO), 1.92 (3H, s, CH_3CONH) ; **^{13}C NMR** (50 MHz, $CDCl_3$) δ 171.3, 171.1, 170.9, 170.5, 170.5, 168.2 (s, $\underline{C}OOCH_3$, 4 $CH_3\underline{C}OO$, $CH_3\underline{C}ONH$), 152.1 (t, $^3J_{C-F}$ = 6.0 Hz), 132.6 (d), 126.7(td, $^3J_{C-F}$ = 5.5 Hz) 126.0.(t, $^2J_{C-F}$ = 22.6 Hz), 124.7 (d), 120.0 (d) (arom.), 111.8 (td, $^1J_{C-F}$ = 235.7 Hz, CHF_2), 100.9 (s, C-2), 73.9 (d, C-6), 69.4, 69.1, 67.7 (d, C-4, C-7, C-8), 62.5 (t, C-9), 53.5 (d, C-5) 49.8 (q, CH_3O), 38.5 (t, C-3), 23.6 , 21.5, 21.3, 21.2, 21.2 (q, 5 $\underline{C}H_3CO$) ; **Anal.** Calcd for $C_{27}H_{33}F_2NO_{13}$: C, 52.51 ; H, 5.39 ; F, 6.15 ; N, 2.27. Found C, 51.71 ; H, 5.53 ; F, 5.75 ; N, 2.28 .

Sodium(2-o-(difluoromethyl)phenyl-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid) onate 7.

To a stirred solution of **6** (82 mg, 0.13 mmol) in methanol (14 mL) was added sodium methoxide (14 mg, 2 eq.). After 2 h, traces of starting material were no longer visible by t.l.c. (eluent : ethyl acetate) and the solution was made neutral with Dowex 50 WX4 (H^+ form) cation exchange resin. After filtration, the solvent was evaporated *in vacuo*, and the residue solubilized in 0.1M aqueous NaOH (45 mL) and stirred 45 mn at room temperature. The solution was neutralised with Dowex 50 WX4 (H^+ form) resin, filtered, and freeze-dried to yield **7** (55 mg, 91 % from **6**). **mp** 176°C (decomp.) ; **IR** (KBr) 3420, 2930, 1640, 1560, 1490, 1460, 1380, 1240, 1020 cm^{-1} ; $[\alpha]_D^{25}$ +21.2° (c 0.6, CH_3OH) ; **^{1}H NMR** (300 MHz, D_2O) δ 7.63-7.22 (4H, M, arom.), 7.10 (t, J_{H-F} = 55.3 Hz, CHF_2), 3.94-3.80 (4H, M, H-5, H-6, H-8, H-9), 3.76 (m, H-4), 3.63-3.56 (2H, M, H-7, H-9'), 2.88 (dd, J_{3e-3a} = 12.6 Hz, J_{3e-4} = 4.7 Hz, H-3e), 2.01 (3H, s, $C\underline{H}_3CONH$), 1.95 (dd, J_{3a-4} = 12.2 Hz, H-3a) ; **^{13}C NMR** (75.5 MHz, D_2O) δ 177.9 (s, $CH_3\underline{C}ONH$), 175.1 (s, C-1), 154.6 (t,$^3J_{C-F}$ = 6.5 Hz), 134.8 (d), 128.8 (td,$^3J_{C-F}$ = 5.4 Hz), 128.7 (t,$^2J_{C-F}$ = 23.0 Hz), 127.3 (d), 123.7 (d) (arom.), 115.0 (td, $^1J_{C-F}$ = 233.4 Hz, CHF_2), 105.7 (s, C-2), 76.3 (d, C-6), 74.5 (d, C-8), 71.0, 70.8 (d, C-4, C-7), 65.5 (t, C-9), 54.5 (d, C-5), 43.3 (t, C-3), 24.9 (q, CH_3) ; **MS** : 480 (M^++Na; 40%), 458 (23%), 352 (13%), 336 (100%), 314 (28%).

10 The α configuration of the compounds **5**, **6** and **7** were assigned on the basis of chemical shifts of H-3 and H-4. [11]

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