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Aromatic sialic acid analogues as potential inhibitors of influenza virus neuraminidase

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Abstract

The influenza virus neuraminidase (NA) is an enzyme essential for viral infection and offers a potential target for antiviral drug development. We aimed our research at the synthesis of non-carbohydrate molecules able to inhibit NA as transition-state analogues. Aromatic sialic acid analogues (compound **5** and compound **10**) were synthesised in good yields starting from commercially available benzoic acids using a suitable synthetic strategy.

Keywords: Sialic acid; Influenza; Neuraminidase

1. Introduction

Influenza viruses are enveloped by a host cell-derived lipid membrane which is penetrated by numerous copies of two distinct types of virally coded molecules: haemagglutinin (HA) and neuraminidase (NA). The HA of influenza viruses binds to terminal sialic acid residues on the cell membrane as the first step of viral infection. Viral attachment is followed by receptor mediated endocytosis, after which the viral and cell membranes fuse themselves, allowing the nucleocapsid to enter the cytoplasm. The NA (EC 3.2.1.18) is an exoglycosidase that catalyses the hydrolysis of the α-(2,3) and α-(2,6) glycosidic linkage between a terminal sialic acid and its adjacent carbohydrate moiety on a variety of glycoconjugates. NA activity is thought to be essential for the maintenance of virus mobility, e.g. by means of the prevention of self-aggregation, to facilitate release of the virus from the cell surface, to prevent virus activity from being destroyed by the mucins which are rich in sialic acids, and it is also involved in mediating membrane fusion [1]. The enzyme mechanism of NA from influenza virus has been investigated by kinetic isotope methods, NMR, and a molecular dynamics simulation of the enzyme–substrate complex [2–5]. Janakiraman et al. [6] proposed a mechanism of influenza virus NA reaction in which the driving force comes solely from the induction and stabilisation of the oxocarbonium ion intermediate (see Fig. 1). According to this mechanism it is possible to inhibit the enzyme by structurally analogue molecules to the hypotetic transition state of considered reaction.

The concept of structural similarity to the transition state has found wide application in drug design over the years. The multitude of enzyme-inhibitor interactions are governed by steric as well as electronic factors. In theory, compounds that closely resemble the transition state structure should give high binding affinity towards the target enzyme. These potential inhibitors, besides, having a high affinity for stable bond with the protein, and they do not need to be transformed in the reaction products.

One of the most potent synthetic inhibitors for NA (K_i of 4 μM [7]), commercially available 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA, see Fig. 1), was obtained by the simple dehydration of sialic acid. According to the NA mechanism action described above, DANA is considered a transition state-like analogue binding to the active site of NA. Several NA inhibitor analogues have been synthesised using DANA as a base molecule. An extremely potent influenza NA

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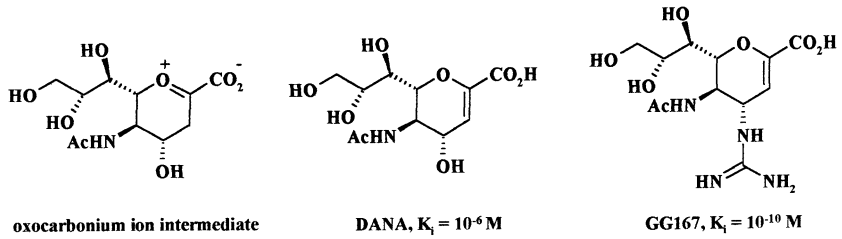


Fig. 1.

inhibitor, with K_i value of 10^{-10} M, is 2,3-didehydro-2,4-dideoxy-4-guanyl-*N*-acetylneuraminic acid, Zanamivir (GG167, see Fig. 1) [8–10]. GG167 is a transition state analogue, representing the optimal conformational state imposed on the sialic acid unit by the enzyme during the catalytic cleavage of the glycosidic linkage.

GG167 exhibited potent antiviral activity against a variety of influenza A and B strains in the cell culture assay; it is currently being evaluated in human clinical trials and has shown efficacy in phase III challenge studies in both prophylaxis and treatment of influenza virus infections [11]. However, poor oral bioavailability and rapid excretion precluded GG167 as a potential oral agent against influenza infection and GG167 has to be administered by either intranasal or inhaled routes in clinical trials. In the case of an influenza epidemic, oral administration may be a more convenient and economical method for treatment and prophylaxis. Therefore, it would be desirable to have a new class of orally active NA inhibitors as potential agents against influenza infection. Moreover, the relative chemical sensitivity (e.g. to acid, base, and heat) and the complex stereochemistry (five chiral centres) for this class of compound seems to make the production of next generation agents problematic. The general approach to the structure-based design of NA inhibitors uses the structure of the DANA–NA complex as a starting point and is based on the development of new classes of lead compounds by using chemically simpler cyclic templates instead of the dihydropyran ring of DANA. It has been proposed that simple non-carbohydrate analogues containing the carboxylate and the acetylamino groups attached to a cyclic backbone 'spacer' would be sufficient to generate lead compounds for further elaboration as NA inhibitors. The spacer would need to correctly orient these groups as found in bound DANA. It has been also required that such compounds adopt a planar structure near the carboxylate to mimic the transition state and be able to present additional side-chain functionality for interaction with other conserved amino acid residues in the sialic acid binding site. Among several chemical classes considered, the benzoic acid seems to be the most suitable starting target; in fact, not only it positions the carboxyl group in the active site correctly thank to its similar size to the DANA sugar ring, but the planarity near the carboxylate mimics correctly that found in the transition-state-like structure of DANA. Furthermore, the benzene ring scaffold gives advantages of non-chirality, chemical and metabolic stability and increased lipophilicity compared with the dihydropyran ring. These factors may be important to improve the deficient pharmacokinetic profiles observed for GG167. Recently, simple aromatic influenza NA inhibitors have been synthesised [12]. These compounds have a comparable affinity for the NA with the sialic acid.

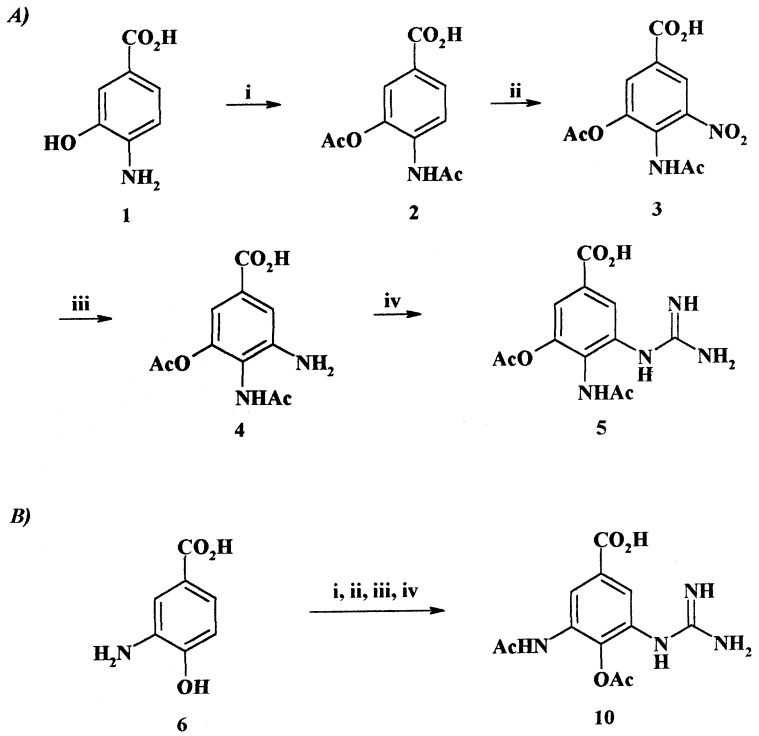
2. Results and discussion

We synthesised two aromatic analogues, compounds **5** and **10**. In these molecules we conserved the functional groups, the carboxylic and the acetamido ones, necessary for the interaction with the NA active site; also we introduced the guanidino group at C-5 and we substituted an acetyl group for the glyceric chain. These products were prepared as illustrated in Scheme 1 (part A and B). Compound **5** has been synthesised utilising as a starting material commercially available 4-amino-3-hydroxybenzoic acid 1 (Scheme 1, part A).

Compound **1** was acetylated with acetic anhydride and an aqueous solution of sodium acetate in presence of hydrochloric acid to give the product **2**. Compound **2** was treated with a solution of the nitrating mixture made from acetic anhydride and nitric acid in dioxane affording nitro derivative **3**, with unsatisfactory yields.

The ^{1}H NMR spectrum of compound **3** shows the de-shielding of the protons at C-2 and at C-6 that give two doublets at δ 7.92 and 8.55, with coupling constant of 1.8 Hz, confirming that the nitration of aromatic ring has occurred in *meta* position as regards the carboxylic group and in *ortho* position as to the acetamido group.

The catalytic reduction of nitro derivative **3**, carried out with hydrazine in ethanol in the presence of palladium on calcium carbonate, affords compound **4**. The guanidino group at C-5 was introduced by reaction of compound **4** with cyanamide in solution of hydrochloric acid affording the objective molecule **5**.



Scheme 1. (i) Ac_2O, $AcONa_{aq}$, 2 N HCl; (ii) Ac_2O, HNO_3, dioxane; (iii) N_2H_4, Pd–C, EtOH; (iv) NH_2CN, 6 N HCl.

Compound **10** was prepared by using a similar synthetic strategy (Scheme 1, part B).

The main difference between two syntheses regards the nitration step: in fact the compound **2** nitration affords the nitro derivative **3** in very low yields whereas compound 8 was obtained as the only reaction product in quantitative yield.

3. Experimental

^{1}H NMR spectra were measured with a Varian Gemini 200 MHz spectrometer and chemical shifts are expressed in ppm from Me_4Si. 4-Amino 3-hydroxybenzoic acid (**1**) and 3-amino-4-hydroxybenzoic acid (**6**) were obtained from Aldrich Chemical Co.

Product purification was obtained by solid–liquid column chromatography on Merck 0.063–0.20 nm silica gel. The elution mixtures were determined case by case. Merck TLC plates coated with Kiesel-Gel 60 F_{254} were employed to monitor the reactions, sprinkling with 2 N H_2SO_4 and then heating at 120°C.

3.1. 3-Acetoxy-4-(acetylamino)-benzoic acid (2)

To a stirred solution of commercially available compound **1** (500 mg) in 2 N HCl (10 ml) at 0°C (ice bath) a solution of NaOAc (5 g) in water was added. To this Ac_2O (2.5 ml) was added. The mixture was stirred at 0°C for 5 min, and it was then allowed to warm slowly to room temperature as the ice bath melted. After 4 h a light brown precipitate had formed. This was filtered, washed with water (25 ml), and air-dried to provide **2** (450 mg, 58% yield); it is pure enough to be utilised in the following synthetic passages. ^{1}H NMR (DMSO-d_6): δ 2.12 (s, 3H, NHAc), 2.33 (s, 3H, OAc), 7.67 (d, 1H, arom., $J = 1.9$ Hz), 7.77 (dd, 1H, arom., $J = 1.9$, 8.4 Hz), 8.15 (d, 1H, arom., $J = 8.4$ Hz), 9.62 (s, 1H, NH). *Anal.* Calc. for $C_{11}H_{11}NO_5$: C, 55.70; H, 4.67; N, 5.90. Found: C, 55.60; H, 4.80; N, 5.81%.

3.2. 3-Acetoxy-4-(acetylamino)-5-nitrobenzoic acid (3)

Compound **2** (500 mg) was suspended with stirring in a mixture of Ac_2O (4 ml) and dioxane (3 ml). This was cooled to 0°C, and a cold solution of the nitrating mixture made from Ac_2O (1.5 ml) and concentrated HNO_3 (1.5 ml) was added slowly to the mixture containing **2**. The reaction mixture was then warmed to 30–35°C until the reaction was complete as evidence by TLC (9:1 $CHCl_3 + CH_3OH$). The reaction mixture was poured onto ice/water (100 ml), extracted with EtOAc (4×20 ml), dried on Na_2SO_4, and concentrated to dryness on a rotary evaporator. The residue was purified by chromatography eluting with chloroform + methanol (9:1) to give compound **3** (262 mg, 44% yield) as an oil. ^{1}H NMR (CD_3OD): δ 2.10 (s, 3H, NHAc), 2.30 (s, 3H, OAc), 8.05 (d, $J = 1.5$ Hz, 1 H, arom.), 8.35 (d, $J = 1.5$ Hz, 1H, arom.). *Anal.* Calc. for $C_{11}H_{10}N_2O_7$: C, 46.82; H, 3.57; N, 9.93. Found: C, 46.71; H, 3.68; N, 9.81%.

3.3. 3-Acetoxy-4-(acetylamino)-5-aminobenzoic acid (4)

Compound **3** (300 mg) was dissolved in EtOH (20 ml) and catalytic quantity of Pd–C was added to it. To this mixture hydrazine hydrate (0.1 ml) was added dropwise. The reaction mixture was heated at 80°C for 3 h. The Pd–C was filtered and the ethanol was concentrated under vacuum to give free amine **4** as a pale yellow oil (260 mg, 97% yield). For the characterisation of **4**, 30 mg was purified by chromatography eluting with chloroform + methanol (8:2). ^{1}H NMR (D_2O): δ 2.22 (s, 3H, NHAc), 2.30 (s, 3H, OAc), 7.40–7.45 (m, 2H, aromatic). *Anal.* Calc. for $C_{11}H_{12}N_2O_5$: C, 52.38; H, 4.80; N, 11.11. Found: C, 52.27; H, 4.88; N, 11.03%.

3.4. 3-Acetoxy-4-(acetylamino)-5-guanidinobenzoic acid (5)

Compound **4** (200 mg) was dissolved in 6 N HCl (4 ml); NH_2CN (140 mg) was added. The reaction mixture was heated at 100°C with stirring for 30 min. The mixture was cooled in an ice bath, diluted with water (4 ml), acidified with concentrated HCl (4 ml) and cooled at −20°C. After 3 h the solid crystallised, was filtered on gooch and was washed with 2 N HCl (5 ml). After chromatographic purification using as eluent chloroform + methanol (8:2) compound **5** (160 mg, 67% yield) was obtained as a white solid. ^{1}H NMR (DMSO-d_6): δ 2.14 (s, 3 H, $NCOCH_3$), 2.30 (s, 3 H, AcO), 7.50 (br s, 2 H, NH_2), 7.89 (m, 2 H, aromatic), 9.1 (s, 1H, NH), 10.01 (s, 1H, NH). *Anal.* Calc. for $C_{12}H_{14}N_4O_5$: C, 48.98; H, 4.80; N, 19.04. Found: C, 48.77; H, 4.95; N, 18.95%.

3.5. 3-(Acetylamino)-4-acetoxybenzoic acid (7)

To a stirred solution of commercially available compound **6** (500 mg) in 2 N HCI (10 ml) at 0°C (ice bath) a solution of NaOAc (5 g) in water was added. To this Ac_2O (2.5 ml) was added. The mixture was stirred at 0°C for 5 min, and it was then allowed to warm slowly to room temperature as the ice bath melted. After 4 h a light brown precipitate had formed. This was filtered, washed with water (25 ml), and air-dried to provide **7** (462 mg, 60% yield); it is pure enough to be utilised in the following synthetic passages. ^{1}H NMR (DMSO-d_6): δ 2.17 (s, 3H, NHAc), 2.35 (s, 3H, OAc), 7.69 (d, 1H, arom., $J = 1.8$ Hz), 7.79 (dd, 1H, arom., $J = 1.8$, 7.4 Hz), 8.17 (d, 1H, arom., $J = 7.4$ Hz), 9.30 (s, 1H, NH). *Anal.* Calc. for $C_{11}H_{11}NO_5$: C, 55.70; H, 4.67; N, 5.90. Found: C, 55.59; H, 4.77; N, 5.94%.

3.6. 3-(Acetylamino)-4-acetoxy-5-nitrobenzoic acid (8)

Compound **7** (500 g) was suspended with stirring in a mixture of Ac_2O (4 ml) and dioxane (3 ml). This was cooled to 0°C, and a cold solution of the nitrating mixture made from Ac_2O (1.5 ml) and concentrated HNO_3 (1.5 ml) was added slowly to the mixture containing **7**. The reaction was then warmed to 30–35°C until the reaction was complete as shown by TLC (9:1 $CHCl_3 + CH_3OH$). The reaction mixture was poured onto ice/water (100 ml), extracted with EtOAc (4 × 20 ml), dried on Na_2SO_4, and concentrated to dryness on a rotary evaporator to give crude **8** (510 mg, 86% yield). Compound **8** is pure enough to be utilised in the following synthetic passages. For its characterisation, a crude sample (30 mg) was purified by chromatography eluting with chloroform + methanol (9:1) to give compound **8** (23 mg) as an oil. ^{1}H NMR (CD_3OD): δ 2.13 (s, 3H, NHAc), 2.35 (s, 3H, OAc), 8.10 (d, $J = 1.5$ Hz, 1H, arom.), 8.42 (d, $J = 1.5$ Hz, 1H, arom.). *Anal.* Calc. for $C_{11}H_{10}N_2O_7$: C, 46.82; H, 3.57; N, 9.93. Found: C, 47.70; H, 3.65; N, 9.82%.

3.7. 3-(Acetylamino)-4-acetoxy-5-aminobenzoic acid (9)

Compound **8** (500 g) was dissolved in EtOH (20 ml) and a catalytic quantity of Pd–C was added to it. To this mixture hydrazine hydrate (0.1 ml) was added dropwise. The reaction mixture was heated at 80°C for 3 h. The Pd–C was filtered and the ethanol was concentrated under vacuum to give the free amine **9** as a pale yellow oil (422 mg, 94% yield). For the characterisation of **9**, 30 mg was purified by chromatography eluting with chloroform + methanol (8:2). ^{1}H NMR (D_2O): δ 2.25 (s, 3H, NHAc), 2.30 (s, 3H, OAc), 7.37–7.42 (m, 2H, arom.). *Anal.* Calc. for $C_{11}H_{12}N_2O_5$: C, 52.38; H, 4.80; N, 11.11. Found: C, 52.25; H, 4.88; N, 11.01%.

3.8. 3-(Acetylamino)-4-acetoxy-5-guanindinobenzoic acid (10)

Compound **9** (300 g) was dissolved in 6 N HCl (4.5 ml); NH_2CN (150 mg) was added. The reaction mixture was heated at 100°C with stirring for 30 min. The mixture was cooled in an ice bath, diluted with water (5 ml), acidified with concentrated HCl (5 ml) and cooled at −20°C. After 3 h the solid crystallised, was filtered on gooch and was washed with 2 N HCl (5 ml). After chromatographic purification using as eluent chloroform + methanol (8:2) compound 10 (232 mg, 66% yield) was obtained as a white solid. ^{1}H NMR (DMSO-d_6): δ 2.17 (s, 3H, $NCOCH_3$), 2.30 (s, 3H, OAc), 7.52 (br s, 2H, NH_2), 7.78 (m, 2H, arom.), 8.9 (s, 1H, NH), 10.0 (s, 1H, NH). *Anal.* Calc. for $C_{12}H_{14}N_4O_5$: C, 48.98; H, 4.80; N, 19.04. Found: C, 48.80; H, 4.87; N, 18.96%.This work is part of a project supported by Istituto Pasteur–Fondazione Cenci Bolognetti, Università di Roma, 'La Sapienza'.

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