Structures of Aromatic Inhibitors of Influenza Virus Neuraminidase[†,‡]

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ABSTRACT: Neuraminidase (NA), a surface glycoprotein of influenza virus, is a potential target for design of antiinfluenza agents. The crystal structure of influenza virus neuraminidase showed that in the active site 11 residues are universally conserved among all strains known so far. Several potent inhibitors based on the carbohydrate compound 2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid (DANA) have been shown to bind to the conserved active site and to reduce virus infection in animals when administered by nasal spray. Inhibitors of this type are, however, rapidly excreted from physiological systems and may not be effective in order to provide long-time protection. A new class of specific NA inhibitors, which are benzoic acid derivatives, has been designed on the basis of the three-dimensional structure of the NA−DANA complex and modeling of derivatives of 4-(acetylamino)benzoic acid in the NA active site. Intermediates were synthesized and were shown to moderately inhibit the NA activity and to bind to the NA active site as predicted. These rudimentary inhibitors, 4-(acetylamino)-3-hydroxy-5-nitrobenzoic acid, 4-(acetylamino)-3-hydroxy-5-aminobenzoic acid, and 4-(acetylamino)-3-aminobenzoic acid, and their X-ray structures in complexes with N2 (A/Tokyo/3/67) and B/Lee/40 neuraminidases have been analyzed. The coordinates of such inhibitors complexed with NA were used as the starting model for further design of more potent benzoic acid inhibitors. Because the active site residues of NA are invariant, the designed aromatic inhibitors have the potential to become an antiviral drug against all strains of influenza virus.

Neuraminidase and Its Active Site. The orthomyxovirus influenza has two major surface membrane-bound glycoproteins, the hemagglutinin and the neuraminidase (acylneuraminyl hydrolase). Both of these proteins are essential to virus replication. Hemagglutinin (HA) attaches the virus to its receptor on the cell surface, which is a terminal sialic acid (NANA).[1] Neuraminidase (NA) leaves a glycosidic bond of the terminal sialic acid (Klenk *et al.*, 1955; Gottschalk, 1957) and thus is described as a receptor-destroying enzyme. Classification of influenza virus strains by antigenic cross-reactivity (World Health Organization, 1980) has revealed nine antigenic-distinct subtypes of neuraminidase, two of which (N1 and N2) have been found in human viruses. Flu epidemics result either from point mutations of the viral genome associated with sequence changes in the surface glycoproteins (antigenic drift) or from reassorting of the viral genome resulting in switch of subtypes. The active site of NA, however, is invariant for all strains of influenza virus (Burmeister *et al.*, 1992; von Itzstein *et al.*, 1993), and inhibition of the NA enzymatic activity stops virus infection (Liu & Air, 1993). It seems that the active site, a deep pocket on the NA surface, is an ideal target for drug design.

The NA head can be proteolytically cleaved from the viral surface and crystallized. The crystal structures of NA from viruses of types A and B have been reported (Baker *et al.*, 1987; Bossart-Whitaker *et al.*, 1993; Burmeister *et al.*, 1992; Janakiraman *et al.*, 1994; Tulip *et al.*, 1991; Varghese & Colman, 1991; Varghese *et al.*, 1983). All NA structures are very similar even though the sequence identity is only about 30%. The NA is a tetramer with a molecular mass of about 240 kDa that is anchored to the virus envelope by a long stalk and a transmembrane domain. Each of the identical monomers incorporates six four-stranded β sheets arranged in a propeller-like manner (Figure 1). Sialic acid binds to an active site in a crater at the top (distant from the virus surface) of the NA. This site is lined with an unusually large number of charged amino acids (Colman *et al.*, 1983).

MATERIALS AND METHODS

Design. When DANA, a sialic acid transition-state analog, is bound to the NA active site, the plane of the acetylamino group is perpendicular to the plane of the tetrahydropyran ring and bisects the O−C=O bond of the carboxylate. This produces an amide N to carboxylate C distance of 5.5 Å and an amide O to carboxylate C distance of 6.2 Å. We proposed that simple noncarbohydrate analogs containing the carboxylate and the acetylamino groups attached to a cyclic backbone "spacer" would be sufficient to generate lead compounds for further elaboration as neuraminidase inhibitors. The spacer would need to correctly orient these groups as found in bound DANA. It was also required that such

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[‡] Coordinates have been deposited in the Brookhaven Protein Data Bank under the file names 1IVB, 1IVC, 1IVD, 1IVE, 1IVF, and 1IVG.

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[1] Abbreviations: NA, neuraminidase; NANA, *N*-acetylneuraminic acid; DANA, 2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid; BANA, benzoic acid derived inhibitor of neuraminidase; K_i, inhibition constant; IC, inhibitory concentration; F_o, observed structure factor; F_c, calculated structure factor.

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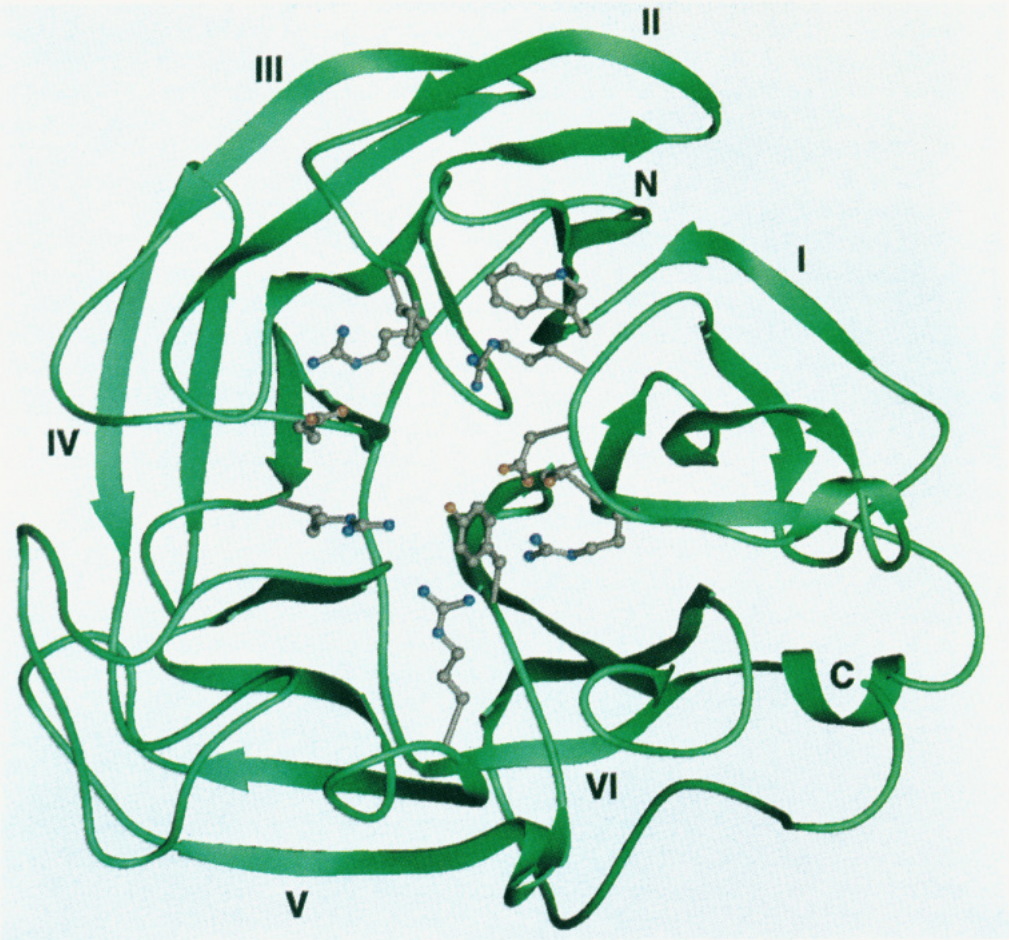

FIGURE 1: Ribbon drawing (Carson, 1987) of the N2 (A/Tokyo/3/67) influenza virus neuraminidase monomer with the 11 active site residues depicted in a ball and stick fashion. The four-stranded β sheets are numbered I through VI; the amino and carboxyl termini are designated as N and C. The 4-fold axis that generates the whole neuraminidase head is perpendicular to the plane of the figure and is placed in the lower right corner of the figure.

compounds adopt a planar structure near the carboxylate to mimic the transition state and be capable of presenting additional side-chain functionality for interaction with other conserved amino acid residues in the sialic acid binding site. Candidate classes of potential inhibitors were first evaluated for proper spacing of the amide and carboxyl groups using the software Sybyl 6.03 (Sybyl, 1993). Utilizing the molecular mechanics routine within Sybyl, Maximim 2, molecules were energy minimized, and the amide N to carboxylate C distance was determined. Among several chemical classes considered, the benzene ring spacer was the first choice. The measured distances in the modeled 4-(acetylamino)benzoic acid, energy minimized from a starting geometry approximating that for bound DANA, were 5.7 Å for the amide N to carboxylate C distance and 6.3 Å for the amide O to carboxylate C distance. The correct presentation of functionality was further verified by superimposing proposed compounds with DANA using the FIT procedure provided in Sybyl 6.03 and determining the rms deviation for fit atoms. In addition to proper positioning of these key groups, the benzoic acid class offered the potential advantages of inherently simple stereochemistry (no chiral carbons occur at side-chain to ring branches), relative conformational rigidity (the predicted positioning of side-chain functionality is simplified), relative ease of synthesis, and prospects for good metabolic disposition (many useful drugs are based upon benzene). A similar molecular mechanics modeling approach was then utilized to compare the orientation of additional side-chain functionality on proposed benzoic acids with comparable groups on DANA. Finally, the designed structures were inspected for reasonable steric and electrostatic complementarity while positioned in the neuraminidase binding site using the software FRODO (Jones, 1985, 1978).

Chemistry. Preparation of 3-Acetoxy-4-(acetylamino)-benzoic Acid (2). To a stirred solution of commercially available compound **1** (0.50 g, 3.3 mmol) in 2 N HCl (10 mL) at 0 °C (ice bath) was added a solution of NaOAc (5.0 g, 61 mmol) in water (25 mL). To this was added Ac_2O (5.4 g, 53 mmol). 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): mp 219−221 °C (CH_3OH/H_2O); IR (KBr) 3400−2950, 1735, 1660 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.17 (s, 3 H, $COCH_3$), 2.35 (s, 3 H, $COCH_3$), 7.69 (d, 1 H, aromatic, $J = 1.8$ Hz), 7.79 (dd, 1 H, aromatic, $J = 7$ and 1.8 Hz), 8.17 (d, 1 H, aromatic, $J = 7$ Hz), 9.3 (s, 1 H, NH).

Preparation of 3-Acetoxy-4-(acetylamino)-5-nitrobenzoic Acid (3). Compound **2** (1.00 g, 4.21 mmol) was suspended with stirring in a mixture of Ac_2O (8.33 mL, 8.99 g, 88.2 mmol) and dioxane (6.6 mL). This was cooled to 0 °C, and a cold solution of the nitrating mixture made from Ac_2O

(3.33 mL, 3.59 g, 35.2 mmol) and concentrated HNO_3 (3.33 mL) was slowly added to the mixture containing **2**. The reaction mixture was then warmed to 30−35 °C until the reaction was complete as evidenced by TLC. The reaction mixture was poured onto ice/water (100 mL), extracted with EtOAc (4 × 50 mL), dried ($NaSO_4$), and concentrated to dryness on a rotary evaporator to give crude **4** (1.05 g, 89.9%) as an oil. Titration with $CHCl_3$ gave a solid: mp 196−201 °C (dioxane/hexane); IR (KBr) 3400−3000, 1700, 1670 cm^{-1}; ^1H NMR (CD_3OD) δ 8.42−8.33 (d, J = 1.5 Hz, 1 H, aromatic), 8.05−8.12 (d, J = 1.5 Hz, 1 H, aromatic), 2.35 (s, 3 H, OCOCH3), 2.13 (s, 3 H, $NCOCH_3$).

Preparation of 4-(Acetylamino)-3-hydroxy-5-nitrobenzoic Acid (BANA105). Compound **3** (0.850 g, 3.54 mmol) was dissolved in 0.1 N NaOH (80 mL), and the mixture was stirred at room temperature for 4 h. This was acidified with concentrated HCl (2 mL), diluted with water (20 mL), and extracted with ethyl acetate (3 × 60 mL). The combined extracts were dried (Na_2SO_4) and concentrated on a rotary evaporator to give crystalline BANA105 (0.712 g, 98.4% yield): mp 256−259 °C (methanol); IR (KBr) 3400−3100, 1640, 1590 cm^{-1}; ^1H NMR (DMSO-d_6) δ 10.93 (s, 1 H, COOH), 9.90 (s, 1 H, NH), 7.79−7.69 (m, 3 H, aromatic), 2.04 (s, 3 H, $NCOCH_3$). Anal. Calcd for $C_9H_8N_2O_6$: C, 45.00; H, 3.33; N, 11.66. Found: C, 45.06; H, 3.38; N, 11.77.

Preparation of 3-Amino-4-(acetylamino)-5-hydroxybenzoic Acid Hydrochloride (BANA106). Compound BANA105 (100 mg, 0.416 mmol) was dissolved in ethanol (3 mL), and Pd−C (100 mg) was added to it. To this mixture was added hydrazine hydrate (55% hydrazine, 0.10 mL, 55 mg, 1.7 mmol) dropwise. The reaction mixture was heated at reflux for 1 h. The Pd−C was filtered and the ethanol was concentrated under vacuum to give free amine BANA106 as a pale yellow oil (85 mg, 97% yield). The oil was dissolved in ethanol (3 mL), HCl (gas) was bubbled through the solution for a few minutes, and ether (10 mL) was added. No precipitate formed, so the solution was concentrated to dryness to give **6** (100 mg) as the hydrochloride salt: mp 220 °C (dec); IR (KBr) 3400−2600, 1710, 1600 cm^{-1}; ^1H NMR (D_2O) δ 7.45−7.40 (m, 2 H, aromatic), 2.22 (s, 3 H, $NCOCH_3$). Anal. Calcd for $C_9H_{10}N_2O_4$·HCl: C, 43.81; H, 4.46; N, 11.35. Found: C, 44.16; H, 4.62; N, 11.21.

Preparation of 4-(Acetylamino)-3-nitrobenzoic Acid (5). Commercially available compound **4** (5.00 g, 27.9 mmol) was gradually stirred into a paste prepared by adding finely pulverized potassium nitrate (6.70 g, 66.3 mmol) to concentrated H_2SO_4 (30 mL), and the mixture was stirred at −10 to 0 °C in a salt/ice bath for 1 h. The syrupy mass was then slowly poured onto cracked ice. After being allowed to stand for 1 h the yellow precipitate was filtered, washed on the filter with cold water, and air-dried to give **5** (5.27 g, 84.3% yield): mp 215−220 °C (ethanol); IR (KBr) 3200−2800, 1650, 1550, 1430 cm^{-1}; ^1H NMR (DMSO-d_6) δ 10.57 (s, 1 H, COOH), 8.36 (s, 1 H, aromatic), 8.2 (δ, J = 8.5 Hz, 1 H, aromatic), 7.82 (d, J = 8.5 Hz, 1H, aromatic), 2.11 (s, 3 H, $COCH_3$).

Preparation of 4-(Acetylamino)-3-aminobenzoic Acid Hydrochloride (BANA108). To a stirred mixture of **5** (1.00 g, 4.46 mmol) and 10% Pd−C (1.0 g) in ethanol (10 mL) and 5% HCl (1.2 mL) was added dropwise hydrazine hydrate (55% hydrazine, 1.0 mL, 17 mmol). The mixture was stirred at room temperature for 1 h, the catalyst was filtered, and the filtrate was concentrated under reduced pressure. Compound BANA108 (0.875 g, 100%) was obtained as a white solid residue: mp 220−223 °C (methanol/hexane); IR (KBr) 3400−3200, 1630 cm^{-1}; ^1H NMR (CD_3OD) δ 7.49−7.29 (m, 2 H, aromatic), 7.20−7.12 (m, 1 H, aromatic), 2.16 (s, 3 H, $COCH_3$). Anal. Calculated for $C_9H_{10}N_2O_3$·Cl: C, 46.45; H, 4.73; N, 12.04. Found: C, 46.55; H, 5.26; N, 12.39.

Inhibition Assay. The inhibitory activity of the compounds on influenza virus neuraminidase is tested *in vitro* using two different assays and substrates, the fluorogenic 4-methylumbelliferyl-NANA and the high molecular weight fetuin, with released sialic acid detected by the thiobarb standard colorimetric assay (Aymard-Henry *et al.*, 1973; Lentz *et al.*, 1987). All inhibitors were tested with viruses containing N2 (A/Tokyo/3/67) and type B (B/Memphis/3/89) neuraminidases.

Crystallization and Inhibitor Soaking. Native crystals of A/Tokyo/3/67 NA were grown by the hanging drop method (McPherson, 1985) using purified NA heads as described (M. J. Jedrzejas, N. M. Janakiraman, L. Zhou, W. G. Laver, and M. Luo, unpublished experiments). By X-ray methods, the space group of our N2 crystals (A/Tokyo/3/67) was determined to be orthorhombic $C222_1$ with the unit cell dimensions of a = 120.97 Å, b = 141.14 Å, c = 142.16 Å. The N2−inhibitor complexes were prepared by soaking the native N2 $C222_1$ crystals in a roughly 5 mM solution of the compound of interest for about 8 h. Inhibitor concentrations higher than 5 mM destroyed the crystals. The soaking buffer contained 0.10 M sodium phosphate, 0.15 M sodium chloride, 5% DMSO (to increase solubility of inhibitor), and 12.5% PEG 4000, at pH 6.3. The crystallization of B/Lee/40 NA was described in Lin *et al.* (1990). The B/Lee/40−BANA105 complex was prepared in fashion similar to those of N2−BANA. The B/Lee/40 soaking solution contained 2.0 M sodium nitrate, 5 mM calcium chloride, 0.9% saline, 0.1% azide, 5% DMSO, and 15% PEG 4000. The tetragonal unit cell of B/Lee/40 crystals is $a = b$ = 124.54 Å and c = 71.82 Å, and the space group is $P42_12$ (Janakiraman *et al.*, 1994).

X-ray Diffraction and Refinement. The diffraction data were collected at room temperature on a SIEMENS multiple wire area detector mounted on a RIGAKU rotating anode X-ray generator (RU-200) using Cu Kα radiation. The data were processed using the XENGEN package (Howard *et al.*, 1985). The coordinates of Jedrzejas *et al.* (unpublished experiments) were used for the native A/Tokyo/3/67 NA model, and the model of Janakiraman *et al.* (1994) was used for the native B/Lee/40. The inhibitors were incorporated into the native structures by building the molecules into $||F_o| - |F_c||$ electron density maps after initial rigid body refinements of the protein component. The complexes were then refined following the X-PLOR protocol (Brunger, 1993). The topology and the parameter files for inhibitors were created on the basis of literature values and a small molecule crystal structure of the 4-(acetylamino)-3-hydroxy-5-nitrobenzoic acid for this purpose (M. J. Jedrzejas, S. Singh, W. J. Brouillette, G. M. Air, and M. Luo, unpublished experiments). The charge distribution on these molecules was calculated using the semiempirical procedures of MOPAC 6.0 (Dewar, 1983; M. J. Jedrzejas, unpublished experiments). All structures of the N2−BANA complexes presented here were refined using only half of the asymmetric

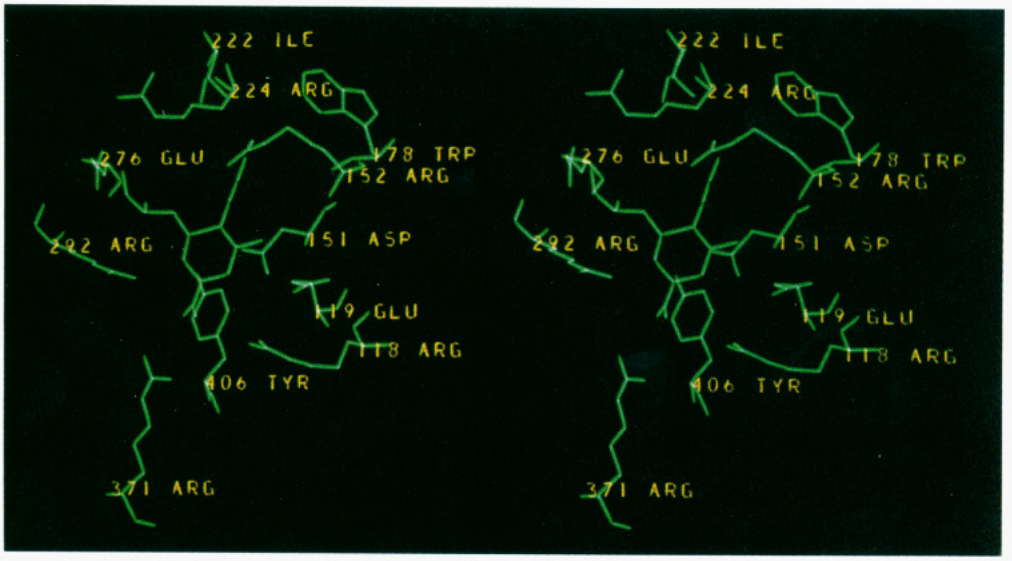

FIGURE 2: DANA in the active site of the N2 (NA/Tokyo/3/67) neuraminidase enzyme. The 11 active site residues are shown and labeled.

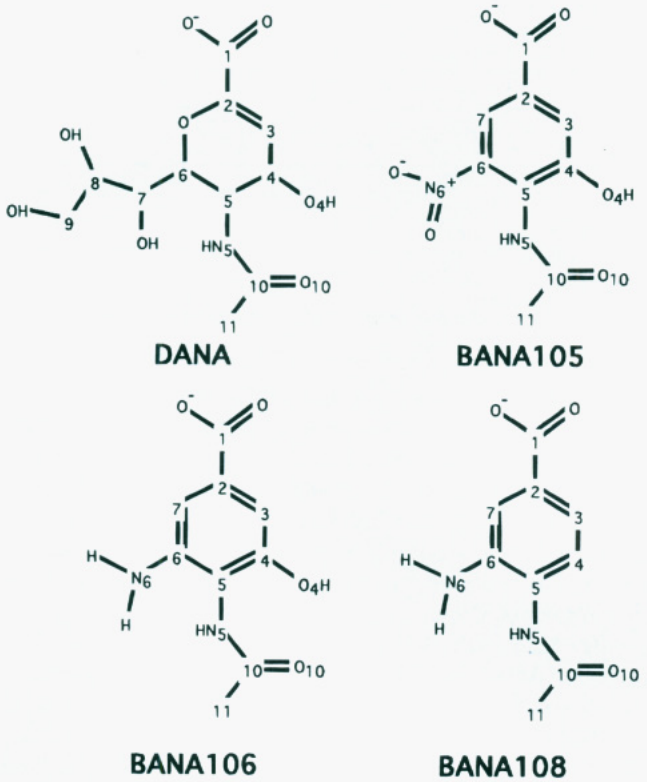

FIGURE 3: Molecular structure diagrams and the numbering schemes of DANA (2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid), 4-(acetylamino)-3-hydroxy-5-nitrobenzoic acid (BANA105), 4-(acetylamino)-3-hydroxy-5-aminobenzoic acid (BANA106), and 4-(acetylamino)-3-aminobenzoic acid (BANA108). The numbering scheme for BANA inhibitors is based on DANA's numbering scheme.

unit (one monomer of the neuraminidase protein) utilizing the noncrystallographic 2-fold axis to generate the whole asymmetric unit. For map calculations the data between 6.5 Å and the highest available resolution of the data (1.8 or 2.4 Å) were used. Finally, conjugate gradient and molecular dynamics refinements were performed by X-PLOR (Brunger, 1993) using data between 6.5 Å and the maximum resolution available and reflections with $F_o/\sigma(F_o) > 4.0$. The *B*-factor and occupancy refinement (Brunger, 1993) followed. The addition of water molecules using standard criteria succeeded for the most important structures (Table 1). The final maps were calculated using phases derived from refined coordinates without the inclusion of inhibitor atoms.

RESULTS

Inhibitor Design. 2-Deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid (DANA) has been shown to be a transition-state analog inhibitor of NA (Miller *et al.*, 1978), and this was used as the framework structure for the design of more potent inhibitors (von Itzstein *et al.*, 1993). Replacement of a hydroxyl group with a guanidino group at the C4 position of DANA produced a potent inhibitor having an inhibition binding constant (K_i) of 10^{-10} M. DANA and DANA-like inhibitors are, however, excreted from physiological systems very rapidly, thereby reducing their clinical efficacy (von Itzstein *et al.*, 1993). Furthermore, the synthesis of DANA analogs is relatively difficult since DANA contains five chiral centers and is unstable to acid or base, limiting the variety of structures that can practically be explored. A different inhibitor with a longer retention time in physiological systems, chemically more accessible and, at the same time, having the high potency of the DANA-like inhibitors, will be a more desirable pharmaceutical agent.

Evaluation of the DANA−NA interactions revealed that the carboxylate of DANA is held strongly by three arginine residues (Arg 118, 292, and 371). These charge−charge interactions dominate the energetic contribution to the tight inhibitor binding (Taylor & von Itzstein, 1994) and should be present in any newly designed inhibitors. Secondly, the acetylamino group is the only substituent which occupies a hydrophobic pocket (formed mainly by Ile 222 and Trp 178). In addition, the carbonyl oxygen of the acetylamino group forms a hydrogen bond with Arg 152. The intact acetylamino group is important for tight binding. On the other hand, the OH group at C4 and the glycerol group at C6 of DANA are the first target positions for further modification. The C4 position of DANA was utilized to add the guanidino group that afforded an inhibitor in the subnanomolar range (Taylor & von Itzstein, 1994).

Our approach was to construct new classes of noncarbohydrate molecules containing, in place of DANA's tetrahydropyran ring, a spacer that can preserve the charge

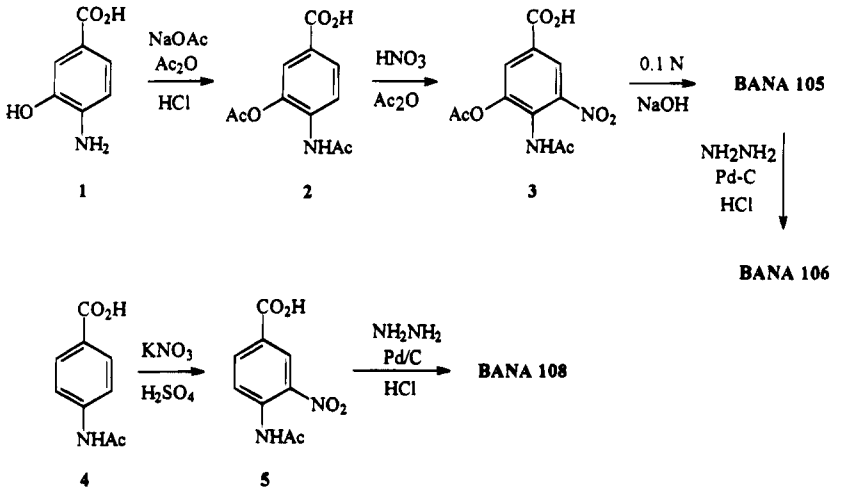

FIGURE 4: Schematic representation of the synthesis of BANA105, BANA106, and BANA108 compounds.

interactions of the carboxyl group at one end and the interaction of the acetylamino methyl group to the hydrophobic pocket and the acetylamino carbonyl oxygen interaction with Arg 152 at the other end (Figure 2). Ideally, this spacer should permit relatively simple yet versatile chemistry to be carried out for placing various substituents at C4 and C6 positions. A benzene ring is one of the best candidates which meets the criteria. Not only does its similar size to the DANA sugar ring correctly position the carboxyl and acetylamino groups in the active site, but the planarity near the carboxylate correctly mimics that found in the transition-state-like structure of DANA (Janakiraman *et al.*, 1994). Furthermore, it was chosen also for its inherent simple stereochemistry (no chiral atoms at ring−side-chain junctions) and conformational rigidity.

Initial modeling of the 4-(acetylamino)benzoic acid derivatives in the active site of the native NA was performed in order to predict possible interactions between the protein and inhibitor. A series of aromatic compounds were then synthesized and tested. We describe here the structures of three such compounds, 4-(acetylamino)-3-hydroxy-5-nitrobenzoic acid (BANA105), 4-(acetylamino)-5-amino-3-hydroxybenzoic acid (BANA106), and 4-(acetylamino)-3-aminobenzoic acid (BANA108), bound in the active site of N2 NA and BANA105 in B/Lee/40 NA. Molecular structure diagrams of these compounds together with the numbering schemes are provided in Figure 3. These compounds are not strong inhibitors of influenza virus neuraminidase, but their inhibitory activity is comparable to or better than that of sialic acid.

Chemical Synthesis and in Vitro Testing. BANA105, BANA106, and BANA108 were synthesized from commercially available benzoic acids as shown in Figure 4. For the preparations of BANA105 and BANA106, 4-amino-3-hydroxybenzoic acid (**1**) was diacetylated using acetic anhydride to give **2** in 58% yield. This was nitrated to provide **3** in 90% yield, and the acetate ester was selectively hydrolyzed under basic conditions to provide BANA105 in 98% yield. The reduction of the nitro group via transfer hydrogenation gave BANA106 in 84% yield. The preparation of BANA108 utilized commercially available 4-(acetylamino)benzoic acid, which was nitrated to provide **5** in 84% yield. This was then reduced using transfer hydrogenation, as before, to give a quantitative yield of BANA108.

Table 1: IC_{50} (mM) Inhibitory Concentrations of the Inhibitors DANA, NANA, BANA105, BANA106, and BANA108 with N2 (A/Tokyo/3/67) and B/Mem/89 Neuraminidases Utilizing Two Substrates, the Fluorogenic 4-Methylumbelliferyl-NANA (MUN) and the High Molecular Weight Fetuin

	MUN		fetuin	
inhibitor	N2 (A/Tokyo/3/67)	B/MEM/89	N2 (A/Tokyo/3/67)	B/MEM/89
DANA	0.015	0.015	–[a]	–
NANA	–	>10	–	–
BANA105	0.75	0.75	1	1
BANA106	>10	10	>10	>10
BANA108	>10	>20	>10	–

[a] A dash indicates that these values of inhibition constants were not measured.

The *in vitro* assay for the inhibitory activity of the compounds on influenza virus neuraminidase utilizes two substrates, the fluorogenic 4-methylumbelliferyl-NANA and the high molecular weight fetuin (Aymard-Henry *et al.*, 1973; Lenz *et al.*, 1987). The IC_{50} inhibitory concentrations measured for all three inhibitors, BANA105, BANA106, and BANA108, with the reference values for DANA and NANA are shown in Table 1.

Structure. The structure of the native N2 neuraminidase protein (A/Tokyo/3/67) in a new crystal form (Jedrzejas *et al.*, unpublished experiments), grown by the hanging drop method (McPherson, 1985), and structures of several N2−BANA and B/Lee/40−BANA105 complexes were solved by X-ray crystallography. The space group of the new N2 crystals is orthorhombic $C222_1$ with the unit cell dimensions of a = 120.97 Å, b = 141.14 Å, and c = 142.16 Å. The initial N2 atomic model for X-ray structure determination was obtained by molecular replacement methods (Rossman & Blow, 1962) using the coordinates of Varghese *et al.* (1991). For the B/Lee/40 NA structure, coordinates from Janakiraman *et al.* (1994) were used. The NA−inhibitor complexes were prepared by soaking the native N2 or B/Lee/40 crystals in a solution of the above inhibitors. The final results of refinement of the inhibitor complexes with N2 and B/Lee/40 NA are listed in Table 2. The structure of the N2−DANA complex was also obtained for comparison.

DISCUSSION

Comparison of Inhibitors Bound to the Active Site. The aromatic BANA inhibitors occupy the same site as DANA

Table 2: Summary of Refinement Results for Complexes of Inhibitors BANA105, BANA106, BANA108, and DANA with N2 (A/Tokyo/3/67) and B/Lee/40 NA

protein−inhibitor	no. of refl	space group and unit cell dimensions (Å)	resol (Å)	R_{sym}[a] (%)	crystal R-factor[b] (%)	no. of water molecules	rms[c] bonds (Å)	rms[c] angles (deg)
N2 native[d]	38 881	$C222_1$; $a = 120.97$, $b = 141.14$, $c = 142.16$	1.8	9.7	21.6	125	0.011	1.84
N2−BANA105	33 264	$C222_1$; $a = 119.93$, $b = 139.87$, $c = 140.01$	1.8	11.9	23.4	125	0.013	2.04
N2−BANA106	28 488	$C222_1$; $a = 120.35$, $b = 139.72$, $c = 140.23$	2.4	8.7	19.9	0	0.013	2.02
N2−BANA108	25 898	$C222_1$; $a = 121.88$, $b = 140.88$, $c = 141.49$	2.4	9.3	21.7	0	0.015	1.70
N2−DANA	23 041	$C222_1$; $a = 119.38$, $b = 139.63$, $c = 140.13$	2.4	13.1	16.3	125	0.012	1.97
B/Lee/40−BANA105	15 271	$P42_12$; $a = b = 124.54$, $c = 71.82$	2.4	10.4	18.9	0	0.016	2.14

[a] $R_{sym} = 100(\sum I_i - \langle I \rangle)/\sum I_i)$, where $\sum I_i$ is the sum of all observations of all reflections. [b] R-factor $= 100(\sum||F_o| - |F_c||/\sum|F_o|)$, $[F_o/\sigma(F_o) > 4.0$, data 6.0−1.9 (2.4) Å]. [c] rms = root-mean-square deviation. [d] The results from the refinement of the native N2 structure are included for comparison.

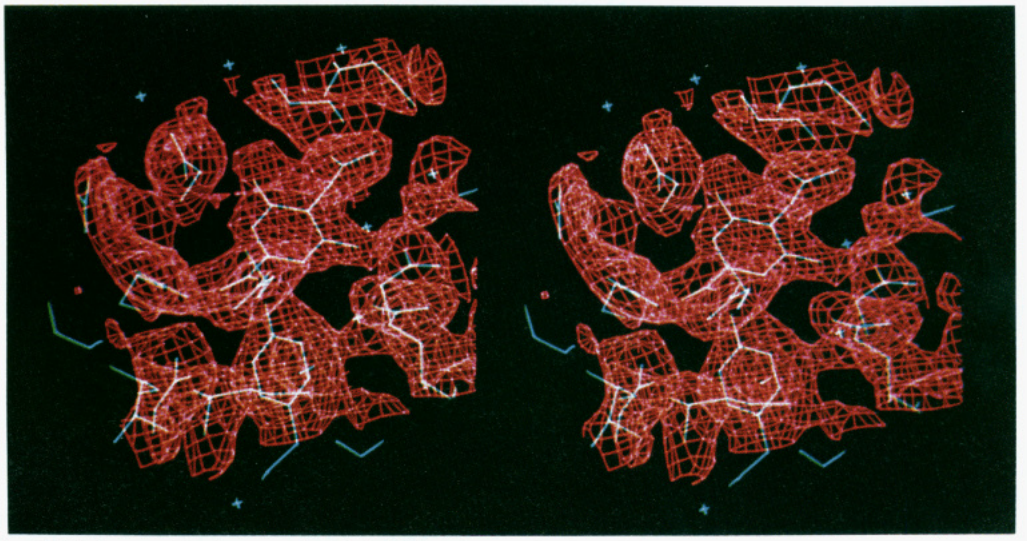

FIGURE 5: $|2|F_o| - |F_c||$ electron density map for the inhibitor 4-(acetylamino)-3-hydroxy-5-aminobenzoic acid (BANA106) in the active site of the N2 protein. The map is drawn at the 1.0σ level. The map was calculated from phases obtained from refined coordinates without the inclusion of inhibitor coordinates. The data between 6.5 Å and the highest resolution of the data (2.4 Å) were used. The benzene ring density is doughnut-like shaped with all substituents very well defined within the density.

Table 3: Interactions between BANA105, BAMA106, BANA108, and DANA Inhibitors and the N2 (A/Tokyo/3/67) and B/Lee/40 NA

inhibitor's group	inhibitor's atom	residue name	atom name	distance (Å)				
				BANA105−N2	BANA106−N2	BANA108−N2	DANA−N2	BANA105−B/Lee/40
CO_2	O1A	Arg 371	NH1	3.65	3.07	3.42	2.96	3.08
		Arg 292	NH1	3.44	3.77	3.51	3.81	3.19
	O1B	Arg 371	NH2	3.72	4.35	3.87	3.92	3.45
		Arg 118	NH1	3.00	4.20	3.38	3.56	3.36
		Arg 118	NH2	3.15	2.86	5.35	4.41	2.69
ring	center	Tyr 406	OH	3.24	3.05	3.26	3.11	3.16
acetylamino	O10	Arg 152	NH2	2.57	3.11	3.08	3.19	2.87
OH	O4	Asp 151	OD2	3.61	3.55		3.26	3.17

in the active site of NA. There are, however, some differences in the orientation of the ring and the side-chain groups. The $|2|F_o| - |F_c||$ electron density maps at the 1.0σ level for the three N2−BANA inhibitors are shown in Figure 5. The electron density maps were calculated using phases obtained from the refined complex coordinates omitting the inhibitor. Substituents of the benzene ring are well-defined within the density. The interactions between BANA inhibitors and active site residues of N2 or B/Lee/40 NA complexes are tabulated in Table 3.

The mode of binding for all three BANA inhibitors is cognate. Figure 6 shows all three inhibitors overlaid together inside the N2 active site (the protein structures were aligned to each other by minimizing rms differences of their Cα atom positions to account for unit cell length differences). BANA105 and BANA106 are coplanar whereas BANA108 tilts upward at the NH_2 side by about 10° due to lack of constraints. Carboxyl groups of these inhibitors are approximately parallel to the $-NHC(NH)NH_2$ plane of Arg 371 (same as for DANA) to accommodate this charge-charge interaction. The carbonyl oxygen of the acetylamino group points towards Arg 152 to optimize the hydrogen bond interactions. For BANA105, the electron density for NO_2 is, as expected, substantially larger than that for the OH group on the opposite side of the benzene ring, and therefore, the nitro group of BANA105 is unambiguously placed near Glu 276 and the OH group is near Asp 151. BANA105 bound in B/Lee/40 is identical to that in N2. The amino group of BANA108 is clearly located on the same side of the active site as the nitro group of BANA105. For BANA106, however, it is impossible to incontestably distinguish between the amino and the hydroxyl groups since the electron densities of these two groups are very similar. By analogy to BANA108, the amino group of BANA106 was placed on

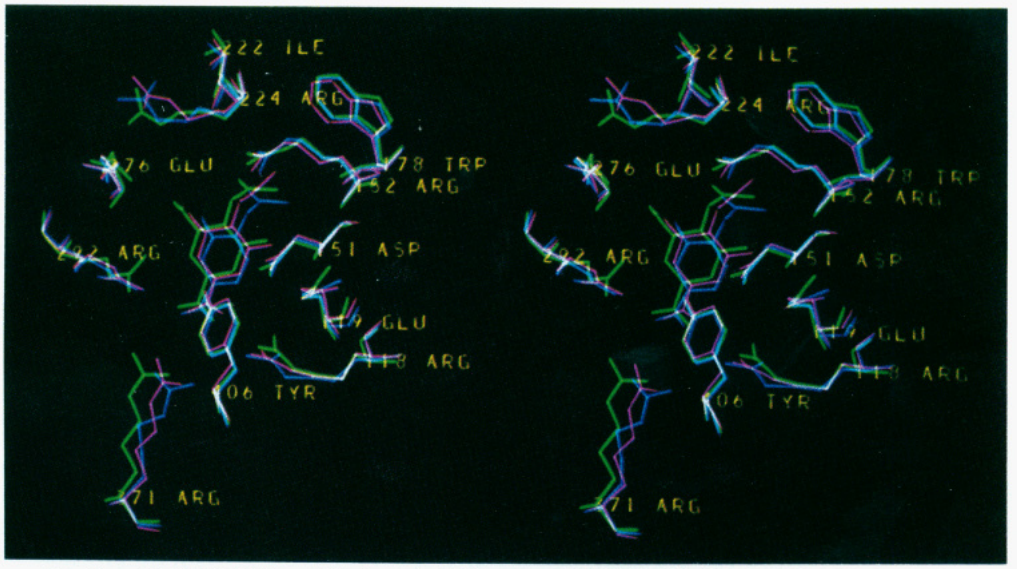

FIGURE 6: Binding of the three BANA inhibitors 105 (green), 106 (red), and 108 (blue) inside the N2 (A/Tokyo/3/67) neuraminidase active site. Only active site residues are shown.

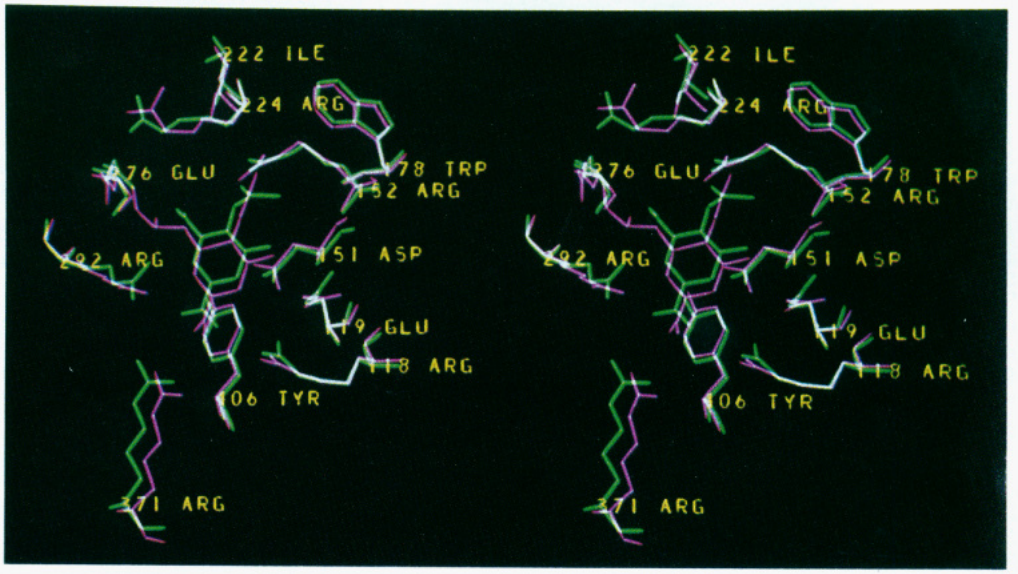

FIGURE 7: Comparison of binding of BANA105 (green) and DANA (red) to N2 (A/Tokyo/3/67) neuraminidase. Only active site residues are shown.

the same side of the BANA108 amino group. The BANA108 inhibitor lacking a group at C4 is moved away from Glu 276 when compared to BANA105, which has a hydroxyl group at this position. In addition, the benzene ring may be involved in aromatic interactions with Tyr 406. The center of the inhibitor ring is on average 3.18 Å away from the OH group of the tyrosine.

A comparison of BANA105 and DANA inhibitors bound to N2 NA (Figure 7) showed that the benzene ring of BANA105 is nearly parallel to the tetrahydropyran ring of DANA, and the major interactions that include carboxyl group−Arg 371, 118, 292, the acetylamino group interaction with the hydrophobic pocket, and the acetylamino carbonyl−Arg 152 interaction are preserved. The plane of CO_2 is parallel to the plane of $-NHC(NH)NH_2$ of Arg 371. BANA105 is shifted toward Glu 276 when compared with DANA, but there is no interaction contact involved between the nitro group of BANA105 and Glu 276. The glycerol group on DANA makes hydrogen bonds with Glu 276 at this position.

Binding Constants. The affinity of these three BANA inhibitors is correlated to their interactions with the active site of NA. In the BANA108 complex there are no extra interactions other than those of the carboxylate and the acetylamino groups. Its benzene ring is not constrained to the optimal binding position (a 10° rotation when compared to DANA). As a result, it is the weakest inhibitor of the three. In the BANA106 complex, the addition of a hydroxy group at the C4 position eliminates the rotation of the benzene ring and also resulted in an increase in binding affinity by hydrogen bonding to Asp 151. The presence of an amino group in BANA106 did not introduce any additional interactions. The enhanced inhibitory binding of BANA105 when compared to BANA106, on the other hand, must be due to the presence of the nitro group at the C6 position since it has the same OH interactions as BANA106. The contribution of the NO_2 group is not totally evident but appears in part to result from a slight repositioning of this inhibitor. Changes in solvation may also contribute to this enhancement. On the basis of the above three preliminary

inhibitors and computational chemistry methods a series of more potent inhibitors are being designed.

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