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Hydrophobic Benzoic Acids as Inhibitors of Influenza Neuraminidase

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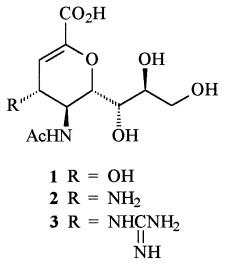
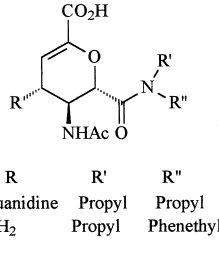
Abstract—Neuraminidase (NA) plays a critical role in the life cycle of influenza virus and is a target for new therapeutic agents. A new benzoic acid inhibitor (**11**) containing a lipophilic side chain at C-3 and a guanidine at C-5 was synthesized. The X-ray structure of 4-(*N*-acetylamino)-5-guanidino-3-(3-pentyloxy)benzoic acid in complex with NA revealed that the lipophilic side chain binds in a newly created hydrophobic pocket formed by the movement of Glu 278 to interact with Arg 226, whereas the guanidine of **11** interacts in a negatively charged pocket created by Asp 152, Glu 120 and Glu 229. Compound **11** was highly selective for type A (H2N2) influenza NA (IC_{50} 1 μM) over type B (B/Lee/40) influenza NA (IC_{50} 500 μM).

Introduction

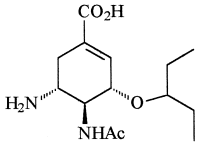
New and emerging strains of influenza virus cause serious disease each year.[1] Although vaccination is available, it is often ineffective due to the high antigenic changes exhibited by the influenza virus. Therefore, efforts have focused on rational drug design to develop new agents against potential targets within the virus. The enzyme sialidase, or neuraminidase (NA), a surface glycoprotein, plays a critical role in the life cycle of influenza virus and has been the focus of new drug development. NA mediates the cleavage of terminal sialic acid residues from the cell surface glycoproteins and glycolipids, liberating the virus from the infected cell's surface to facilitate spread. This process may also enhance viral movement through the mucus of the respiratory tract to infect epitheliel cells.[2–4] NA consists of a symmetrical tetrameric head, which projects from



1 R = OH
2 R = NH_2
3 R = $NHCNH_2$ (=NH)



	R	R'	R"
4a	Guanidine	Propyl	Propyl
4b	NH_2	Propyl	Phenethyl



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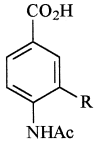
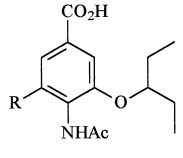


6 R = $NHCOCH(CH_2CH_3)_2$
7 R = $NHCH(CH_2CH_3)_2$
8 R = $OCH(CH_2CH_3)_2$
9 R = $OCH(CH_2CH_2CH_3)_2$
BANA 101 R = OH
BANA 108 R = NH_2
BANA 113 R = Guanidine



10 R = NH_2
11 R = $NHCNH_2$ (=NH)

Key words: Neuraminidase; influenza virus; sialidase; structure-based drug design.
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the viral membrane through a centrally attached stalk. Each of the four subunits of the head contains a catalytic site and a calcium binding site. The stalk of the NA has been proteolytically cleaved to release the heads, which can be crystallized. The released enzyme retains catalytic activity. Based on antigenic cross reactivity, nine subtypes of NA have been identified for influenza A whereas only one subtype exists for influenza B. The crystal structures of NA's from both types A and B reveal that the catalytic site is completely conserved among all influenza subtypes.[5–7] A symmetrical folding pattern of six four-stranded β-sheets in a propeller fashion forms the active site, which is composed primarily of charged amino acids.[5,8–13] Site-specific mutagenesis of these residues usually results in inactivation of the enzyme, indicating that the virus may not easily escape NA targeted drug therapy by mutation.[14,15] In animals administration of NA-specific antibodies limits viral replication and infection, indicating that this enzyme represents a suitable target for antiviral drug development.[16] Furthermore, a NA minus mutant was unable to replicate. Currently three NA inhibitors have entered clinical trials.[6,28,30]

In earlier studies it was found that 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (**1**) (neu5Ac2en), an analogue of sialic acid, is an inhibitor of NA with a K_i of 4 μM.[17,18] Based on the structural information provided by the crystal structure of **1** complexed with NA several potent inhibitors were designed. Both amine **2** and guanidine **3** are more potent inhibitors than **1** with K_i values of 10^{-8} and 10^{-10} M, respectively.[6,19,20] However, these compounds are not orally active, they are stereochemically complex, and their synthesis can be lengthy. In an attempt to develop chemically simple inhibitors, we prepared several achiral benzoic acids, among which 4-*N*-acetylamino-3-guanidinobenzoic acid (BANA 113) was a modest inhibitor with an IC_{50} value of 10 μM.[22–24]

The crystal structure of **BANA 113** complexed with NA(Fig. 2) revealed that it interacts with NA in a similar fashion to **3** with the exception of the guanidino group (Fig. 1). In compound **3**, the guanidine interacts

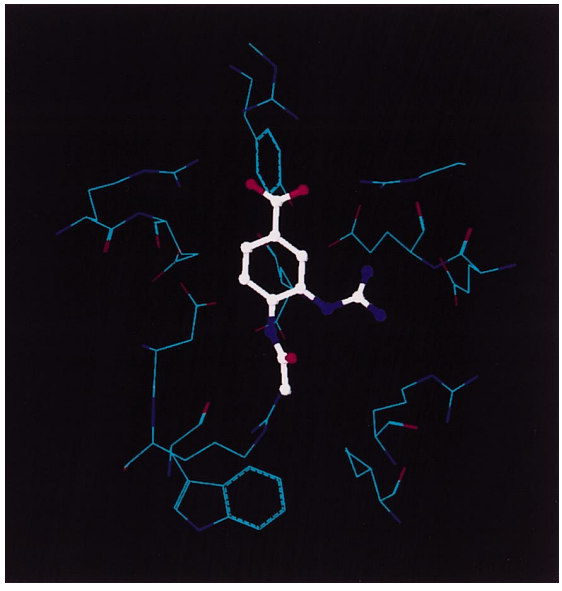


Figure 2. X-ray crystal structure of the binding site region for the complex formed between **BANA 113** and N9 neuraminidase.

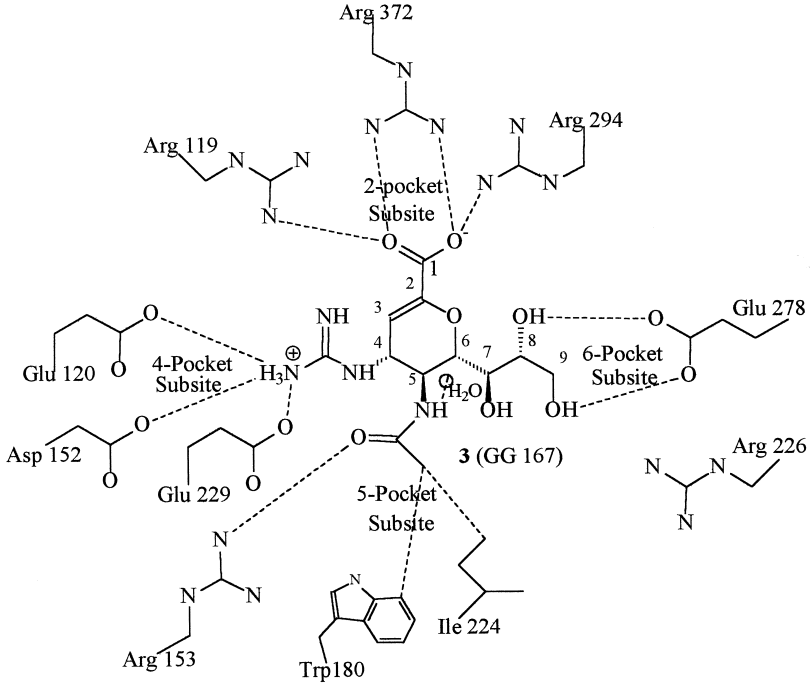


Figure 1. Cartoon showing the interactions of **3** (GG167) in the neuraminidase binding site (N9 numbering). Note that Tyr 406 lies under **3** and is not shown.

electrostatically with Asp 152, Glu 229 and Glu 120 (C4 subsite), and the glycerol forms hydrogen bonds with Glu 278 (C6 subsite).[21] However, in **BANA 113** the guanidine occupies the glycerol binding subsite (C6 subsite) of **3**, forming a salt bridge with Glu 278.

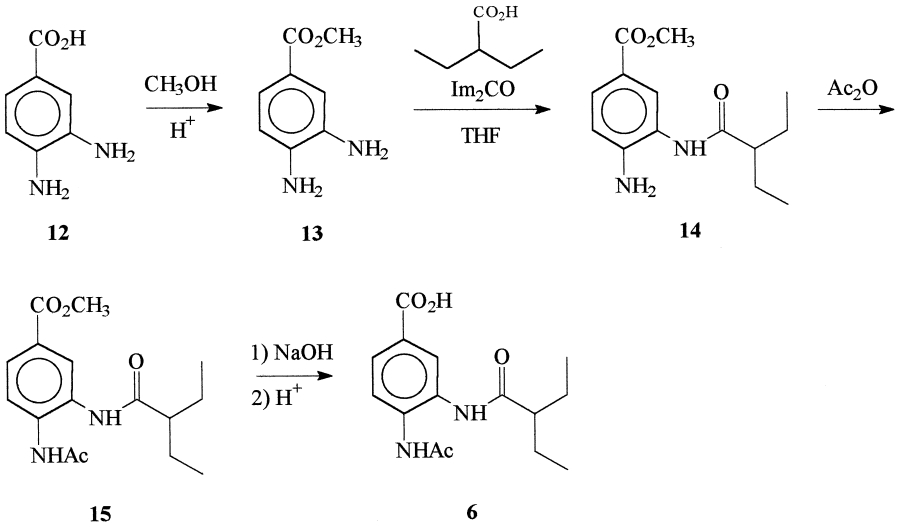
Recent studies indicate that the replacement of the glycerol side chain of **3** with hydrophobic side chains, as in **4a**, results in potent inhibitory activity, although these compounds are highly selective for type A over type B NA.[25,26] On the other hand the cyclohexene analogue **5** displayed broad spectrum activity against both influenza A and B sialidases.[28,29] The crystal structure of **4b** in complex with NA revealed that it binds to the enzyme in essentially the same way as **3**, except the hydrophobic side chain interacts in a hydrophobic pocket (C6 subsite) resulting from the movement of Glu 278 to interact with Arg 226. In order to determine the effect of hydrophobic substituents in place of, and in addition to, the guanidino grouping of **BANA 113**, we carried out the synthesis and in vitro evaluation of benzoic acids **6**–**11**.

Chemistry

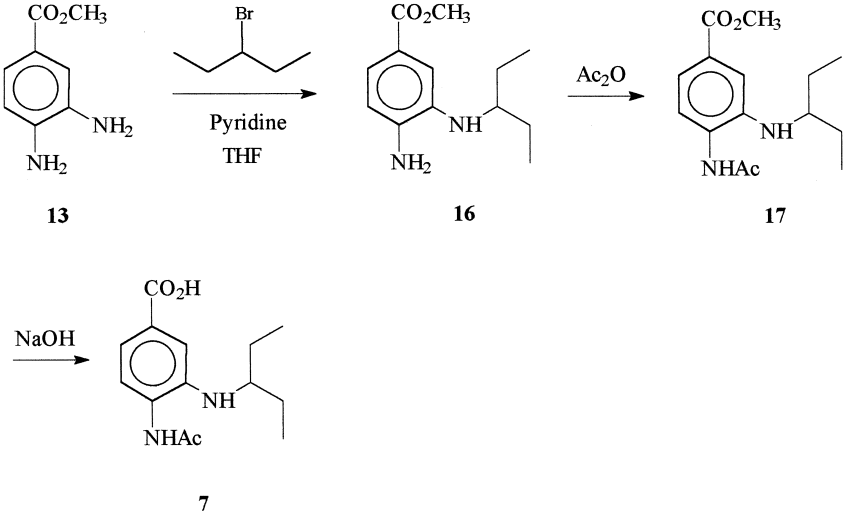
The synthesis of hydrophobic amide **6** is described in Scheme 1. The Fischer esterification of 3,4-diaminobenzoic acid (**12**) gave **13**, which underwent regioselective acylation, using 2-ethylbutyric acid, on the 3-amino group to provide amide **14**. This intermediate was then *N*-acetylated and the ester saponified to provide target **6**.

Similarly, the synthesis of hydrophobic aniline **7** is summarized in Scheme 2. Ester **13** underwent regioselective alkylation, using 3-bromopentane, on the 3-amino group to give intermediate **16**. This underwent *N*-acetylation to yield **17**, which was saponified to form the final product **7**.

The synthesis of **8** and **9**, as shown in Scheme 3, began with the Fischer esterification of 4-amino-3-hydroxybenzoic acid (**18**). Selective alkylation of the resulting phenol (**19**) with 3-bromopentane or 4-bromoheptane in the presence of K_2CO_3 provided the methyl 3-alkoxy-4-



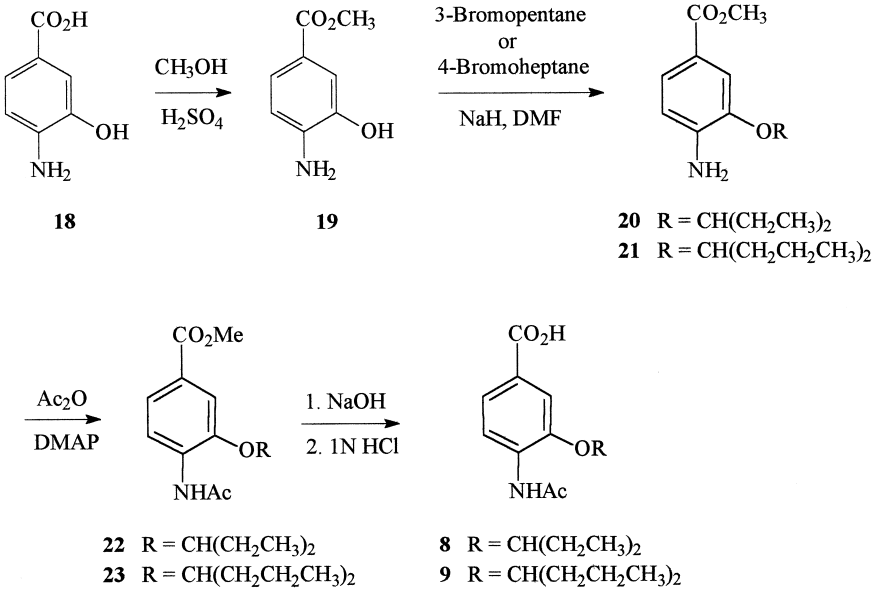
Scheme 1.



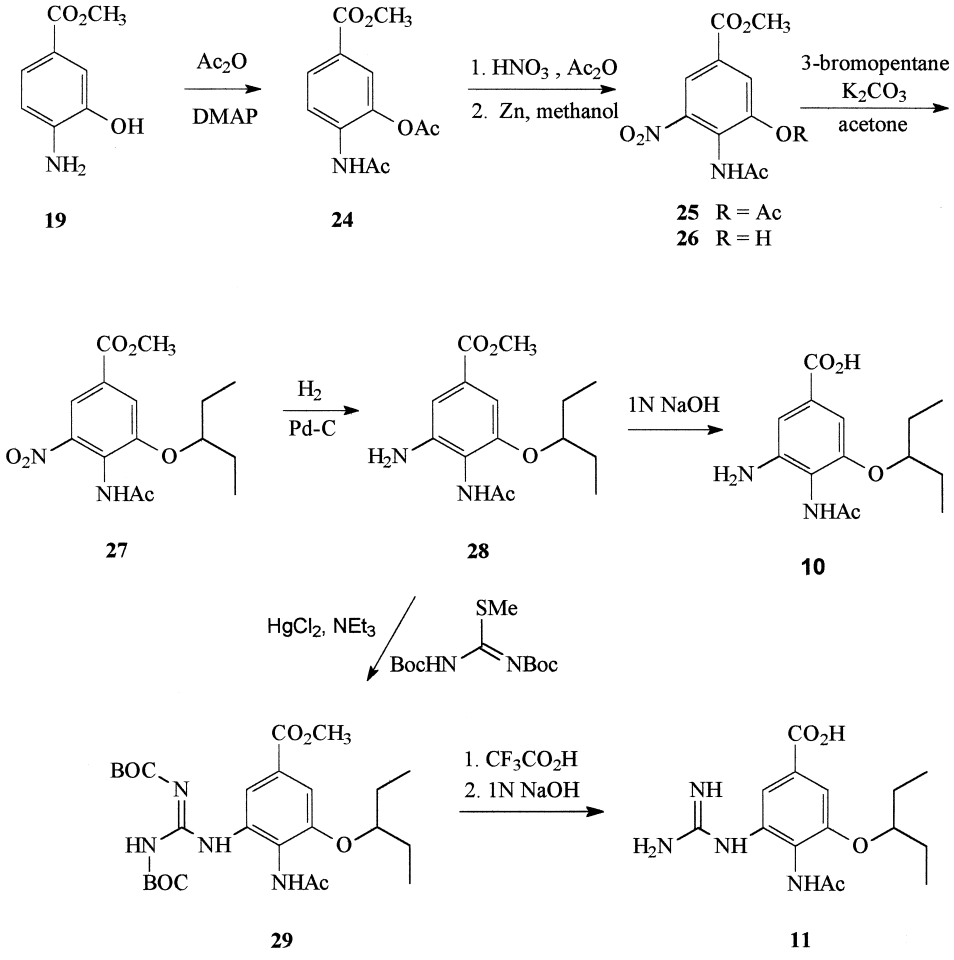
Scheme 2.

aminobenzoates **20** and **21** in 90% yield. The *N*-acetylation of each in the presence of acetic anhydride and DMAP to give **22** and **23**, followed by saponification, provided **8** and **9**.

The synthesis of 4-(*N*-acetylamino)-5-guanidino-3-(3-pentyloxy)benzoic acid (**11**) is summarized in Scheme 4. Acetylation of methyl 4-amino-3-hydroxybenzoate (**19**) in the presence of acetic anhydride and DMAP gave **24**



Scheme 3.



Scheme 4.

in quantitative yield. The nitration of **24** using HNO_3 and acetic anhydride provided the desired regioisomer **25**. Generation of the phenol **26** in the presence of Zn and methanol followed by *O*-alkylation with 3-bromopentane provided methyl 4-(*N*-acetylamino)-5-nitro-3-(3-pentyloxy)benzoate (**27**) in 80% yield. The reduction of the nitro functionality using catalytic hydrogenation gave the amine **28.** Saponification of the ester in **28** provided target **10**. Alternatively, compound **28** underwent guanylation with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea to provide **29**. The acidic removal of the Boc groups followed by basic hydrolysis of the ester provided the target molecule **11**.

Biological Assays

All compounds were evaluated for in vitro inhibitory activity using purified NA from the H2N2 and H1N9 strains of influenza A and from B/Lee/40. Since in our experience the potency of benzoic acid inhibitors for N9 (an avian strain) correlates well with N2 (infects humans), only selected compounds were tested against H2N2. The assay was based upon a published method.[32]

X-ray Crystallography

Purified N9 NA was crystallized using the hanging drop method, and the resulting crystals were diffracted strongly to at least 2 Å resolution. Inhibitor complexes with enzyme were prepared by soaking the crystal in a 1–2 mM solution of the inhibitor in stabilization buffer for at least 24 h.

Results and Discussion

As depicted in Figure 1, the crystal structure of **3** in complex with NA[21] revealed that the C-8 and C-9 hydroxyl groups interact with Glu 278, while the carbon framework of the glycerol side chain makes hydrophobic interactions with Arg 226. Initially, when **BANA 113** was designed, the guanidino group was expected to mimic the interactions of the guanidine in compound **3**. The guanidine group in **3** interacts in the C-4 subsite containing Asp 152, Glu 229 and Glu 120. However, in **BANA 113** the guanidino group occupies the glycerol subsite (C-6 subsite) of **3** and interacts with Glu 278 (Fig. 2). This may be due to the structural constraints of the planar benzene ring. However, the presence of an additional substituent, such as a lipophilic side chain at the 5-position of **BANA 113**, should occupy the glycerol binding subsite of **3** and thus force the guanidino group into the C-4 subsite. To test the feasibility of this approach, the simple model compounds **6–9** were synthesized. Compounds **6–8** all contain the 3-pentyl side chain, differing only in the mode of connection to the benzene ring, while compound **9** contains the more hydrophobic 4-heptyl group.

The results of enzyme inhibition assays for **6–9** are given in Table 1. Those compounds containing the 3-pentyl group (**6–8**) were equally effective inhibitors of influenza A NA (IC_{50} = 25–37 μM for N9), while compound **9**, which contained a 4-heptyl group, was roughly 10 times less effective (IC_{50} = 230 μM). Comparison of the activity of compounds **6–8** to their parent structures (**BANA 101** or **BANA 108**) reveals that the hydrophobic 3-pentyl grouping enhances activity by at least 400 times. Also of interest was the observation that compounds **6–9** exhibited 20- to >100-fold selectivity for NA from influenza A as compared to influenza B.

In order to confirm the expected orientation of the hydrophobic group in the glycerol binding subsite, the crystal structure of the complex of **8** with NA was determined (Fig. 3). As revealed by the crystal structure, the interactions of the carboxylic acid and the acetamido groups were similar to those observed for **3**. The carboxylate interacts in a strongly positively charged

Table 1. In vitro inhibitory effects of benzoic acid analogues on influenza A and B neuraminidases (IC_{50}, μM),[a] as compared with sialic acid derivatives

Compound	H2N2	H1N9	B/Lee/40
3	5×10^{-3}		4×10^{-3}
4	$1\times10-^{3}$		0.5
6		27	>670
7		25	2300
8	15	37	>5000
9		230	>10,000
10	8	15	2000
11	1	3	500
BANA 101[b]	>10,000		>10,000
BANA 108[b]	>10,000		>20,000
BANA 113[b]	10		10[c]

[a] IC_{50} values are the mean of duplicate experiments. In all cases each IC_{50} value differed from its duplicate by less than twofold.
[b] Data taken from ref 20.
[c] Determined using B/Mem/89 neuraminidase.

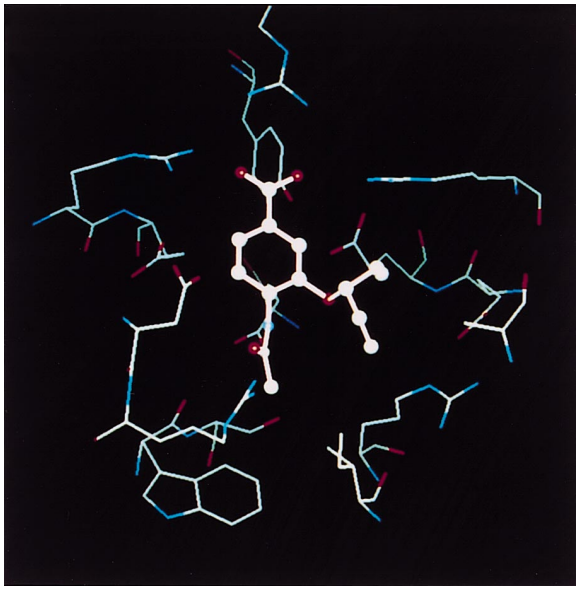


Figure 3. X-ray crystal structure of the binding site region for the complex formed between benzoic acid **8** and N9 neuraminidase.

pocket created by three arginines (Arg 119, Arg 294, Arg 372). The carbonyl of the *N*-acetyl group hydrogen bonds to Arg 153 and the methyl group occupies a hydrophobic pocket created by Trp 180 and Ile 224. However, the pentyloxy group interacts in a hydrophobic pocket lined by Glu 278, Arg 226, Ala 246 and Ile 224. In **BANA 113** the guanidine occupies this same subsite and makes electrostatic interactions with Glu 278. In the complex with inhibitor **8** this interaction is not available, and Glu 278 adopts a different conformation in which it interacts with Arg 226, creating a hydrophobic pocket into which the pentyloxy group binds. Despite the identical location of the conserved amino acids that form the first shell in influenza A and B sialidases, these compounds selectively inhibit sialidase A. This result is consistent with earlier reports on hydrophobic carboxamide analogues of sialic acid.[27] For example, inhibitors **4a** and **4b** exhibit a magnitude of selectivity for influenza A over influenza B similar to that observed for **6**–**8**. In the crystal structures of **4b** with influenza A and B sialidases, the movement of Glu 278 (Glu 276 in influenza B) was accompanied by formation of a salt bridge with Arg 226 (as also observed for **8** in complex with flu B). In influenza A sialidase, this rearrangement resulted in minimal disruption of the surrounding protein structure, whereas in B sialidase this movement created distortions in the protein backbone near Glu 278 and in the second amino acid shell, since this region contains differences among non-conserved amino acids as compared to type A. Thus salt bridge formation in influenza B sialidase, in comparison to influenza A, is believed to be associated with additional energy penalties, resulting in a much weaker inhibition constant. We anticipate that the same factors account for the type A selectivity of inhibitors **6**–**9**.

In light of these results, compounds **10** and **11**, which contain amino and guanidino side chains, respectively, at the 5-position were synthesized. Compound **10** inhibited influenza A NA with an IC_{50} value of 15 μM and compound **11** with an IC_{50} value of 3 μM. The activity of **11** represents a smaller enhancement in activity than was anticipated if the hydrophobic interactions of **8** were maintained and the guanidino group properly oriented into the comparable binding subsite of **3**. Thus the crystal structure of inhibitor **11** in complex with the enzyme was determined. As shown in Figure 4, interactions of the carboxylate, pentyloxy and acetamido groups were preserved as also observed for compound **8**. Additionally, as anticipated, the guanidine in compound **11** occupies the guanidine subsite (C-4 subsite) of **3**, interacting electrostatically with Glu 229 and Asp 152. This result was due to the structural constraints imposed by the 3-pentyloxy group, which occupies the large hydrophobic pocket created by Glu 278, Arg 226, Ala 246 and Ile 224.

We previously reported[31] a similar result upon evaluating 4-(*N*-acetylamino)-3,5-diguanidinobenzoic acid in comparison to **BANA 113**. While the reasons for the poor improvement in binding of benzoic acids which orient the guanidino group into the C-4 subsite of **3** remain unclear, some possibilities may be noted. For example, the conformation of the guanidine required for bound **11** may be a higher energy one.

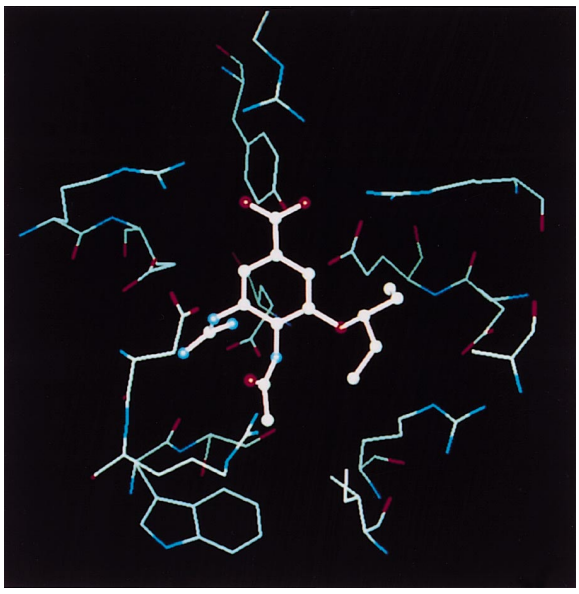


Figure 4. X-ray crystal structure of the binding site region for the complex formed between benzoic acid **11** and N9 neuraminidase.

This study clearly demonstrates that properly substituted benzoic acids interact with neuraminidase in a similar manner to non-aromatic inhibitors, and that benzoic acids provide an attractive structural template for developing more effective agents. Since hydrophobic groupings on this template can significantly enhance binding, the presence of a hydrophobic grouping and an alternative polar side chain for interaction in the C-4 subsite (other than guanidine) may provide more potent benzoic acid inhibitors.

Experimental

General methods

Melting points were obtained on an electrothermal melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker ARX 300. Infrared spectra were recorded on a Bruker FT infrared spectrophotometer. Combustion analyses were provided by Atlantic Microlabs of Atlanta, GA. Mass spectra were obtained on a Perkin–Elmer API triple-quadrupole mass spectrometer using positive electrospray ionization. Analytical chromatography was performed on Whatman PE Sil G/UV silica gel plates. Flash chromatography was performed using J.T. Baker silica gel (40 μM). Tetrahydrofuran (THF) was distilled from sodium metal/benzophenone. Dichloromethane (CH_2Cl_2) and benzene were distilled from calcium hydride. Anhydrous *N,N′*-dimethylformamide (DMF) was purchased from Aldrich in sure-seal containers. Methanol was distilled from $Mg(OMe)_2$. All other commercially obtained reagents were used as received. The H2N2 neuraminidase (A/Singapore/1/57) was obtained from Dr.

Robert Webster at St. Jude Children's Hospital, Memphis, TN. The H1N9 neuraminidase was obtained from Dr. Graeme W. Laver at John Curtin School of Medical Research, Australian National University, Canberra 2601, Australia.

4-(*N*-Acetylamino)-3-[*N*-(2-ethylbutanoyl)amino]benzoic acid (6). Compound **15** (0.15 g, 0.49 mmol) was dissolved in 1 N sodium hydroxide (3.5 mL) and stirred at room temperature for 2 h. To the pale orange reaction mixture was carefully added glacial acetic acid, to pH 5, and the reaction mixture was then cooled to 0°C. The white precipitate was filtered and washed with cold water to provide 0.14 g (100%) of **6** as a white solid: mp 286–291°C. MS *m*/*z* 293 (M + 1); ^{1}H NMR (300 MHz, DMSO) δ 0.89 (t, 6H, *J* = 7.3 Hz), 1.40–1.61 (m, 4H), 2.03 (s, 3H), 2.26–2.32 (m, 1H), 7.57 (d, 1H, *J* = 8.1 Hz), 7.54 (d, 1H *J* = 8.1 Hz), 7.92 (s, 1H), 9.92 (s, 1H), 10.01 (s, 1H). Anal. calcd for $C_{15}H_{20}N_2O_4 \cdot 0.25H_2O$: C, 60.71; H, 6.91; N, 9.44. Found: C, 60.94; H, 6.94; N, 9.49.

4-(*N*-Acetylamino)-3-[*N*-(3-pentyl)amino]benzoic acid (7). Compound **17** (0.105 g, 0.380 mmol) was dissolved in 1 N sodium hydroxide. After 6 h the orange mixture was carefully acidified with acetic acid to pH 4.5–5 and then cooled in the refrigerator. The precipitate was filtered and washed with cold water to provide 0.080 g (80%) of **7** as a light brown solid. R_f 0.16 (50% ether–chloroform); mp 145–148°C (water). MS *m*/*z* 265 (M + 1). ^{1}H NMR (300 MHz, DMSO) δ 0.89 (t, 6H, *J* = 7.3 Hz), 1.45–1.58 (m, 4H), 2.09 (s, 3H), 3.24 (m, 1H), 4.76 (d, 1H, *J* = 7.9 Hz), 7.12 (dd, 1H, *J* = 1.6 and 8.0 Hz), 7.17 (s, 1H), 7.37 (d, 1H, *J* = 8.0 Hz), 12.3 (br, 1H). Anal. calcd for $C_{14}H_{20}N_2O_3$: C, 61.54; H, 7.33; N, 10.26. Found: C, 61.39; H, 7.33; N, 10.21.

4-(*N*-Acetylamino)-3-(3-pentyloxy)benzoic acid (8). A suspension of **22** (130 mg, 0.460 mmol) in 0.2 mL of methanol and 0.8 mL of 1N NaOH was stirred at room temperature for 15 h. The methanol was evaporated under vacuum below 40°C and the reaction mixture was diluted with 0.5 mL of 1 N NaOH and water (5 mL). The aqueous layer was washed with 5 mL of ether and then acidified with 1 N HCl, during which time the product precipitated. The mixture was cooled at 0°C for 30 min, filtered, and air dried to give **8** (100 mg, 82.0%): mp 149–152°C (ether). MS *m*/*z* 266 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.5 (d, 1H, *J* = 8.4 Hz), 8.0 (br, 1H), 7.74 (dd, 1H, *J* = 1.7 and 8.4 Hz), 7.58 (d, 1H, *J* = 1.7 Hz), 4.33 (m, 1H), 2.24 (s, 3H), 1.7 (m, 4H), 0.99 (t, 6H); ^{13}C NMR ($CDCl_3$) δ 171.5, 168.9, 146.0, 133.5, 124.3, 123.9, 118.9, 113.6, 81.9, 26.0, 25.13, 9.6. Anal. calcd for $C_{14}H_{19}NO_4 \cdot 0.5H_2O$: C, 61.31; H, 7.38; N, 5.10. Found: C, 61.73; H, 7.04; N, 5.21.

4-(*N*-Acetylamino)-3-(4-heptyloxy)benzoic acid (9). A suspension of **23** (300 mg, 0.980 mmol) in 1.5 mL of methanol and 2.0 mL of 1 N NaOH was stirred at room temperature for 12 h. The methanol was evaporated under vacuum below 40°C, and the reaction mixture was diluted with 1 mL of 1 N NaOH and water (5 mL). The aqueous layer was washed with 5 mL of ether and then acidified with 1 N HCl, during which time the product precipitated. The mixture was cooled for 30 min, filtered and air dried to give **9** (280 mg, 98.0%) as a white solid: mp 165–167°C (chloroform). MS *m*/*z* 294 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.3 (br, 1H), 8.5 (d, 1H, *J* = 8.5 Hz), 8.0 (br, 1H), 7.74 (dd, 1H, *J* = 1.6 and 8.5 Hz), 7.6 (d, 1H, *J* = 1.6 Hz), 4.4 (m, 1H), 2.2 (s, 3H), 1.68 (m, 4H), 1.4 (m, 4H), 0.95 (t, 6H, *J* = 7.3 Hz); ^{13}C NMR (CD_3OD) δ 171.6, 169.5, 148.9, 133.7, 127.4, 123.6, 121.6, 114.9, 80.5, 37.1, 24.4, 19.7, 14.6. Anal. calcd for $C_{16}H_{23}NO_4 \cdot 0.25H_2O$: C, 64.50; H, 7.80; N, 4.70. Found: C, 64.14; H, 7.71; N, 4.71.

4-(*N*-Acetylamino)-5-amino-3-(3-pentyloxy)benzoic acid (10). A solution of **28** (100 mg, 0.340 mmol) in 0.5 mL of methanol and 2 mL of 0.5 N NaOH was stirred at room temperature for 2 h. The reaction mixture was acidified with glacial acetic acid, the methanol was removed under vacuum, and the residual aqueous layer was cooled to give a precipitate. This was filtered and dried to give **10** (60 mg, 63%): mp 165–170°C (decomposed). MS *m*/*z* 281 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.5 (br, 1H), 7.1 (br, 1H), 7.0 (br, 1H), 5.5 (br, 3H), 4.25 (m, 1H), 2.2 (s, 3H), 1.65 (m, 4H), 0.95 (t, 6H). Anal. calcd for $C_{14}H_{20}N_2O_4 \cdot 0.5H_2O$: C, 58.13; H, 7.27; N, 9.67. Found: C, 57.79; H, 7.12; N, 9.42.

4-(*N*-Acetylamino)-5-guanidino-3-(3-pentyloxy)benzoic acid (11). A solution of **29** (100 mg, 0.18 mmol) and trifluoroacetic acid (150 mg) in 0.5 mL of CH_2Cl_2 was stirred at room temperature for 4 h. Trifluoroacetic acid (500 mg) and CH_2Cl_2 (0.5 mL) were added and stirring was continued for another 5 h. The solvent and excess trifluoroacetic acid were removed under vacuum to give 86 mg of white solid which was dissolved in methanol (1 mL) and 1 N NaOH (2 mL). The resulting suspension was stirred at room temperature for 16 h. The methanol was evaporated below 40°C and the aqueous layer was neutralized with glacial acetic acid. The solvent was evaporated to dryness, and the crude solid residue was dissolved in 1 mL of water and cooled to give **11** (40 mg, 66%) as a white precipitate: mp 221–223°C. MS *m*/*z* 323 (M + 1); ^{1}H NMR (300 MHz, D_2O) δ 7.51 (d, 1H), 7.35 (d, 1H), 4.3 (m, 1H), 2.1 (s, 3H), 1.6 (m, 4H), 0.85 (t, 6H). Anal. calcd for $C_{15}H_{22}N_4O_4 \cdot 1.25H_2O$: C, 52.25; H, 7.11; N, 16.25. Found: C, 52.24; H, 6.87; N, 16.15.

Methyl 3,4-diaminobenzoate (13). 3,4-Diaminobenzoic acid (**12**, 20.0 g, 0.130 mmol) was dissolved in dry methanol (460 mL) and concentrated sulfuric acid (17 mL). The mixture was stirred at reflux under a nitrogen atmosphere. After 15 h the dark reaction mixture was cooled to room temperature and quenched with 10% sodium bicarbonate (400 mL) to pH 7.5. Ethyl acetate (600 mL) was added, the mixture shaken in a separatory funnel, and the aqueous layer was further extracted with ethyl acetate (3×200 mL). The combined organic layers were washed with 5% sodium bicarbonate (3×200 mL) and water (3×200 mL), and then dried over sodium sulfate. The solvent was removed in vacuo. The crude material was purified by recrystallization from benzene: hexane to provide 14.6 g (67.0%) of **13** as a pale yellow solid: mp 103–105°C (benzene:hexane). R_f 0.50 (80% EtOAc:hexane); MS *m*/*z* 167 (M + 1); ^{1}H NMR (300

MHz, $CDCl_3$) 3.25–4.0 (br s, 4H), 3.85 (s, 3H), 6.68 (d, 1H), 7.41 (d, 1H), 7.46 (dd, 1H). Anal. calcd for $C_8H_{10}N_2O_2$: C, 57.82; H, 6.07; N, 16.86. Found: C, 58.02; H, 6.05; N, 16.92.

Methyl 4-amino-3-[*N*-(2-ethylbutanoyl)amino]benzoate (14). 2-Ethylbutyric acid (0.38 mL, 3.0 mmol) was dissolved in dry tetrahydrofuran (2.5 mL). *N,N'*-Carbonyldiimidazole (0.54 g, 3.3 mmol) was then added and bubbling was observed. After 10 min, methyl 3,4-diaminobenzoate (**13**, 0.50 g, 3.0 mmol) was added and the yellow reaction mixture was stirred at room temperature. After 30 h, water (3 mL) and ethyl acetate (3 mL) were added. The mixture was filtered to provide 0.64 g (80%) of **14** as a white solid: mp 200–203°C (water). R_f 0.47 (50% ether–chloroform); MS m/z 265 (M + 1); ^{1}H NMR (300 MHz, DMSO) δ 0.86 (t, 6H, J = 7.4 Hz), 1.49 (m, 4H), 2.25 (m, 1H), 3.75 (s, 3H), 5.63 (br s, 2H), 6.75 (d, 1H, J = 8.5 Hz), 7.52 (dd, 1H, J = 2.0 and 8.4 Hz), 7.86 (d, 1H, J = 2.0 Hz), 9.20 (s, 1H). Anal. calcd for $C_{14}H_{20}N_2O_3$: C, 63.64; H, 7.58; N, 10.61. Found: C, 63.58; H, 7.67; N, 10.67.

Methyl 4-(*N*-acetylamino)-3-[*N*-(2-ethylbutanoyl)amino]-benzoate (15). Aniline **14** (0.15 g, 0.57 mmol) was dissolved in acetic anhydride (2.17 mL, 23.0 mmol) and the white reaction mixture was stirred at room temperature. After 1.5 h, ethyl acetate (38 mL) and water (24 mL) were added. The organic layer was washed with water (3×24 mL). The combined aqueous layers were extracted with ethyl acetate (2×35 mL) and the organic layers were dried (sodium sulfate), filtered and the solvent was removed in vacuo to provide 0.15 g (88%) of **15** as a white solid: mp 138–142°C. R_f 0.53 (ether); MS m/z 307 (M + 1); ^{1}H NMR (300 MHz, DMSO) δ 0.89 (t, 6H, J = 7.3 Hz), 1.53 (m, 4H), 2.09 (s, 3H), 2.25 (m, 1H), 3.84 (s, 3H), 7.75 (m, 2H), 8.12 (s, 1H), 9.51 (br s, 2H). Anal. calcd for $C_{16}H_{22}N_2O_4$: C, 62.74; H, 7.19; N, 9.15. Found: C, 62.58; H, 7.25; N, 9.11.

Methyl 4-amino-3-[*N*-(3-pentyl)amino]benzoate (16). Methyl 3,4-diaminobenzoate (**13**, 2.00 g, 12.0 mmol) was dissolved in dry tetrahydrofuran (6 mL). To the dark solution was added 3-bromopentane (1.82 g, 12.1 mmol) and pyridine (2.35 g, 29.7 mmol). The dark reaction mixture was stirred under reflux. After 14 h, the mixture was cooled to room temperature and ethyl acetate (127 mL) and water (127 mL) were added. The organic layer was washed with water (3×125 mL). The combined aqueous layers were extracted with ethyl acetate (3×75 mL). The combined organic layers were dried over sodium sulfate, filtered and the solvent was removed in vacuo to provide a crude brown solid. Purification of the crude product by flash chromatography on silica using 40% ethyl acetate:hexane provided 1.17 g (41.0%) of **16** as an amber oil. Intermediate **16** was carried forward without additional purification. R_f 0.57 (40% ethyl acetate:hexane); MS m/z 237 (M + 1); ^{1}H NMR (300 MHz, DMSO) δ 0.96 (t, 6H, J = 7.3 Hz), 1.63 (m, 4H), 3.35 (br s, 1H), 3.92 (s, 3H), 6.67 (d, 1H, J = 8.0 Hz), 7.48 (m, 2H).

Methyl 4-*N*-acetylamino 3-[*N*-(3-pentylamino)]benzoate (17). Compound **16** (0.155 g, 0.560 mmol) was dissolved in dry tetrahydrofuran (2.3 mL) and cooled to 0°C with an ice bath. To the red solution was added acetic anhydride (1 mL) and the reaction mixture was allowed to equilibrate to room temperature. After 18 h, ethyl acetate (39 mL) and water (23 mL) were added to the light-red reaction mixture. The organic layer was washed with water (3×23 mL) and the combined aqueous layers were extracted with ethyl acetate (2×23 mL) and dried over sodium sulfate. After filtration the solvent was removed in vacuo. The residue was purified by recrystallization from ethyl acetate:hexane to provide 0.122 g (68.0%) of **17** as off-white crystals: mp 107–109°C (EtOAc:hexane). R_f 0.50 (60% ethyl acetate:hexane); MS m/z 278 (M + 1); ^{1}H NMR (300 MHz, DMSO) δ0.89 (t, 6H, J = 7.4 Hz), 1.52 (m, 4H), 2.10 (s, 3H), 3.25 (m, 1H), 3.80 (s, 3H), 4.82 (d, 1H, J = 7.8 Hz), 7.13 (d, 1H, J = 8.1 Hz), 7.16 (s, 1H), 7.43 (d, 1H, J = 8.1 Hz), 9.24 (br s, 1H). Anal. calcd for $C_{15}H_{22}N_2O_3$: C, 64.73; H, 7.97; N, 10.06. Found: C, 64.54; H, 7.97; N, 9.98.

Methyl 4-amino 3-hydroxybenzoate (19). Acid **18** (10.0 g, 65.0 mmol) was dissolved in a mixture of dry methanol (700 mL) and concentrated sulfuric acid (15 mL). The dark reaction mixture was stirred under reflux under a nitrogen atmosphere. After 14 h, the dark mixture was quenched with solid sodium bicarbonate until pH 7–8, and ethyl acetate was added (600 mL). This was separated and the aqueous layer was extracted with additional ethyl acetate (3×200 mL). The combined organic layers were washed with 5% sodium bicarbonate (2×100 mL), water (2×100 mL), and dried over sodium sulfate. After filtration the solvent was removed in vacuo to provide 9.06 g (83.0%) of **19** as a brown–red solid: mp 114–116°C. MS m/z 168 (M + 1); ^{1}H NMR (300 MHz, DMSO) δ 3.85 (s, 3H), 4.18 (br s, 2H), 6.68 (d, 1H), 7.50 (d, 1H), 7.58 (m, 1H). Anal. calcd for $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.55; H, 5.38; N, 8.30.

Methyl 4-amino-3-(3-pentyloxy)benzoate (20) Method A. A suspension of aminophenol **19** (500 mg, 3.00 mmol), bromopentane (500 mg, 3.30 mmol) and K_2CO_3 (500 mg, 3.60 mmol) in 10 mL of acetone was stirred at reflux for 12 h. Bromopentane (500 mg, 3.30 mmol) and K_2CO_3 (250 mg, 1.80 mmol) were added and the reaction continued for another 6 h, after which additional bromopentane (500 mg, 3.30 mmol) and K_2CO_3 (250 mg, 1.80 mmol) were added. Stirring was continued for another 6 at reflux. The reaction mixture was evaporated to dryness. The crude material was suspended in 15 mL of 2 N NaOH and the product extracted into ethyl acetate (3×25 mL). The combined organic layers were washed with water (3×10 mL), dried (Na_2SO_4) and evaporated to give **20** (600 mg, 85.0%) as an oil which solidified upon standing: mp 45–46°C (ether:hexane).

Method B. To a suspension of 60% NaH (145 mg, 6.00 mmol) in 1 mL of dry DMF was added a solution of aminophenol **19** (500 mg, 3.00 mmol) in 1.5 mL of DMF followed by 3-bromopentane (453 mg, 3.00 mmol) in 1.5 mL of DMF. After 3 h of stirring additional NaH (145 mg, 6.00 mmol) and additional 3-bromopentane (453 mg, 3.00 mmol) were added, and the stirring was continued for another 9 h. The DMF was removed under vacuum and the crude reaction mixture

was suspended in 15 mL of 2 N NaOH. The basic aqueous layer was extracted with chloroform (3×25 mL) and the combined organic layers were dried (Na_2SO_4) and evaporated to give **20** (600 mg, 85.0%) as an oil which solidified upon standing: mp 45–46°C (ether:hexane). MS *m/z* 238 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.5 (dd, 1H, *J* = 1.8 and 8.5 Hz), 7.45 (d, 1H, *J* = 1.8 Hz), 6.65 (d, 1H, *J* = 8.5 Hz), 4.2–4.3 (m, 3H), 3.85 (s, 3H), 1.7 (m, 4H), 0.95 (t, 6H, *J* = 7.4 Hz). Anal. calcd for $C_{13}H_{19}NO_3$: C, 65.82; H, 8.01; N, 5.90. Found: C, 65.85; H, 8.06; N, 5.97.

Methyl 4-amino-3-(3-heptyloxy)benzoate (21). To a suspension of 60% NaH (450 mg, 18.8 mmol) in 1 mL of dry DMF were added a solution of aminophenol **19** (1.00 g, 5.98 mmol) in 1.5 mL of DMF and 4-bromoheptane (1.60 g, 8.93 mmol) in 2.5 mL of DMF. After 3 h of stirring NaH (400 mg, 16.6 mmol) and 4-bromoheptane (500 mg, 2.79 mmol) were added, and the stirring was continued for an additional 9 h. The DMF was removed under vacuum and the crude reaction mixture was suspended in 15 mL of 2 N NaOH. The basic aqueous layer was extracted with chloroform (3×25 mL) and the combined organic layers were dried (Na_2SO_4) and evaporated to give **21** (1.10 g, 69.0%) as an oil, which was carried forward in this form without additional purification. MS *m/z* 238 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.5 (dd, 1H, *J* = 1.7 and 8.5 Hz), 7.45 (d, 1H, *J* = 1.7 Hz), 6.65 (d, 1H, *J* = 8.5 Hz), 4.4 (m, 1H), 4.25 (br, 2H) 3.85 (s, 3H), 1.65 (m, 4H), 1.4 (m, 4H), 0.92 (t, 6H, *J* = 7.5 Hz).

Methyl 4-(*N*-acetylamino)-3-(3-pentyloxy)benzoate (22). A mixture of **20** (300 mg, 1.20 mmol) and DMAP (25 mg) in 1 mL of acetic anhydride was stirred at room temperature for 12 h. The reaction mixture was diluted with 50 mL of CH_2Cl_2 and the organic layer was washed with 1 N HCl (15 mL), H_2O (15 mL) and saturated $NaHCO_3$ solution (15 mL), dried (Na_2SO_4) and evaporated to give an oil. This was purified by flash column chromatography on silica gel (ether) to give **22** (325 mg, 92.0%) as an oil which solidified upon standing: mp 88–89°C (hexane). MS 280 *m/z* (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (d, 1H, *J* = 8.5 Hz), 8.0 (br, 1H), 7.65 (dd, 1H, *J* = 1.8 and 8.5 Hz), 7.52 (d, 1H, *J* = 1.8 Hz), 4.25 (m, 1H), 3.88 (s, 3H), 2.2 (s, 3H), 1.7 (m, 4H), 0.95 (t, 6H, *J* = 7.4 Hz); ^{13}C NMR ($CDCl_3$) δ 168.4, 166.7, 145.9, 132.9, 124.8, 122.9, 118.7, 113.2, 81.7, 52.0, 25.9, 25.0, 9.5. Anal. calcd for $C_{15}H_{21}NO_4$: C, 64.51; H, 7.52; N, 5.02. Found: C, 64.51; H, 7.53; N, 5.08.

Methyl 4-(*N*-acetylamino)-3-(4-heptyloxy)benzoate (23). A mixture of **21** (1.00 g, 3.70 mmol) and DMAP (10 mg) in 2 mL of acetic anhydride was stirred at room temperature for 2 h. The reaction was diluted with 50 mL of ether, washed with water (3×10 mL), dried (Na_2SO_4) and evaporated to give **23** (1.10 g, 95.0%) as a white solid: mp 66–68°C (hexane). MS *m/z* 308 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.4 (d, 1H, *J* = 8.5 Hz), 7.9 (br, 1H), 7.6 (dd, 1H, *J* = 1.7 and 8.5 Hz), 7.5 (d, 1H, *J* = 1.7 Hz), 4.4 (m, 1H), 3.89 (s, 3H), 2.2 (s, 3H), 1.66 (m, 4H), 1.48 (m, 4H), 0.94 (t, 6H, *J* = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 168.7, 167.1, 146.3, 133.2, 125.1, 123.2, 119.0, 113.3, 79.4, 52.3, 36.3, 25.3, 18.9, 14.4. Anal. calcd for $C_{17}H_{25}NO_4$: C, 66.45; H, 8.14; N, 4.56. Found: C, 66.52; H, 8.14; N, 4.56.

Methyl 4-(*N*-acetylamino)-3-acetyloxybenzoate (24). A suspension of phenol **19** (3.00 g, 18.0 mmol) and DMAP (50 mg) in 40 mL of acetic anhydride was stirred at room temperature for 12 h, during which time the product precipitated. The reaction mixture was diluted with 100 mL of water, filtered, and the collected solid air dried to give **24** (4.50 g, 100%) as a white solid: mp 156–159°C (ether). MS *m/z* 252 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.4 (d, 1H, *J* = 8.6 Hz), 7.9 (dd, 1H, *J* = 1.9 and 8.6 Hz), 7.82 (d, 1H, *J* = 1.9 Hz), 7.38 (br, 1H), 3.9 (s, 3H), 2.4 (s, 3H), 2.2 (s, 3H). Anal. calcd for $C_{12}H_{13}NO_5$: C, 57.37; H, 5.18; N, 5.57. Found: C, 57.61; H, 5.08; N, 5.41.

Methyl 4-(*N*-acetylamino)-3-acetyloxy-5-nitrobenzoate (25). An ice cold solution of **24** (1.00 g, 4.00 mmol) in a mixture of dioxane (8 mL) and acetic anhydride (8 mL) was treated slowly with ice cold nitrating reagent (4 mL of HNO_3 and 4 mL of acetic anhydride). The resulting mixture was stirred at 0°C for 15 min and at room temperature for 5 h. The reaction mixture was then diluted with 50 mL of ice water and the product was extracted into ethyl acetate (3×50 mL). The combined organic layers were washed with ice cold water (3×30 mL), dried (Na_2SO_4) and evaporated under vacuum to give a yellow oil. The oil was triturated with hexane (3×5 mL), the hexane was discarded, and the residue was dried on a high vacuum pump, which resulted in partial solidification. The semisolid was triturated with ether, filtered, and washed with small amounts of ether to give **25** (0.50 g, 42.7%): mp 139–141°C. MS *m/z* 297 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, 1H, *J* = 1.6 Hz), 8.48 (br, 1H), 8.13 (d, 1H, *J* = 1.6 Hz), 3.96 (s, 3H), 2.3 (s, 3H), 2.2 (s, 3H). Anal. calcd for $C_{12}H_{12}N_2O_7$: C, 48.65; H, 4.05; N, 9.46. Found: C, 48.43; H, 4.26; N, 9.21.

Methyl 4-(*N*-acetylamino)-3-hydroxy-5-nitrobenzoate (26). A solution of **25** (780 mg, 2.60 mmol) in 25 mL of methanol was treated with activated Zn (1.00 g). The resulting mixture was then stirred at room temperature for 1.5 h. The reaction mixture was filtered, the solvent was removed under vacuum and the resulting oil was dissolved in 100 mL of ethyl acetate. The organic layer was then washed with 0.5 N HCl (3×10 mL), dried (Na_2SO_4) and evaporated to give a solid which was suspended in ether and filtered to give **26** (600 mg, 89.7%): MS *m/z* 253 (M−1); ^{1}H NMR (300 MHz, CD_3OD) δ 10.25 (br, 1H), 9.45 (br, 1H), 8.4 (d, 1H, *J* = 1.9 Hz), 7.9 (d, 1H, *J* = 1.9 Hz), 4.0 (s, 3H), 2.4 (s, 3H). This intermediate was carried forward without additional purification.

Methyl 4-(*N*-acetylamino)-5-nitro-3-(3-pentyloxy)benzoate (27). A solution of phenol **26** (480 mg, 1.89 mmol), 3-bromopentane (800 mg, 5.30 mmol) and K_2CO_3 (530 mg, 3.80 mmol) in 10 mL of acetone was stirred at reflux for 2 h. Additional 3-bromopentane (260 mg, 1.72 mmol) was added and the stirring continued for another 2 h. After this time 3-bromopentane (820 mg, 5.42 mmol) and K_2CO_3 (450 mg, 3.25 mmol) were again added and the stirring continued for 12 h. The acetone was removed under vacuum, and the crude mixture was suspended in 100 mL of ethyl acetate and washed with water (3×15 mL). The aqueous layer was extracted with

ethyl acetate (25 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated to give a solid. This was purified by flash chromatography on silica gel (2:1, ether:hexane) to give **27** (500 mg, 82.0%): mp 103–105°C (ether). MS *m/z* 325 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.1 (d, 1H, *J* = 1.7 Hz), 7.78 (br, 1H), 7.73 (d, 1H, *J* = 1.7 Hz), 4.37 (m, 1H), 3.89 (s, 3H), 2.2 (s, 3H), 1.7 (m, 4H), 0.95 (t, 6H, *J* = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 167.99, 164.8, 150.8, 144.1, 126.6, 124.3, 117.6, 116.6, 82.6, 52.6, 25.6, 23.4, 9.2. Anal. calcd for $C_{15}H_{20}N_2O_6$: C, 55.55; H, 6.17; N, 8.64. Found: C, 55.69; H, 6.17; N, 8.62.

Methyl 4-(*N*-acetylamino)-5-amino-3-(3-pentyloxy)-benzoate (28). A suspension of **27** (300 mg, 0.920 mmol) and 10% Pd/C (275 mg) in 15 mL of methanol was shaken in the presence of hydrogen gas (30 psi) for 1 h. The reaction mixture was diluted with 25 mL of methanol, filtered, and the filter was washed well with methanol (25 mL). The methanol was evaporated to dryness under vacuum to give **28** (270 mg, 99.0%) as an oil which was crystallized from ether:hexane: mp 128–129°C. MS *m/z* 295 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (br, 1H), 7.10 (s,1H), 7.01 (s, 1H), 4.46 (br, 2H), 4.25 (m, 1H), 3.89 (s, 3H), 2.25 (s, 3H), 1.67 (m, 4H), 0.95 (t, 6H, *J* = 7.4 Hz). Anal. calcd for $C_{15}H_{22}N_2O_4$: C, 61.22; H, 7.48; N, 9.52. Found: C, 61.07; H, 7.52; N, 9.41.

Methyl 4-(*N*-acetylamino)-5-[*N,N′*-di(*tert*-butyloxycarbonyl)guanidino]-3-(3-pentyloxy)benzoate (29). To an ice cold solution of **28** (270 mg, 0.920 mmol) in 3 mL of dry DMF was added 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (423 mg, 1.37 mmol), triethylamine (190 mg, 1.80 mmol) and $HgCl_2$ (374 mg, 1.37 mmol). The resulting mixture was stirred at 0°C for 3 h and at room temperature for 3 h. The DMF was evaporated below 50°C and the crude product was suspended in 100 mL of ether, filtered and evaporated to give an oil. This was purified by flash chromatography on silica gel (2:1, ether:hexane) to give **29** (450 mg, 92.0%) as a colorless oil which was crystallized from ether:hexane: mp 131-133° C. MS *m/z* 537 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.5 (br, 1H), 10.3 (br, 1H), 8.1 (s, 1H), 7.5 (br, 1H), 7.4 (s, 1H), 4.26 (m, 1H), 3.9 (s, 3H), 2.2 (s, 3H), 1.7 (m, 4H), 1.5 (s, 9H), 1.48 (s, 9H), 0.94 (t, 6H); ^{13}C NMR ($CDCl_3$) δ 168.7, 166.1, 162.8, 153.6, 152.5, 133.5, 128.3, 124.1, 118.5, 110.7, 83.3, 81.0, 79.3, 51.9, 27.7, 25.3, 22.9, 9.0. Anal. calcd for $C_{26}H_{40}N_4O_8$: C, 58.20; H, 7.46; N, 10.45. Found: C, 58.34; H, 7.49; N, 10.54.

X-ray crystallography

Purified N9 NA was crystallized using the hanging drop technique in which droplets of protein solution on siliconized cover slips are inverted on a linbro plate. The droplet consisted of equal volumes of protein solution (10–15 mg/mL in water) and potassium phosphate buffer (1.7 M, pH 6.6). This mixture was equilibrated through the vapor phase with a reservoir of 1.9 M potassium phosphate buffer at pH 6.8. Large rhombic dodecahedral crystals grew in a few days. The space group has been identified as cubic, *I*432, with cell dimension $\alpha = 183.8$ Å. The crystals diffract strongly to at least 2 Å resolution on a rotating anode source.

The complexes of the inhibitor with NA were prepared by diffusing the compound into N9 crystals in a stabilization buffer which was the same as the reservoir solution used in crystallization. The final concentration of the inhibitor in the solution was 1–2 mM. The N9 crystals were allowed to equilibrate with the inhibitor compound for at least 24 h before data collection.

All X-ray intensity measurements were recorded with a Nicolet/Siemens X-100 multiwire area detector on a Rigaku RU-300 rotating anode X-ray generator operating at 100 mA and 50 kV with 0.3×0.3 focus and a Cu anode. The data collection parameters were crystal to detector distance of 16 cm, swing angle of 28°, frame width of 0.1°, and exposure time of 240–300 s. Each crystal provided about 800–900 frames of data before radiation damage deteriorated the diffraction quality.

The X-ray intensity data were processed using the XENGEN suite of programs. The integrated intensities were then scaled and merged to produce a final data set containing only unique reflections. The completeness of data to 2.1 Å was generally about 90%. The refinement of the inhibitor complexes was performed using the X-PLOR simulated annealing protocol. Diffraction data in the 10–2.1 Å resolution range were used in the refinement with a 2σ cutoff on F_0s. The starting model for the refinement of the inhibitor complexes was the 1.9 Å refined uncomplexed NA native structure. The water molecules in the active site of the native structure were removed prior to the refinement of the complex structure. A full cycle of the X-PLOR simulated annealing protocol was carried out on this model. F_0-F_c and $2(F_0-F_c)$ difference Fourier maps were calculated, and a model of the inhibitor molecule was fitted into the electron density in the active site. Water molecules were also placed in the active site based on the difference electron density maps, and these positions were compared to the active site water structure in the native NA model. At the end of the simulated annealing protocols and positional and temperature factor refinement, the crystallographic R-factor converged to 17–19% at 2 Å resolution for the inhibitor-complex structures.

Enzyme Assays

All compounds were evaluated for in vitro inhibitory actions using the method reported by von Itzstein et al.[32] The NA from the H1N9 strain of influenza was obtained using the method described by Laver et al.[33] The assay employed a spectrofluorometric technique that uses 2′-(methylumbelliferyl)-α-D-acetylneuraminic acid as substrate. Cleavage of this substrate by NA produces a fluorescent product which can be quantified. The assay mixture contained inhibitors at various concentrations (4–6 points) and enzyme in 32.5 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffer, 4 mM $CaCl_2$ at pH 6.5 (total volume = 80 μL). The reaction was started by the addition of 20 μL of the substrate to give a final concentration of 75 μM. After 10 min at 37°C, 2.4 mL of 0.1 M glycine:NaOH (pH 10.2) was added to 0.1 mL of the reaction mixture to terminate

the reaction. A blank was run with the same substrate solution excluding the enzyme. Fluorescence was read using an Aminco-Bowman fluorescence spectrophotometer (excitation, 360 nm; emission, 450 nm), and substrate blanks were subtracted from the sample readings. The IC_{50} was calculated by plotting percent inhibition versus the inhibitor concentration, and determination of each point was performed in duplicate. In all cases each IC_{50} value differed from its duplicate by less than twofold.

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