2332 J. Med. Chem. 1999, 42, 2332−2343

Potent Inhibition of Influenza Sialidase by a Benzoic Acid Containing a 2-Pyrrolidinone Substituent

Venkatram R. Atigadda,[†] Wayne J. Brouillette,[*,†,‡] Franco Duarte,[†] Shoukath M. Ali,[†] Yarlagadda S. Babu,[§] Shanta Bantia,[§] Pooran Chand,[§] Naiming Chu,[§] John A. Montgomery,[§] David A. Walsh,[§] Elise A. Sudbeck,[‡] James Finley,[‡] Ming Luo,[‡] Gillian M. Air,[⊥] and Graeme W. Laver[∥]

Department of Chemistry and Center for Macromolecular Crystallography, University of Alabama at Birmingham, Birmingham, Alabama 35294, BioCryst Pharmaceuticals, Inc., 2190 Parkway Lake Drive, Birmingham, Alabama 35244, Department of Biochemistry and Molecular Biology, University of Oklahoma, Oklahoma City, Oklahoma 73190, and John Curtin School of Medical Research, Australian National University, Canberra 2601, Australia

Received December 16, 1998

On the basis of the lead compound 4-(*N*-acetylamino)-3-guanidinobenzoic acid (BANA 113), which inhibits influenza A sialidase with a K_i of 2.5 μM, several novel aromatic inhibitors of influenza sialidases were designed. In this study the *N*-acetyl group of BANA 113 was replaced with a 2-pyrrolidinone ring, which was designed in part to offer opportunities for introduction of spatially directed side chains that could potentially interact with the 4-, 5-, and/or 6-subsites of sialidase. While the parent structure 1-(4-carboxy-2-guanidinophenyl)pyrrolidin-2-one (**8**) was only a modest inhibitor of sialidase, the introduction of a hydroxymethyl or bis(hydroxymethyl) substituent at the C5′ position of the 2-pyrrolidinone ring resulted in inhibitors (**9** and **12**, respectively) with low micromolar activity. Crystal structures of these inhibitors in complex with sialidase demonstrated that the substituents at the 5′-position of the 2-pyrrolidinone ring interact in the 4- and/or 5-subsites of the enzyme. Replacement of the guanidine in **12** with a hydrophobic 3-pentylamino group resulted in a large enhancement in binding to produce an inhibitor (**14**) with an IC_{50} of about 50 nM against influenza A sialidase, although the inhibition of influenza B sialidase was 2000-fold less. This represents the first reported example of a simple, achiral benzoic acid with potent (low nanomolar) activity as an inhibitor of influenza sialidase.

Introduction

Influenza is a major respiratory tract disease affecting millions of people each year. Despite considerable knowledge of viral infectivity, current therapeutic measures do not control the disease. Vaccination has provided only limited control because of the ease with which the virus can mutate to escape the immune system, and the vaccines must be reformulated each year because of this high antigenic drift. Options for the therapeutic treatment of influenza are currently limited to amantadine and rimantadine, which act by interfering with the M2 protein ion channel function that is found only in influenza A. Besides the insensitivity of influenza B viruses, clinical use of these agents is also limited because of the rapid emergence of resistance. New influenza drugs with broad-spectrum activity are needed.

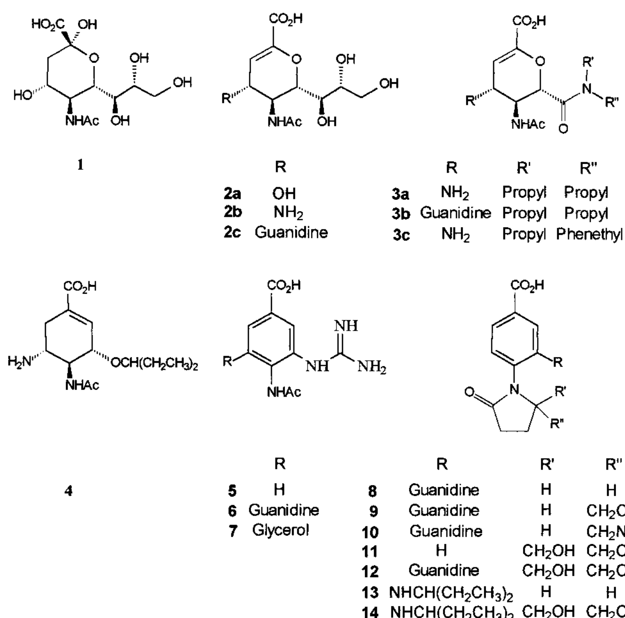
Influenza is an RNA virus that contains two major surface glycoproteins, neuraminidase (NA), or sialidase, and hemagglutinin (HA). These proteins are essential for infection. The virus infects the epithelial cells of the upper respiratory tract by binding to the cell surface receptor sialic acid, and it subsequently undergoes endocytosis into the cell. This binding and endocytosis process is mediated by HA. After viral replication, the progeny virions must be released from the cell to repeat the cell cycle of infection. Influenza neuraminidase mediates the release of virus from the cell surface by cleavage of α-glycosidic bonds to the cell surface sialic acids.[1−3] This prevents viral aggregation and allows viral release from infected cells. This action of NA may also facilitate viral mobility through the mucus of the respiratory tract.

The life cycle of the influenza virus provides several targets for drug development, and neuraminidase offers one attractive site for therapeutic intervention in influenza infections. For influenza A, nine subtypes of neuraminidase have been identified, whereas only one subtype is known for influenza B. Despite this diversity, the catalytic site for all influenza A and B neuraminidases is completely conserved, even though overall amino acid sequence identities may be only 30%.[4−6] The neuraminidase is a tetramer with *C*-4 symmetry and has an approximate molecular weight of 250 000. It consists of a globular head anchored to the membrane through a central stalk. Each of the four subunits of the head is a glycosylated polypeptide and contains a catalytic site. Proteolytic cleavage of the stalk has produced catalytically and antigenically active heads.[1] Heads from several viruses have been crystallized and the X-ray crystal structures determined.[5] These structures reveal that the catalytic site is strain-invariant and contains 18 conserved amino acid residues. Mutation of these conserved amino acid residues in the active

* Corresponding author.
† Department of Chemistry, University of Alabama.
‡ Center for Macromolecular Crystallography, University of Alabama.
§ BioCryst Pharmaceuticals, Inc.
⊥ University of Oklahoma.
∥ Australian National University.

10.1021/jm980707k CCC: $18.00 © 1999 American Chemical Society
Published on Web 06/04/1999

Chart 1



site inactivates the enzyme, suggesting that the virus may not easily escape therapeutic intervention through mutation.[1]

Most of the known inhibitors of neuraminidases are analogues of sialic acid (**1**) (Neu5Ac) (Chart 1), a weak inhibitor (IC_{50} = 10 mM) which is the product of the enzyme reaction. Over 25 years ago the dehydrated analogue, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) (**2a**), was reported to be a much more effective inhibitor (IC_{50} = 10 μM) than sialic acid.[7,8] In recent years several potent inhibitors of neuraminidase have been developed based on the insights provided by its X-ray crystal structure in complex with **2a**.[4,5,9] Compound **2c**, a guanidino analogue of **2a** that is a potent inhibitor of both influenza A and B NA, is currently being evaluated in clinical trials and is effective in both the prevention and the treatment of influenza when administered intranasally.[10] However compound **2c** is inactive when administered orally as a result of poor oral bioavailability and rapid elimination.[10,11] More recently, analogues of **2c** containing hydrophobic groups in place of the glycerol side chain, such as carboxamides **3a** and **3b**, have been described.[12−14] While **3a** and **3b** are highly selective inhibitors of influenza A NA, cyclohexene **4** was reported[14] to exhibit potent activity against both influenza A and B, and the ethyl ester of **4** is orally active.

For convenience we have divided the catalytic site of NA into four subsites named according to the atom number of the pyran ring in sialic acid that bears interacting substituents (Figure 1). Using this description for **2c**, the carboxylic acid at C2 interacts in subsite 2, and this interaction is dominated by three arginine residues (Arg 119, Arg 372, Arg 294). The guanidine interacts in subsite 4 with Asp 152 and Glu 229.[5f] Subsite 5 accommodates the *N*-acetyl methyl group; this contains a small hydrophobic pocket created by Trp 180 and Ile 224 into which extends the methyl group, the amide carbonyl accepts a H-bond from Arg 153, and the N−H donates a H-bond to an ordered water molecule. Finally, the C7 and C8 glycerol hydroxyl groups interact with Glu 278 within subsite 6.

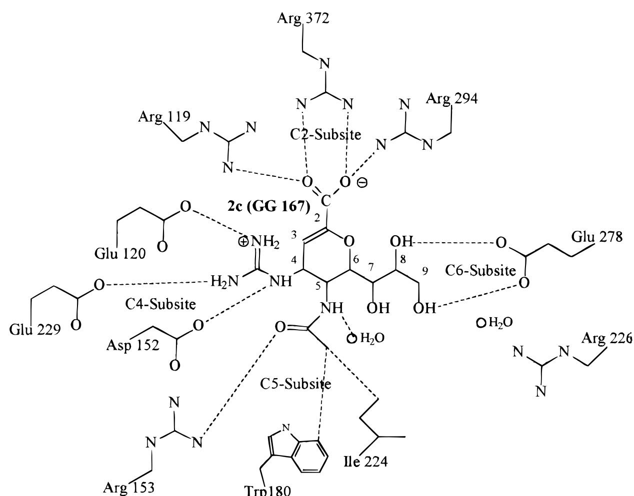


Figure 1. Depiction of the interactions of **2c** (GG167) in the NA binding site (N9 numbering). Note that Tyr 406 lies under **2c** and is not shown.

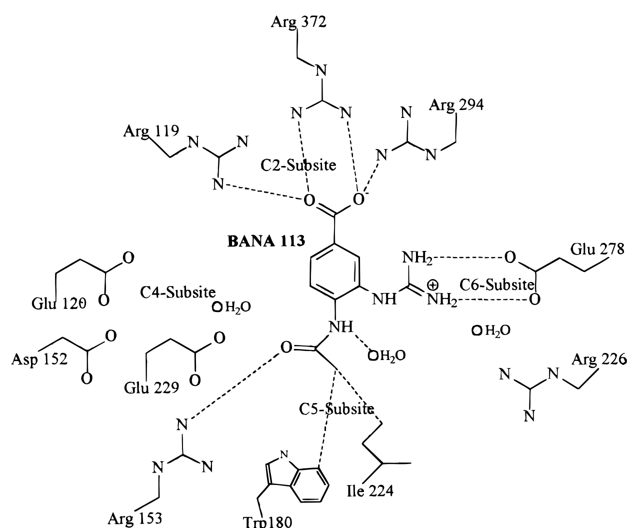


Figure 2. Depiction of the interactions of **5** (BANA 113) in the binding site (N9 numbering). Note that Tyr 406 lies under **5** and is not shown.

In an effort to develop new classes of inhibitors using structure-based drug design, we have described aromatic lead compounds as neuraminidase inhibitors.[15,16] The most potent of these early inhibitors was compound **5** (BANA 113), which has a K_i value of 2.5 μM. Compound **5** binds to the NA enzyme in a unique manner in relation to **2c** (Figure 2). Comparison of the X-ray crystal structures of the NA complex with **5** and **2c** revealed that the guanidino functionalities interact in a completely different manner, while the carboxylate and *N*-acetyl groups occupy the same subsites. In compound **2c**, the C8 and C9 hydroxyls of the glycerol moiety occupy subsite 6, making hydrogen bonds to Glu 278, whereas the guanidino functionality occupies subsite 4 and interacts with Asp 152 and Glu 229 through salt bridges.[5f] However, in the case of inhibitor **5**, which lacks the glycerol moiety, the guanidine functionality occupies subsite 6 (the glycerol subsite of **2c**) instead of subsite 4 (guanidine subsite of **2c**) and forms a salt bridge with Glu 278. In compound **5** the guanidine has the opportunity to occupy either subsite 4 or subsite 6, requiring only a 180° rotation of the benzene ring to

interconvert these orientations, suggesting more favorable interactions with subsite 6.

Given the large enhancement in potency in going from **2a** to **2c**, we decided to incorporate a second guanidino grouping in benzoic acid **5**, to generate **6**, to force a guanidino group into subsite 4.[17] Indeed, the crystal structure of the complex for **6**/NA revealed that the second guanidino grouping occupied the 4-subsite in a manner similar to that for **2c**, although the inhibitory activity for **6** ($IC_{50} = 250\ \mu M$) was much worse than that for **5** ($K_i = 2.5\ \mu M$). Also, incorporation by others of a glycerol side chain at C5, as in **7,** resulted in the loss of inhibitory activity.[18] Several factors may be responsible for the weaker activity of **6**. (1) The desolvation energy for a second guanidinium group might be much greater than anticipated. (2) The first atom of substituents attached to the benzene ring must be coplanar with that ring, resulting in steric congestion of 3,4,5-trisubstituted benzoic acids. These steric interactions may result in an energetically unfavorable conformation for the guanidine of bound **6**.

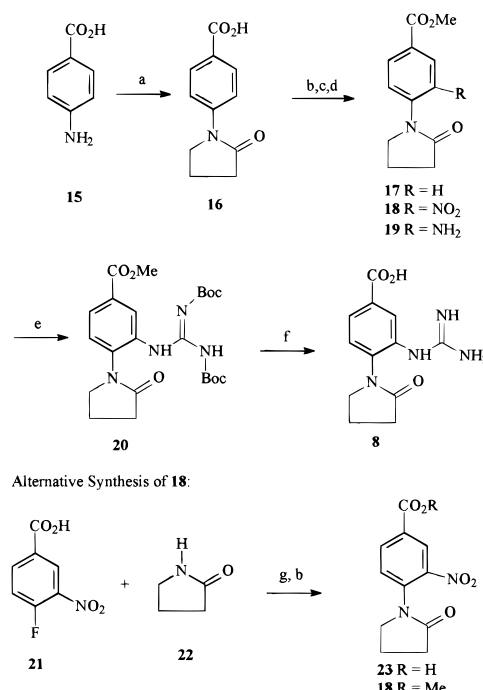
We next investigated the possibility of replacing the *N*-acetyl grouping with a substituent containing side chains for interaction with the 4-subsite. Here we report that a properly substituted 2-pyrrolidinone as replacement for the *N*-acetyl grouping on benzoic acid, when coupled with a hydrophobic substituent, provides a simple, achiral compound that is a low nanomolar inhibitor of NA.

Chemistry

The first goal for modifying **5** was to replace the *N*-acetyl grouping with a compact, cyclic substituent that would offer opportunities for incorporation of spatially directed side chains that could potentially interact with the 4-, 5-, and/or 6-subsites. Computer modeling studies suggested the 2-pyrrolidinone ring for this purpose,[27] and compound **8** was selected as the initial target. The synthesis of **8** is shown in Scheme 1 and began with the alkylation of 4-aminobenzoic acid (**15**) with ethyl 4-bromobutyrate, accompanied by cyclization, in a one-pot procedure in refluxing DMF, to give pyrrolidinone **16** in 66% yield. The use of a base such as triethylamine in this reaction resulted primarily in esterification of the carboxylic acid. Also, attempts to alkylate methyl 4-aminobenzoate in a similar fashion resulted in much poorer yields of **17**. Esterification of the acid **16** followed by nitration using either HNO_3/Ac_2O or NO_2BF_4 resulted in the desired regioisomer **18** in 90% overall yield. Later, **18** was also synthesized in two steps by nucleophilic displacement of commercially available 4-fluoro-3-nitrobenzoic acid (**21**) with 2-pyrrolidinone (**22**), in the presence of NaH, followed by esterification. Catalytic reduction of the nitro group followed by reaction of the amine **19** with *N,N*-bis(*tert*-butoxycarbonyl)thiourea in the presence of mercury chloride provided the guanylated intermediate **20**.[19] Removal of the methyl ester and the *tert*-butoxycarbonyl groups was achieved in one step by refluxing in 1 N HCl to provide the target **8**.

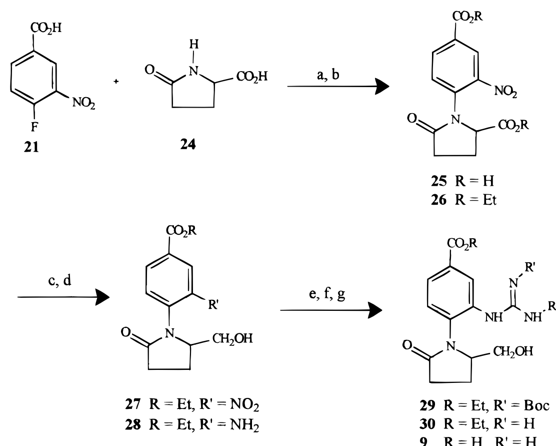
Compounds **9** and **10** were designed as analogues of **8** that contain a side chain on the pyrrolidinone ring capable of interacting with the 4- or 5-subsite. The preparation of **9** is described in Scheme 2. Similarities between intermediates **23** and **25** suggested that, as a first approach, compound **9** could be synthesized by coupling **21** with commercially available 5-(hydroxymethyl)-1-pyrrolidinone. However, coupling attempts using the dianion resulted in alkylation of the alcohol. Therefore, pyrrolidinone carboxylic acid **24** was directly coupled with **21** in the presence of NaH to give the diacid **25** in 50% yield. Fischer esterification of **25** to give diester **26** followed by selective reduction of the aliphatic ester with $NaBH_4$ resulted in the alcohol **27** in 50% yield. Catalytic reduction of the nitro group provided amine **28** which reacted with *N*-*N*-bis(*tert*-butoxycarbonyl)thiourea to yield the guanylated intermediate **29**. Acidic removal of the *tert*-butoxycarbonyl groups followed by saponification gave the target **9**.

Scheme 1[a]



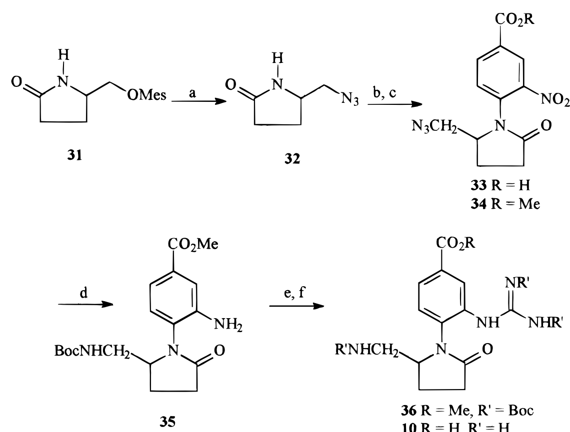
[a] Reagents: (a) ethyl 4-bromobutyrate, DMF; (b) methanol, H^+; (c) HNO_3, Ac_2O, dioxane or NO_2BF_4, CH_2Cl_2; (d) H_2, Pd/C, methanol; (e) *N,N*-bis(*tert*-butoxycarbonyl)thiourea, $HgCl_2$, NEt_3, DMF; (f) HCl; (g) NaH, HMPA.

Scheme 2[a]



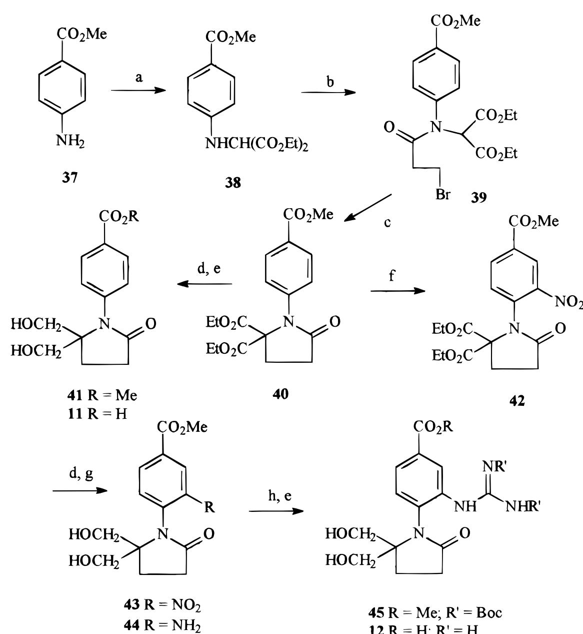
[a] Reagents: (a) NaH, HMPA; (b) ethanol, H^+; (c) $NaBH_4$, ethanol, THF; (d) H_2, Pd/C, ethanol; (e) 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, $HgCl_2$, NEt_3, DMF; (f) CF_3CO_2H; (g) NaOH.

Scheme 3[a]



[a] Reagents: (a) NaN_3, DMF; (b) NaH, 3-nitro-4-fluorobenzoic acid, DMF; (c) methanol, H^+; (d) H_2, Pd/C, di-*tert*-butyl dicarbonate, ethyl acetate; (e) 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, $HgCl_2$, NEt_3, DMF; (f) HCl.

Scheme 4[a]

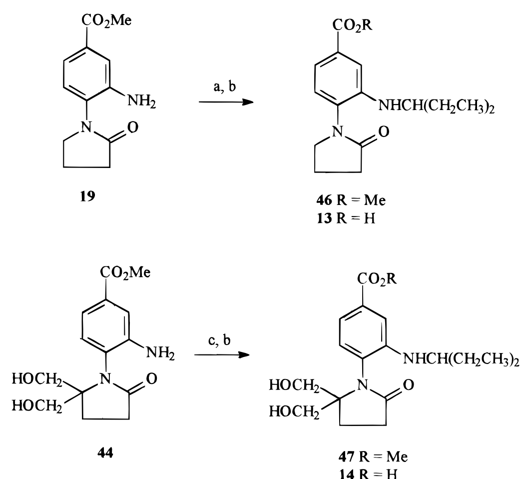


[a] Reagents: (a) diethyl bromomalonate; (b) 3-bromopropionic acid, PCl_3, benzene; (c) NaH, DMF; (d) $NaBH_4$, methanol, THF; (e) NaOH; (f) NO_2BF_4, CH_2Cl_2; (g) H_2, Pd/C, methanol; (h) 1,3-bis(benzyloxycarbonyl)-*S*-methylisothiourea, $HgCl_2$, NEt_3, DMF.

Compound **10** was prepared in an analogous method as shown in Scheme 3. The coupling of **21** with 5-(azidomethyl)-2-pyrrolidinone (**32**), prepared from mesylate **31**, gave intermediate **33**. Fischer esterification to give **34** followed by catalytic reduction using H_2/Pd−C in the presence of di-*tert*-butyl dicarbonate resulted in the reduction of both the nitro and the azide accompanied by protection of the aliphatic amine as the *t*-Boc carbamate to give **35** in quantitative yield. Reaction of the amine **35** with *N,N'*-bis(*tert*-butoxycarbonyl)thiourea resulted in the guanylated intermediate **36**. Acidic hydrolysis of the methyl ester and the *tert*-butoxycarbonyl groups provided compound **10**.

The synthesis of bis(hydroxymethyl)lactam **12** was accomplished as summarized in Scheme 4. Methyl 4-aminobenzoate (**37**) was reacted with diethyl bromomalonate to give **38**, which was acylated with 3-bromopropionic acid and PCl_3 to provide **39**.[20] This underwent cyclization in the presence of NaH to yield lactam **40** in 80% overall yield. The esters of **40** were reduced to the bis(hydroxymethyl) derivative **41**, which underwent saponification to provide target **11**. Initial attempts to nitrate **40** using HNO_3/Ac_2O resulted in poor yields; however, subsequent nitration with nitronium tetrafluoroborate or HNO_3/H_2SO_4 proceeded in greater than 90% yield to give **42**. Reduction of the nitro functionality in intermediate **42** to the amine using catalytic hydrogenation gave only lactam as a result of intramolecular cyclization. Therefore, the aliphatic esters in **42** were first selectively reduced to the alcohols using $NaBH_4$ to give **43**. Reduction of the nitro group then gave amine **44**, which reacted with *S*-methylbenzyloxycarbonylthiourea in the presence of mercuric chloride to provide the guanylated intermediate **45**. The saponification of **45** provided **12**.

Scheme 5[a]



[a] Reagents: (a) 3-pentanone, $TiCl_4$, NEt_3, CH_2Cl_2; (b) NaOH; (c) 3-pentanone, $NaCNBH_4$, acetic acid, dichloroethane.

Initial attempts to introduce lipophilic side chains by reaction of the amine **19** or **44** with alkyl halides failed to provide the desired compounds. However, as shown in Scheme 5, reductive coupling of the amine **19** with 3-pentanone in the presence of $NaCNBH_3$ and $TiCl_4$ followed by saponification resulted in **13** in good yields.[21] Unfortunately, the reductive coupling of **44** with 3-pentanone under identical conditions promoted the intramolecular cyclization of the amine with the pyrrolidinone carbonyl to give an undesired benzimidazole product. But the reductive coupling of amine **44** with 3-pentanone proceeded smoothly in the presence of $NaCNBH_3$ and acetic acid to give the alkylamine **47**. Saponification of the methyl ester provided the target molecule **14**.

Biological Assays

All compounds were evaluated for in vitro inhibitory activity using purified NA from the H1N9 (influenza A) and B/Lee/40 strain. The assay was based upon a published method.[22] The substrate employed was 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUN), whose hydrolysis provides a fluorescent product used for quantitation.

X-ray Crystallography

Purified N9 neuraminidase was crystallized using the hanging drop method, and the resulting crystals

Table 1. In Vitro Inhibitory Effects of Benzoic Acid Analogues on Influenza A and B Neuraminidases

compd	IC_{50} (μM)[a]	
	N9	B/Lee/40
8	250	1000[b]
9[d]	20	10
10[d]	2600	500
11	760	>8000
12	5	8
13	222	>5000
14	0.048	104[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 2-fold. [b] Data for B/Mem/89. [c] Value for B/Mem/89 was 94 μM. [d] Tested as racemic mixtures.

diffracted strongly to at least 2 Å resolution. Inhibitor complexes with N9 neuraminidase 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 shown in Table 1 pyrrolidinone **8**, not surprisingly, was 10−15-fold less active than its *N*-acetyl counterpart **5**, which is consistent with the loss of the hydrogen-bonding interaction of the acetamido group with the ordered water molecule in the 5-subsite. To determine molecular interactions of inhibitor **8**, it was soaked into an N9 crystal of NA, and the structure of the complex was determined (see Figure 3a). In a manner similar to **5**, the carboxylic acid interacts with Arg 119, Arg 294, and Arg 372, and the 3-guanidine forms a salt bridge interaction in subsite 6 with Glu 278. The pyrrolidinone ring occupies subsite 5 and snugly fits into the hydrophobic pocket created by Trp 180 and Ile 224. This ring is oriented roughly perpendicular to the planar benzene ring, and the carbonyl oxygen is located within hydrogen-bonding distance of Arg 153.

Modeling suggested that a hydrogen bond-donating side chain, such as hydroxymethyl or aminomethyl, at the 5′-position of the pyrrolidinone ring in **8** could reach the 4-subsite and replace an ordered water, which may enhance binding. To evaluate this hypothesis **9** and **10** were synthesized. As shown in Table 1 inhibitor **9** was 10 times more effective than the parent compound **8**. However, inhibitor **10** was slightly less active than **8**. To explore structural reasons for the binding differences between these two compounds, **9** and **10** were soaked into N9 crystals and the complex structures were determined. As shown in Figure 3b, these revealed that **9** and **10** interact with the active site of NA in identical fashion. The carboxylate and the guanidine interactions of **9** and **10** with NA are the same as those described for the parent compound **8**, and the hydroxymethyl and aminomethyl groups each replace an ordered water molecule located in the 4-subsite. Although the crystal structures of the complexes do not explain why compound **10** is less active than **9**, a contribution could be the extra energy cost required for desolvation of the charged ammonium group in **10** as compared to the uncharged hydroxy group in **9**.

Further modeling suggested that an additional hydroxymethyl substituent at the C5′ position of inhibitor **9** would be able to interact with, or possibly replace, a water molecule located in the 5-subsite. To evaluate this hypothesis compound **11**, a simple model, was synthesized and evaluated for its biological activity. As indicated **11** (IC_{50} = 0.8 mM) is considerably more active than **16** (10% inhibition at 1 mM). In view of these results, compound **12** was synthesized and evaluated in vitro (the results are summarized in Table 1). The addition of the guanidino grouping in **12** resulted in a 100-fold increase in activity over **11**. However, in comparison to **9** only a modest improvement (2-fold) in activity was achieved by this additional interaction. The crystal structure of the complex was determined by soaking compound **12** into N9 crystals (see Figure 3c). It clearly demonstrates that the additional hydroxymethyl group hydrogen bonds to an ordered water molecule located in the 5-subsite.

As described earlier, one of the most potent inhibitors of neuraminidase is 4-guanidino-Neu5Ac2en (**2c**). More recent studies demonstrated that the replacement of the glycerol functionality with lipophilic side chains, as in **3b**, resulted in potent inhibitors selective for influenza A. Compound **4**, which is a carbocyclic analogue of **3a**, inhibits influenza A NA with an IC_{50} of 1 nM, and it is nearly as effective for influenza B. The ethyl ester of inhibitor **4**, which is orally active, is currently in clinical evaluation. Since the guanidino functionality of **12** occupies the same subsite as the glycerol of inhibitor **2c**, it was anticipated that replacement of the guanidine in inhibitor **12** with lipophilic groups would result in effective inhibitors.

To evaluate this proposal compounds **13** and **14**, which contain a hydrophobic side chain, were synthesized. As shown in Table 1 compound **13** was a relatively modest inhibitor of NA, while **14** exhibited a dramatic improvement in activity against influenza A but had little effect on activity against influenza B. Compound **14** was 100-fold more active than its guanidino counterpart, analogue **12**, and over 15 000-fold more active than **11**, which implies an energetically significant hydrophobic interaction of the *N*-3-pentyl group within the active site. Compound **14** was soaked into an N9 crystal, and the complex structure was determined (see Figure 3d). Inhibitor **14** binds to influenza A neuraminidase in much the same manner as observed for **12**. However, significant differences in the binding of these two compounds were observed for the 6-subsite, where the guanidino group and the pentyl group undergo interaction. For **12** the guanidino group makes a bidentate hydrogen-bonding interaction with Glu 278. For **14** these interactions are not possible for the pentyl group, and the Glu 278 side chain adopts a different conformation to make an intramolecular salt bridge with Arg 226. This interaction creates a small hydrophobic pocket into which one branch of the pentyl side chain binds and the other branch binds in the preexisting, larger hydrophobic pocket created by Ala 246 and Ile 224. The entropy gains as a result of these hydrophobic interactions are likely to be the major contribution for the enhanced NA inhibitory activity (compound **14** is 15 000-fold more active than **11** for the inhibition of influenza A NA).

Despite the essentially identical location of amino acid side chains for the conserved amino acids that form the first amino acid shell in influenza A and B sialidases, compound **14** selectively inhibits (more than 2000-fold) sialidase A. This result is consistent with earlier reports[12,13,24,25] on a class of sialic acid derivatives containing a hydrophobic carboxamide which also oriented the hydrophobic groups in the 6-subsite of neuramini-

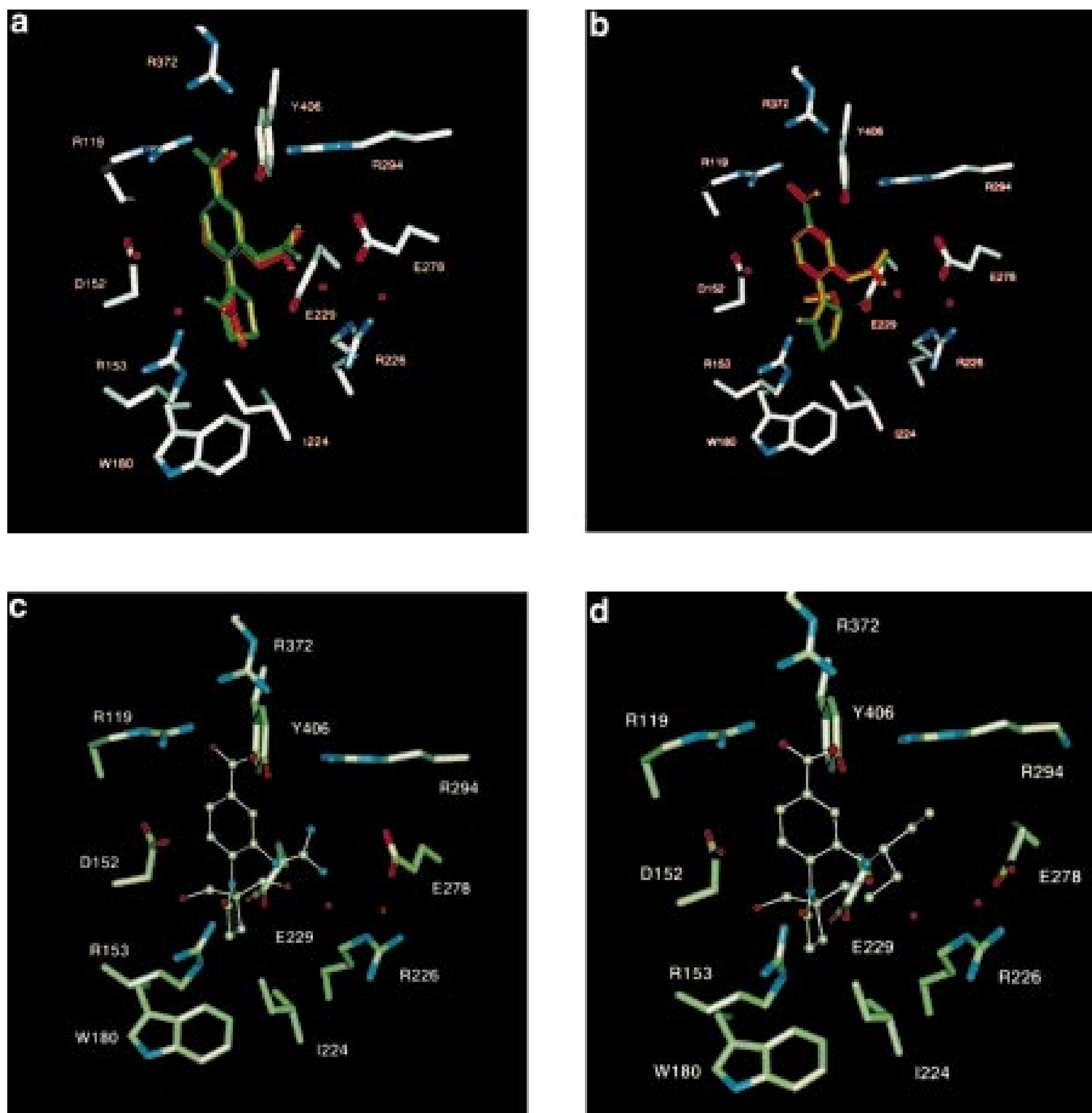


Figure 3. X-ray crystal structures of the active site region for complexes formed between selected pyrrolidinone benzoic acids and N9 neuraminidase: (a) compound **8** (green) and, for comparison, the lead compound **5** (BANA 113; orange); (b) compound **9** (green) and the analogous aminomethyl compound **10** (orange); (c) bis(hydroxymethyl) compound **12**; (d) bis(hydroxymethyl) compound **14**.

dase. For example, structure–activity relationship studies for carboxamide **3a** revealed that these compounds exhibit a magnitude of selectivity for influenza A over influenza B[25]similar to that observed for **14**. The crystal structures of **3c** with both influenza A and B sialidases were determined in order to investigate structural reasons for the selectivity. In the complex of **3c** with both influenza A and B sialidases, the movement of Glu 278 (Glu 276 in influenza B) was observed to form a salt bridge with Arg 226 (as also observed for **14** in complex with influenza A NA). In influenza A sialidase, this salt bridge formation resulted in minimal disruption of the surrounding protein structure (second amino acid shell). However, in influenza B sialidase the movement of Glu 278 (type A numbering) to form the new salt bridge produced distortions in the protein backbone near Glu 278 and in the second amino acid shell, since this region contains differences among nonconserved 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 poorer inhibition constant. Similar selectivity for influenza A neuraminidase has also been observed for selected examples of potent hydrophobic inhibitors based on a cyclohexene template,[26] although structural reasons for the selectivity were not presented. We anticipate that the factors discussed above account for the type A selectivity observed for compound **14**.

In summary, we have developed a highly novel, low nanomolar inhibitor of influenza A neuraminidase. This compound is unique among known potent inhibitors of influenza neuraminidase for several reasons. (1) Compound **14** is the first reported benzoic acid with low nanomolar inhibition. (2) It is the first achiral molecule exhibiting low nanomolar inhibition to be reported from

any structural class. (3) It is the first low nanomolar inhibitor that does not contain a basic side chain (amino or guanidino). The high selectivity of **14** for type A over type B neuraminidase is consistent with the higher energy cost, in type B NA, associated with the movement of Glu 278 to form a new salt bridge in the 6-subsite.

Experimental Section

Melting points were obtained on an Electrothermal melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 300 spectrometer unless otherwise noted. Infrared spectra were recorded on a Bruker infrared spectrophotometer. Combustion analyses were provided by Atlantic Microlabs of Atlanta, GA. Mass spectra were obtained on a Perkin-Elmer SCIEX API triple-quadrupole mass spectrometer using electrospray ionization. Analytical chromatography was performed on Whatman PE Sil G/UV silica gel plates (250 μm). Flash chromatography was performed using J.T. Baker silica gel (40 μm). Tetrahydrofuran was distilled from sodium metal/benzophenone ketyl. Dichloromethane and benzene were distilled from calcium hydride. Anhydrous *N,N*-dimethylformamide was purchased from Aldrich in Sure-Seal containers. Methanol was distilled from Mg(OMe)$_2$. All other commercially obtained reagents were used as received.

1-(4-Carboxyphenyl)pyrrolidin-2-one (16). A solution of **15** (3.00 g, 21.9 mmol) and ethyl 4-bromobutyrate (6.40 g, 32.8 mmol) in 20 mL of DMF was stirred at reflux for 24 h. The DMF was removed under vacuum to give a crude solid which was suspended in 15 mL of water, filtered, and dried. The solid was suspended in a small amount of ether (5 mL), filtered, and dried to give **16** (3.00 g, 66.0%) as a white solid: mp 244–246 °C (ether/ethanol); IR (KBr) 2924–2500, 1709, 1648, 1603 cm^{-1}; MS (electrospray) 204 (M − 1); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.1 (d, 2H, J = 8.9 Hz), 7.7 (d, 2H, J = 8.9 Hz), 3.9 (t, 2H, J = 7.0 Hz), 2.6 (t, 2H, J = 8.0 Hz), 2.2 (m, 2H); ^{13}C NMR (CDCl$_3$) δ 173.61, 166.77, 142.30, 129.54, 129.44, 125.25, 117.63, 47.49, 31.87, 16.79. Anal. ($C_{11}H_{11}NO_3$) C, H, N.

1-(4-Methoxycarbonylphenyl)pyrrolidin-2-one (17). A mixture of **16** (3.00 g, 14.6 mmol) and H_2SO_4 (0.2 mL) in 100 mL of methanol was stirred at reflux for 18 h. The reaction mixture was concentrated to dryness, and the crude residue was dissolved in 100 mL of ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ (3 × 15 mL) and water (10 mL), dried (Na_2SO_4), and evaporated to give **17** (3.00 g, 94.0%) as a white solid: mp 118–120 °C (chloroform/ether); MS (electrospray) 252 (M + 1); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.03 (d, 2H, J = 8.9 Hz), 7.73 (d, 2H, J = 8.8 Hz), 3.91 (s, 3H), 3.89 (t, 2H, J = 7.1 Hz), 2.62 (t, 2H, J = 8.0 Hz), 2.15 (m, 2H); ^{13}C NMR (CDCl$_3$) δ 174.54, 166.49, 143.29, 130.32, 125.29, 118.46, 51.90, 48.34, 32.76, 17.71. Anal. ($C_{12}H_{13}NO_3$) C, H, N.

1-(4-Methoxycarbonyl-2-nitrophenyl)pyrrolidin-2-one (18). Procedure A: An ice-cold mixture of **17** (2.00 g, 9.10 mmol) in 30 mL of CH_2Cl_2 was treated with NO_2BF_4 (2.50 g, 18.8 mmol). The resulting mixture was stirred at 10 °C for 2 h and then at room temperature for 4 h. The reaction mixture was quenched with water (20 mL), and the organic layer was evaporated under vacuum. The white solid which separated was filtered, washed with water, and dried to give **18** (2.20 g, 92.0%): mp 159–161 °C (chloroform/ether); MS (electrospray) 265 (M + 1); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.5 (d, 1H, J = 1.9 Hz), 8.25 (dd, 1H, J = 1.9 & 8.3 Hz), 7.4 (d, 1H, J = 8.3 Hz), 3.96 (s, 3H), 3.95 (t, 2H, J = 6.8 Hz), 2.5 (t, 2H, J = 7.9 Hz), 2.3 (m, 2H); ^{13}C NMR (CD$_3$OD/DMSO) δ 174.62, 164.73, 144.64, 135.73, 134.31, 127.86, 126.11, 125.68, 52.89, 49.35, 31.34, 18.95. Anal. ($C_{12}H_{12}N_2O_5$) C, H, N.

Procedure B: An ice-cold mixture of **17** (1.00 g, 4.57 mmol) in dioxane (6.5 mL) and acetic anhydride (10 mL) was treated with an ice-cold solution of nitrating reagent (4 mL of Ac_2O and 4 mL of HNO_3). The resulting solution was stirred at 35 °C for 5.5 h, after which it was diluted with ice water (50 mL), and the product was extracted into ethyl acetate (3 × 50 mL). This solution was dried (Na_2SO_4) and concentrated to give a yellow oil. The product was purified by flash chromatography on silica gel (10% ethanol/ether) to give **18** (800 mg, 67.0%).

1-(4-Methoxycarbonyl-2-aminophenyl)pyrrolidin-2-one (19). A mixture of **18** (1.00 g, 3.78 mmol) and Pd/C (500 mg) in 50 mL of methanol was shaken on a Parr hydrogenator at 30 psi for 1 h. The reaction mixture was diluted with 50 mL of methanol, and the Pd/C was removed by filtration. The filtrate was evaporated under reduced pressure to give **19** (840 mg, 95.0%) as a white solid: mp 136–138 °C; MS (electrospray) 235 (M + 1); ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.49 (d, 1H, J = 1.8 Hz), 7.47 (dd, 1H, J = 1.8 & 8.1 Hz), 7.1 (d, 1H, J = 8.1 Hz), 4.1 (br, 2H), 3.89 (s, 3H), 3.84 (t, 2H, J = 7.0 Hz), 2.6 (t, 2H, J = 7.9 Hz), 2.2 (m, 2H); ^{13}C NMR (CD$_3$OD) δ 177.49, 168.33, 145.57, 131.27, 129.81, 128.12, 119.67, 118.97, 52.79, 51.12, 32.42, 19.96. Anal. ($C_{12}H_{14}N_2O_3$) C, H, N.

1-[4-Methoxycarbonyl-2-(*N,N*-bis(*tert*-butyloxycarbonyl)guanidino)phenyl]pyrrolidin-2-one (20). An ice-cold mixture of **19** (75 mg, 0.28 mmol) in 1 mL of dry DMF was treated with *N,N*-bis(*tert*-butoxycarbonyl)thiourea (95 mg, 0.34 mmol), triethylamine (116 mg, 1.14 mmol), and $HgCl_2$ (100 mg, 0.36 mmol). The resulting mixture was stirred for 4 h at 0–10 °C. DMF was removed under high vacuum, and the crude mixture was purified by flash chromatography on silica gel (ether) to give **20** (95 mg, 72.0%) as an oil which solidified upon addition of hexane: mp 163–164 °C (ether); MS (electrospray) 477 (M + 1); ^{1}H NMR (300 MHz, CDCl$_3$) δ 11.5 (s, 1H), 10.41 (s, 1H), 8.9 (d, 1H, J = 1.8 Hz), 7.8 (dd, 1H, J = 1.8 & 8.3 Hz), 7.2 (d, 1H, J = 8.3 Hz), 3.91 (s, 3H), 3.75 (t, 2H, J = 6.9 Hz), 2.59 (t, 2H, J = 8.0 Hz), 2.23 (m, 2H), 1.5 (s, 9H), 1.4 (s, 9H); ^{13}C NMR (CDCl$_3$) δ 175.14, 166.21, 163,25, 153.5, 153.09, 134.64, 133.78, 129.86, 126.45, 126.33, 126.26, 83.85, 79.71, 77.64, 77.21, 76.79, 52.22, 49.86, 31.05, 28.11, 28.06, 19.06. Anal. ($C_{23}H_{32}N_4O_7$) C, H, N.

1-(4-Carboxy-2-guanidinophenyl)pyrrolidin-2-one (8). Procedure A: A suspension of **20** (25 mg, 0.050 mmol) in 1.5 mL of 1 N HCl was stirred at reflux for 3.5 h. The solvent was removed under vaccum to give an oily material which solidified upon the addition of ether to give **8** (14 mg, 92.0%) as a white hygroscopic solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.1 (s, 1H), 7.9 (dd, 1H, J = 1.8 & 8.2 Hz), 7.8 (d, 1H, J = 1.8 Hz), 7.6 (d, 1H, J = 8.2 Hz), 7.5 (br, 4H), 3.8 (t, 2H, J = 7.0 Hz), 2.5 (t, 2H, J = 8.0 Hz), 2.15 (m, 2H).

Procedure B: A solution of **20** (200 mg, 0.420 mmol) in 0.5 mL of CH_2Cl_2 was treated with 0.5 mL of trifluoroacetic acid. The mixture was stirred at room temperature for 12 h and concentrated to dryness on high vacuum to give a colorless oil which was dissolved in 2 mL of 1 N NaOH solution. The mixture was stirred at room temperature for 14 h. The pH of the mixture was adjusted to 6 with careful addition of glacial acid and cooled to give a white precipitate which was filtered and dried to give **8** (95 mg, 86.0%): mp 242–243 °C; MS (electrospray) 263 (M + 1); ^{1}H NMR (300 MHz, CD$_3$OD) δ 8.00 (dd, 1H, J = 1.8 & 8.3 Hz), 7.93 (d, 1H, J = 1.8 Hz), 7.48 (d, 1H, J = 8.3 Hz), 3.95 (t, 2H, J = 7.0 Hz), 2.61 (t, 2H, J = 7.9 Hz), 2.30 (m, 2H). Anal. ($C_{12}H_{14}N_4O_3 \cdot H_2O$) C, H, N.

1-(4-Carboxy-2-nitrophenyl)-5-carboxypyrrolidin-2-one (25). A suspension of 60% NaH (1.14 g, 28.4 mmol) in HMPA (3 mL) was treated with a solution of **24** (2.09 g, 16.2 mmol) in HMPA (12 mL). The reaction mixture was heated at 90–95 °C for 15 min and cooled to 45 °C, and then fluoronitrobenzoic acid **21** (1.50 g, 8.10 mmol) in HMPA (8 mL) was added. The reaction mixture was stirred at 160 °C for 23 h. Excess NaH was quenched with cold 1 N HCl (30 mL). The aqueous layer was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with cold water (2 × 20 mL), saturated sodium chloride (2 × 20 mL), and water (2 × 20 mL) and dried (Na_2SO_4), and the solvent was removed in vacuo. The residue was purified by flash chromatography on silica gel (80% ethyl acetate/hexane) to provide **25** (0.965 g, 40.0%) as a light-yellow solid: mp 235–237 °C; IR (KBr) 3300–2750, 1675, 1600, 1350, 1225 cm^{-1}; MS (electrospray) 295 (M + 1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.15–2.20 (m, 1H), 2.40–2.60 (m, 3H), 5.05 (m, 1H), 7.60 (d, 1H), 8.25 (dd,

1H), 8.40 (d, 1H), 9.60 (br s, 2H); ^{13}C NMR (DMSO-d_6) δ 173.90, 171.89, 165.16, 144.26, 134.65, 134.05, 128.97, 125.91, 125.55, 60.92, 29.75, 23.22. Anal. ($C_{12}H_{10}N_2O_7$) C, H, N.

1-(4-Ethoxycarbonyl-2-nitrophenyl)-5-(ethoxycarbonyl)pyrrolidin-2-one (26). A mixture of **25** (0.390 g, 1.33 mmol) in dry ethanol (4 mL) and H_2SO_4 (0.2 mL) was stirred at reflux for 24 h. The ethanol was removed under vacuum, the residue was suspended in 5% $NaHCO_3$ (4 mL), and the product was extracted into ethyl acetate (3 × 10 mL). The combined extracts were dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo to give **26** (0.425 g, 91.0%) as a yellow oil: IR (neat) 3000−2875, 1750, 1625, 1562 cm^{-1}; MS (electrospray) 351 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (t, 3H, *J* = 7.2 Hz), 1.42 (t, 3H, *J* = 7.1 Hz), 2.5−2.8 (m, 3H), 2.3−2.4 (m, 1H), 4.17 (dq, 2H, *J* = 3.0 & 7.1 Hz), 4.46 (q, 2H, *J* = 7.1 Hz), 4.70 (m, 1H), 7.58 (d, 1H, *J* = 8.3 Hz), 8.28 (dd, 1H, *J* = 2.0 & 8.3 Hz), 8.68 (d, 1H, *J* = 1.9 Hz); ^{13}C NMR ($CDCl_3$) δ 174.80, 170.80, 163.81, 145.56, 134.95, 134.30, 130.47, 129.45, 126.71, 62.17, 61.92, 60.29, 29.47, 23.93, 14.15, 13.93. Anal. ($C_{16}H_{19}N_2O_7$) C, H, N.

1-(4-Ethoxycarbonyl-2-nitrophenyl)-5-(hydroxymethyl)pyrrolidin-2-one (27). An ice-cold solution of **26** (2.00 g, 5.70 mmol) in dry ethanol (12.5 mL) and tetradydrofuran (12.5 mL) was treated with sodium borohydride (0.240 g, 6.27 mmol) in portions over a period of 5 min. The reaction mixture was then stirred at room temperature for 5 h. Excess $NaBH_4$ was quenched with cold 1 N HCl (11.0 mL). The yellow mixture was extracted with ethyl acetate (3 × 45 mL). The combined organic layers were washed with saturated sodium chloride (2 × 45 mL), dried (Na_2SO_4), and filtered, and the solvent was removed in vacuo. The crude mixture was purified by flash chromatography on silica gel (80% ethyl acetate/hexane) to provide **27** (0.906 g, 52.0%) as a yellow oil: IR (neat) 3500−3250, 1718, 1615, 1540 cm^{-1}; MS (electrospray) 309 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42 (t, 3H), 2.22−2.78 (m, 4H), 3.74 (dq, 2H), 4.42 (q, 2H), 4.44 (m, 1H), 7.42 (d, 1H), 8.30 (dd, 1H), 8.58 (d, 1H); ^{13}C NMR ($CDCl_3$) δ 175.04, 171.12, 163.76, 145.87, 134.06, 129.50, 127.12, 126.43, 62.63, 61.76, 60.25, 30.91, 20.96, 14.04.

1-(4-Ethoxycarbonyl-2-aminophenyl)-5-(hydroxymethylene)pyrrolidin-2-one (28). A mixture of **27** (0.914 g, 2.97 mmol), ethanol (114 mL), concentrated H_2SO_4 (1.5 mL), and 10% Pd/C (0.32 g) was shaken under 50 psi of hydrogen gas for 1 h. The mixture was filtered through a bed of Celite, washed with hot ethanol (20 mL), and concentrated to dryness on a rotary evaporator to give a semisolid (0.745 g, 95.0%): mp 90−95° C. The material was dissolved in 5% sodium bicarbonate (40 mL), the solution was adjusted to pH 8.0, and sodium chloride (0.50 g) and chloroform (40 mL) were added. The aqueous layer was extracted with chloroform (2 × 40 mL), and the combined organic layers were dried (Na_2SO_4) and concentrated in vacuo to give **28** (0.667 g, 81.0%) as a white, hygroscopic solid: MS (electrospray) 279 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (t, 3H), 2.20−2.80 (m, 4H), 3.50 (dd, 2H), 4.10 (m, 1H), 4.35 (q, 2H), 7.15 (d, 1H), 7.60 (d, 1H), 7.62 (d, 1H).

1-[4-Ethoxycarbonyl-2-(*N,N*-bis(*tert*-butyloxycarbonyl)guanidino)phenyl]-5-(hydroxymethyl)pyrrolidin-2-one (29). An ice-cold solution of **28** (0.315 g, 1.13 mmol) in dry DMF (12 mL) was treated with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (0.493 g, 1.70 mmol), triethylamine (0.358 g, 3.50 mmol), and mercuric chloride (0.369 g, 1.36 mmol). The reaction mixture was stirred under nitrogen for 20 h at room temperature. The reacion mixture was diluted with ethyl acetate (95 mL) and filtered through a bed of Celite. The filtrate was washed with water (50 mL), saturated sodium chloride (50 mL), and water (50 mL). The combined aqueous layers were extracted with ethyl acetate (2 × 25 mL), and the combined organic layers were dried (Na_2SO_4) and concentrated to dryness on a rotary evaporator. The crude yellow oil was purified by flash chromatography on silica gel (80% ethyl acetate/hexane) to give **29** (0.360 g, 61.0%) as a foamy yellow solid: mp 104−110 °C; MS (electrospray) 521 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (t, 3H), 1.50 (m, 18 H), 2.10−2.75 (m, 4H), 3.54 (dd, 2H), 4.21 (br s, 1H), 4.38 (q, 2H), 7.27 (d, 2H), 7.90 (d, 1H), 10.15 (br s, 1H), 11.50 (br s, 1H).

1-(4-Ethoxycarbonyl-2-guanidinophenyl)-5-(hydroxymethyl)pyrrolidin-2-one (30). An ice-cold solution of **29** (0.23 g, 0.44 mmol) in dichloromethane (8 mL) was treated with trifluoroacetic acid (4.65 mL, 60.4 mmol). The resulting mixture was stirred at room temperature for 12 h. The light red mixture was concentrated to dryness, redissolved in dichloromethane (5 mL), and reconcentrated; this process was repeated twice to give the trifluoroacetate salt of **30** (0.20 g, 100%) as an oil: MS (electrospray) 321 (M + 1); ^{1}H NMR (300 MHz, D_2O) δ 1.41 (t, 3H), 2.10−2.22 (m, 1H), 2.40−2.52 (m, 1H), 2.60−2.80 (m, 2H), 3.60 (dd, 2H), 4.38 (m, 1H), 4.44 (q, 3H), 7.60 (d, 1H), 8.15 (m, 2H). For the free base **30**: ^{1}H NMR (300 MHz, D_2O) δ 1.40 (t, 3H), 2.10−2.20 (m, 1H), 2.33−2.48 (m, 1H), 2.53−2.78 (m, 2H), 3.60 (m, 2H), 4.27 (m, 1H), 4.42 (q, 2H), 7.43 (d, 1H), 7.83 (d, 1H), 7.88 (dd, 1H).

1-(4-Carboxy-2-guanidinophenyl)-5-(hydroxymethyl)-pyrrolidin-2-one (9). A solution of **30** (0.19 g, 0.44 mmol) in 1 N sodium hydroxide (5 mL) was stirred at room temperature for 2 h. The pH of the reaction mixture was adjusted to 7.5 with acetic acid. The mixture was concentrated to one-half volume and cooled to give **9** (0.060 g, 47%) as a white solid: mp 253−257 °C (H_2O); IR (KBr) 3500−3150, 1650, 1634 cm^{-1}; MS (electrospray) 293 (M + 1); ^{1}H NMR (300 MHz, DMSO) δ 1.92−2.44 (m, 4H), 4.10 (m, 1H), 7.30 (d, 1H), 7.76 (m, 2H), 7.80 (m, 2H), 7.90−8.65 (m, 2H); ^{1}H NMR (300 MHz, D_2O) δ 2.10−2.20 (m, 1H), 2.38−2.52 (m, 1H), 2.58−2.80 (m, 2H), 3.60 (dd, 2H), 4.33 (m, 1H), 7.52 (d, 1H), 7.94 (m, 1H), 7.97 (dd, 1H). Anal. ($C_{12}H_{16}N_4O_4$) C, H, N.

5-(Azidomethyl)pyrrolidin-2-one (32). A mixture of **31** (140 mg, 0.72 mmol) and sodium azide (100 mg, 1.5 mmol) in 1.5 mL of dry DMF was stirred at 80 °C for 5 h. The solvent was evaporated to dryness under high vacuum, the residue was suspended in chloroform (25 mL), the mixture was filtered, and the filtrate was evaporated to dryness to give an oil. The oil was purified by flash chromatography on silica gel (10% ethanol in chloroform) to give **32** (95 mg, 95%) as an oil: MS (electrospray) 141 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.1 (br, 1H), 3.83 (m, 1H), 3.5 (dd, 1H, *J* = 4.5 & 12.3 Hz), 3.3 (dd, 1H, *J* = 6.7 & 12.3 Hz), 2.36 (m, 2H), 2.28 (m, 1H), 1.8 (m, 1H). Anal. ($C_5H_8N_4O{\cdot}0.25H_2O$) C, H, N.

1-(4-Carboxy-2-nitrophenyl)-5-(azidomethyl)pyrrolidin-2-one (33). To a suspension of 60% NaH (900 mg, 37.5 mmol) in 2 mL of dry DMF was added azide **32** (1.10 g, 7.80 mmol) in 10 mL of DMF. The resulting mixture was stirred for 15 min at 75 °C and cooled to room temperature, and a solution of 4-fluoro-3-nitrobenzoic acid (1.50 g, 8.10 mmol) in 8 mL of DMF was added. The reaction mixture was stirred at room temperature for 20 h. Excess NaH was quenched with 2 N HCl (2 mL), and the solvent was removed under high vaccum below 50 °C. The crude residue was suspended in 10 mL of 1 N HCl, and the product was extracted into ethyl acetate (3 × 50 mL). The extracts were dried (Na_2SO_4) and concentrated to give an oil which was purified by flash chromatography on silica gel (ether) to give **33** (600 mg, 25%) as a pale-yellow oil. This was crystallized from ether/ethanol: mp 119−121 °C; MS (electrospray) 304 (M − 1); ^{1}H NMR (300 MHz, acetone-d_6) δ 8.52 (d, 1H, *J* = 1.9 Hz), 8.33 (dd, 1H, *J* = 1.8 & 8.4 Hz), 7.79 (d, 1H, *J* = 8.4 Hz), 4.7 (m, 1H), 3.72 (d, 2H, *J* = 12.6 Hz), 2.5 (m, 3H), 2.1 (m, 1H); ^{13}C NMR (300 MHz, acetone-d_6) δ 174.66, 165.39, 146.78, 135.72, 134.99, 130.27, 128.41, 127.12, 60.11, 54.47, 30.44, 23.20. Anal. ($C_{12}H_{11}N_5O_5{\cdot}0.25H_2O$) C, H, N.

1-(4-Methoxycarbonyl-2-nitrophenyl)-5-(azidomethyl)-pyrrolidin-2-one (34). A mixture of **33** (520 mg, 1.70 mmol) and H_2SO_4 (0.1 mL) in 40 mL of methanol was stirred at reflux for 12 h. Methanol was removed under vacuum, and the resulting oil was dissolved in 100 mL of ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ (3 × 10 mL) and water (10 mL), dried (Na_2SO_4), and evaporated to give an oil which was purified by flash chromatography on silica gel (ether) to give **34** (520 mg, 96%) as a colorless oil. The oil was crystallized from ether/ethanol: mp 68−70 °C; MS (electrospray) 320 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (d, 1H,

J = 1.9 Hz), 8.32 (dd, 1H, *J* = 1.9 & 8.3 Hz), 7.46 (d, 1H, *J* = 8.3 Hz), 4.36 (m, 1H), 3.98 (s, 3H), 3.57 (dd, 2H, *J* = 5.1 & 12.8 Hz), 2.5 (m, 3H), 2.1 (m, 1H); ^{13}C NMR (acetone-d_6) δ 174.81, 164.77, 146.23, 134.88, 134.67, 129.9, 128.33, 127.15, 59.93, 54.12, 53.23, 30.48, 23.01. Anal. ($C_{13}H_{13}N_5O_5$) C, H, N.

1-(4-Methoxycarbonyl-2-aminophenyl)-5-(*tert*-butyloxycarbonylaminomethyl)pyrrolidin-2-one (35). A suspension of 10% Pd/C (210 mg) in 5 mL of ethyl acetate was shaken on a Parr shaker for 5 min using 30 psi of H_2 gas. Compound **34** (220 mg, 0.69 mmol) and di-*tert*-butyl dicarbonate (155 mg, 0.700 mmol) in 5 mL of ethyl acetate were added, and the resulting mixture was shaken for 2.5 h at 30 psi. The reaction mixture was diluted with 50 mL of methanol, filtered, and evaporated to dryness under vacuum to give **35** (240 mg, 100%) as a colorless oil which was used in the next reaction without further purification: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.55 (m, 2H), 7.12 (d, 1H), 5.58 (br, 1H), 4.28 (m, 1H), 4.15 (br s, 2H), 3.90 (s, 3H), 3.45 (m, 1H), 2.85 (t, 0.5H), 2.8 (t, 0.5H), 2.6 (t, 2H), 2.35 (m, 1H) 2.1 (m, 1H), 1.4 (s, 9H).

1-[4-Methoxycarbonyl-2-(*N,N*-bis(*tert*-butyloxycarbonyl)guanidino)phenyl]-5-(*tert*-butyloxycarbonylaminomethyl)pyrrolidin-2-one (36). An ice-cold solution of **35** (245 mg, 0.70 mmol) in 3 mL of dry DMF under nitrogen was treated with 1,3-bis(*tert*-butoxycarbonyl)-2-thiopseudourea (232 mg, 0.840 mmol), triethylamine (215 mg, 2.10 mmol), and $HgCl_2$ (230 mg, 0.84 mmol). The reaction mixture was then stirred at 0−10 °C for 4 h. The DMF was removed under vacuum, and the crude product was purified by flash chromatography on silica gel (ether) to give **36** (280 mg, 60%) as a colorless oil. The oil was crystallized from ether/hexane: mp 134−136 °C; MS (electrospray) 606 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.5 (br, 1H), 10.05 (br, 1H) 8.55 (br, 1H), 7.9 (d, 1H, *J* = 8.0 Hz), 7.25 (d, 1H, *J* = 8.2 Hz),5.35 (br, 1H), 4.4 (m, 1H), 3.95 (s, 3H), 3.75 (m, 1H), 3.3 (m, 1H), 2.6 (m, 2H), 2.4 (m, 1H), 1.95 (m, 1H), 1.55 (s, 9H), 1.45 (s, 9H), 1.35 (s, 9H). Anal. ($C_{29}H_{43}N_5O_9$) C, H, N.

1-(4-Carboxy-2-guanidinophenyl)-5-(aminomethyl)pyrrolidin-2-one (10). A suspension of **36** (33 mg, 0.050 mmol) in 1.5 mL of 1 N HCl was stirred at reflux for 4 h. The solvent was removed under vacuum to give an oil which was crystallized from ethanol/ether to give **10** (16 mg, 84%) as a white hygroscopic solid: mp 205 °C (foamed); MS (electrospray) 292 (M + 1); ^{1}H NMR (300 MHz, D_2O) δ 8.18 (m, 2H), 7.62 (d, 1H), 4.6 (m, 1H), 3.15 (m, 2H), 2.75 (m, 2H), 2.65 (m, 1H), 2.15 (m, 1H). Anal. ($C_{13}H_{19}N_5O_3Cl_2{\cdot}0.5H_2O{\cdot}0.5CH_3CH_2OH$) C, H, N.

Diethyl 2-(4-Carboxymethylphenylamino)malonate (38). A mixture of **37** (3.10 g, 20.5 mmol) and diethyl bromomalonate (2.45 g, 10.2 mmol) was heated at 115 °C for 16 h in an oven. The reaction mixture was cooled, suspended in benzene (20 mL), and filtered. The filtrate was diluted with additional benzene (50 mL) and washed with 2 N HCl (3 × 10 mL) and water (10 mL). The organic layer was dried (Na_2SO_4) and evaporated to give an oil, which solidified upon standing. The crude product was recrystallized from ether/hexane to give **38** (2.7 g, 85%): mp 66−69 °C (ether/hexane); MS (electrospray) 310 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.9 (d, 2H, *J* = 8.8 Hz), 6.6 (d, 2H, *J* = 8.8 Hz), 5.2 (br d, 1H, *J* = 7.2 Hz), 4.8 (d, 1H, *J* = 7.2 Hz), 4.3 (q, 4H), 3.8 (s, 3H), 1.3 (t, 6H); ^{13}C NMR ($CDCl_3$) δ 166.93, 166.86, 149.04, 131.42, 120.04, 112.19, 62.51, 59.80, 51.48, 13.87. Anal. ($C_{15}H_{19}NO_6$) C, H, N.

***N*-[Bis(ethoxycarbonyl)methyl]-*N*-(3-bromopropionyl)-4-(methoxycarbonyl)aniline (39).** A mixture of **38** (1.10 g, 3.50 mmol), 3-bromopropionic acid (1.10 g, 7.20 mmol), and phosphorus trichloride (1 mL) in 12 mL of benzene was stirred at reflux for 30 h. The reaction mixture was diluted with 50 mL of benzene and washed with saturated $NaHCO_3$ (3 × 15 mL), water (10 mL), 1 N HCl (10 mL), and water (10 mL). The organic layer was dried (Na_2SO_4) and evaporated to give pure **39** (1.4 g, 89%) as an oil which was used in the next reaction without further purification: MS (electrospray) 444 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (d, 2H, *J* = 8.3 Hz), 7.54 (d, 2H, *J* = 8.3 Hz), 5.46 (s, 1H), 4.2 (m, 4H), 3.95 (s, 3H), 3.56 (t, 2H, *J* = 6.7 Hz), 2.69 (t, 2H, *J* = 6.9 Hz), 1.2 (t, 6H).

1-(4-Methoxycarbonylphenyl)-5,5-bis(ethoxycarbonyl)pyrrolidin-2-one (40). A suspension of 60% NaH (70 mg, 2.9 mmol) was washed with hexane (3 × 1 mL) to remove mineral oil. The NaH in 10 mL of dry DMF was treated with **39** (517 mg, 1.10 mmol) in 7 mL of DMF. The reaction mixture was stirred at room temperature for 2 h. Excess NaH was quenched with 1 mL of 1 N HCl. The solvent was removed under high vacuum, the resulting crude oil was suspended in 50 mL of ethyl acetate, and the organic layer was washed with water (3 × 10 mL), dried (Na_2SO_4), and evaporated to give pure **40** (415 mg, 98%) as an oil: MS (electrospray) 364 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.0 (d, 2H, *J* = 8.4 Hz), 7.4 (d, 2H, *J* = 8.4 Hz), 4.2 (qq, 4H), 3.9 (s, 3H), 2.7 (m, 2H), 2.6 (m, 2H) 1.2 (t, 6H); ^{13}C NMR δ 174.64, 167.75, 165.95, 140.82, 129.65, 128.56, 126.81, 74.11, 62.25, 51.79, 29.04, 28.94, 13.36. Anal. ($C_{18}H_{21}NO_7$) C, H, N.

1-(4-Methoxycarbonylphenyl)-5,5-bis(hydroxymethyl)pyrrolidin-2-one (41). To an ice-cold solution of **40** (500 mg, 1.37 mmol) in 6 mL of dry THF and 6 mL of dry methanol was added $NaBH_4$ (200 mg, 5.20 mmol) in small portions over a period of 30 min. The reaction mixture was then stirred at 0−10 °C for 4 h. Additional $NaBH_4$ (75 mg, 1.98 mmol) was added, and the stirring continued for 6 h more at 0−10 °C. The reaction mixture was quenched with 5 mL of 0.5 N HCl, organic solvent was evaporated, and the product was extracted into ethyl acetate (5 × 25 mL). The extracts were dried (Na_2SO_4) and evaporated to give 450 mg of crude oil, which was purified by flash chromatography on silica gel (10% ethanol in ether) to give **41** (330 mg, 86%) as a solid: mp 133−135 °C; MS (electrospray) 280 (M + 1); ^{1}H NMR (300 MHz, CD_3OD) δ 8.10 (d, 2H, *J* = 8.3 Hz), 7.43 (d, 2H, *J* = 8.4 Hz), 3.92 (s, 3H), 3.38 (dd, 4H, *J* = 7.2 & 11.9 Hz), 2.58 (t, 2H, *J* = 8.5 Hz), 2.29 (t, 2H, *J* = 8.5 Hz). Anal. ($C_{14}H_{17}NO_5$) C, H, N.

1-(4-Carboxyphenyl)-5,5-bis(hydroxymethyl)pyrrolidin-2-one (11). A solution of **41** (60 mg, 0.21 mmol) in 2 mL of 1 N NaOH was stirred at room temperature for 3.5 h. The pH of the reaction mixture was adjusted to 2 with 1 N HCl; the product was extracted into ethyl acetate (5 × 10 mL), dried, and evaporated to provide a solid residue. The residue was suspended in ether and filtered to give **11** (40 mg, 70%): mp 195−197 °C (ether/ethanol); MS (electrospray) 264 (M − 1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.99 (br, 1H), 7.97 (d, 2H, *J* = 8.4 Hz), 7.35 (d, 2H, *J* = 8.4 Hz), 5.05 (br, 2H), 3.25 (dd, 4H, *J* = 11.3 & 16.1 Hz), 2.4 (t, 2H, *J* = 8.6 Hz), 2.1 (t, 2H, *J* = 8.5 Hz). Anal. ($C_{13}H_{15}NO_5$) C, H, N.

1-(4-Methoxycarbonyl-2-nitrophenyl)-5,5-bis(ethoxycarbonyl)pyrrolidin-2-one (42). An ice-cold solution of **40** (450 mg, 1.23 mmol) in 5 mL of CH_2Cl_2 was treated with nitronium tetrafluoroborate (550 mg, 4.10 mmol). The resulting mixture was stirred at 5−10 °C for 3 h. The reaction mixture was evaporated to dryness, and the crude mixture was dissolved in 50 mL of ethyl acetate. The organic layer was washed with water (3 × 15 mL), dried (Na_2SO_4), and evaporated to give **42** (440 mg, 95%) as an oil which solidified upon standing: mp 81−82 °C (ether); MS (electrospray) 409 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.7 (d, 1H, *J* = 1.9 Hz), 8.35 (dd, 1H, *J* = 1.9 & 8.4 Hz), 7.85 (d, 1H, *J* = 8.4 Hz), 4.35 (m, 2H), 4.1 (m, 2H), 3.95 (s, 3H), 3.15 (m, 1H), 2.7 (m, 2H), 2.5 (m, 1H), 1.3 (t, 3H), 0.98 (t, 3H). Anal. ($C_{18}H_{20}N_2O_9$) C, H, N.

1-(4-Methoxycarbonyl-2-nitrophenyl)-5,5-bis(hydroxymethyl)pyrrolidin-2-one (43). An ice-cold solution of **42** (4.10 g, 10.0 mmol) in a mixture of 50 mL of dry THF and 50 mL of dry methanol was treated with small portions of $NaBH_4$ (1.80 g, 47.0 mmol) over a period of 1 h. Stirring continued for 4 h after which additional $NaBH_4$ (0.500 g, 13.2 mmol) was added. After another 4 h of stirring more $NaBH_4$ (0.500 g, 13.2 mmol) was added, and the stirring continued for 6 more h at 0° C. The reaction mixture was then quenched with 1 N HCl (25 mL) and concentrated to about 20 mL volume under vaccum. The product was extracted into chloroform (5 × 50 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to give an oil which was purified by flash chromatography on silica gel (10% ethanol in ether) to give **43** (2.00 g, 62.0%) as an off-white solid: mp 144−145 °C; MS (electro-

spray) 325 (M + 1); ^{1}H NMR (300 MHz, CD_3OD) δ 8.58 (d, 1H, J = 1.9 Hz), 8.29 (dd, 1H, J = 1.9 & 8.3 Hz), 7.73 (d, 1H, J = 8.3 Hz), 3.95 (s, 3H), 3.8 (dd, 2H, 11.9 & 52 Hz), 3.6 (dd, 2H, 11.3 & 29.5 Hz), 2.55 (m, 2H), 2.3 (m, 1H), 2.0 (m, 1H,); ^{13}C NMR (CD_3OD) δ 179.73, 166.42, 149.62, 137.01, 135.49, 133.08, 132.28, 127.81, 73.79, 66.41, 64.98, 53.72, 31.44, 27.44. Anal. ($C_{14}H_{16}N_2O_7$) C, H, N.

1-(4-Methoxycarbonyl-2-aminophenyl)-5,5-bis(hydroxymethyl)pyrrolidin-2-one (44). A suspension of **43** (0.80 g, 2.5 mmol) in 25 mL of methanol was treated with 10% Pd/C (500 mg), and the resulting mixture was placed on a Parr shaker at 50 psi of H_2 gas for 1 h. The reaction mixture was diluted with 50 mL of methanol, filtered, and evaporated to dryness under vacuum to give an oil which was solidified upon the addition of ether to give pure **44** (630 mg, 87%): mp 192–194 °C; MS (electrospray) 295 (M + 1); ^{1}H NMR (300 MHz, CD_3OD) δ 7.52 (d, 1H, J = 1.9 Hz), 7.34 (dd, 1H, J = 1.9 & 8.1 Hz), 7.23 (d, 1H, J = 8.2 Hz), 3.9 (s, 3H), 3.63–3.32 (m, 4H), 2.67–2.5 (m, 2H), 2.4–2.3 (m, 2H); ^{13}C NMR (CD_3OD) δ 179.43, 168.55, 148.19, 132.37, 131.90, 126.75, 119.74, 119.07, 73.14, 64.07, 64.02, 52.77, 31.94, 25.60. Anal. ($C_{14}H_{18}N_2O_5$· 0.25CH_3OH) C, H, N.

1-[4-Methoxycarbonyl-2-(*N,N*-bis(benzyloxycarbonyl)guanidino)phenyl]-5,5-bis(hydroxymethyl)pyrrolidin-2-one (45). An ice-cold solution of **44** (254 mg, 0.860 mmol) in 3 mL of dry DMF was treated with bis(benzyloxycarbonyl)-*S*-methylthiourea (425 mg, 1.20 mmol), $HgCl_2$ (330 mg, 1.20 mmol), and triethylamine (250 mg, 2.40 mmol). The resulting mixture was stirred at 0 °C for 3 h and the temperature slowly raised to room temperature, where the mixture stirred for 12 h. DMF was removed under vacuum, and the crude mixture was purified by flash chromatography on silica gel (ether) to give **45** (370 mg, 71%) as a colorless oil: MS (electrospray) 605 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.8 (br, 1H), 10.1 (br, 1H), 7.9 (d, 1H), 7.5 (d, 1H), 7.35 (m, 11H), 5.2 (m, 4H), 3.9 (s, 3H), 3.75 (m, 1H), 3.5 (m, 1H), 3.35 (m, 2H), 2.6 (m, 2H), 2.25 (m, 1H), 2.0 (m, 1H).

1-(4-Carboxy-2-guanidinophenyl)-5,5-bis(hydroxymethyl)pyrrolidin-2-one (12). A solution of **45** (350 mg, 0.57 mmol) in 1 mL of methanol was treated with 1 N NaOH (2.5 mL). The resulting suspension was stirred at room temperature for 16 h. The reaction mixture was adjusted to pH 3 with 1 N HCl, and the product was chromatographed on an ion-exchange column (Dowex; 1.4 N NH_4OH) to give **12** (110 mg, 59.0%) as the ammonium salt: MS (electrospray) 323 (M + 1); ^{1}H NMR (300 MHz, D_2O) δ 7.95 (d, 1H, J = 1.8 Hz), 7.93 (dd,1H, J = 1.9 & 8.2 Hz), 7.45 (d, 1H, J = 8.2 Hz), 3.7 (dd, 2H, J = 8.1 & 12.0 Hz), 3.55 (d, 1H, J = 12.0 Hz), 3.29 (d, 1H, J = 12 Hz), 2.7 (m, 2H), 2.35 (m, 2H). Anal. ($C_{14}H_{21}N_5O_5$·H_2O) C, H, N.

1-[4-Methoxycarbonyl-2-(3-pentylamino)phenyl]pyrrolidin-2-one (46). A solution of **19** (500 mg, 2.10 mmol), 3-pentanone (190 mg, 2.20 mmol), and triethylamine (440 mg, 4.30 mmol) in 10 mL of dry CH_2Cl_2 was treated with $TiCl_4$ (3.5 mL, 3.50 mmol). The resulting mixture was stirred at room temperature for 18 h, and $NaCNBH_3$ (410 mg, 6.50 mmol) was added. Stirring was continued for 3 more h. The reaction mixture was quenched with 1 N NaOH (2 mL), and the CH_2Cl_2 was evaporated under vacuum. The product was extracted into ethyl acetate (3 × 30 mL), dried (Na_2SO_4), and evaporated. The resulting oil was purified by flash chromatography on silica gel (10% ethanol in ether) to give **46** (160 mg, 25.0%): MS (electrospray) 305 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (d, 1H, J = 1.8 Hz), 7.33 (dd, 1H, J = 1.8 & 8.0 Hz), 7.08 (d, 1H, J = 8.0 Hz), 3.95 (br d, NH, J = 7.6 Hz), 3.89 (s, 3H), 3.74 (t, 2H, J = 6.9 & 6.9 Hz), 3.35 (m, 1H), 2.60 (t, 2H, J = 7.8 Hz), 2.22 (m, 2H), 1.57 (m, 4H), 0.91 (t, 6H); ^{13}C NMR δ 174.65, 166.83, 143.42, 129.64, 128.53, 125.93, 117.33, 113.10, 54.67, 51.79, 50.04, 31.18, 26.25, 19.05, 9.75. Anal. ($C_{17}H_{24}N_2O_3$· 0.75H_2O) C, H, N.

1-[4-Carboxy-2-(3-pentylamino)phenyl]pyrrolidin-2-one (13). A solution of **46** (150 mg, 0.490 mmol) in 1 mL of methanol was treated with 1 N NaOH (2 mL), and the resulting mixture was stirred at room temperature for 16 h. The reaction mixture was acidified with excess glacial acetic acid, and the product precipitated. The mixture was filtered, and the collected solid was washed with water and dried to give pure **13** (125 mg, 87.0%): mp 178–180 °C (water); MS (electrospray) 291 (M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44 (d, 1H, J = 1.2 Hz), 7.37 (dd, 1H, J = 1.2 & 8.1 Hz), 7.09 (d, 1H, J = 8.1 Hz), 3.75 (t, 2H, J = 6.9 & 7.0 Hz), 3.34 (m, 1H), 2.65 (t, 2H, J = 8.0 & 8.0 Hz), 2.23 (m, 2H), 1.55 (m, 4H), 0.92 (t, 6H, J = 7.4 & 7.4 Hz); ^{13}C NMR ($CDCl_3$) δ 175.64, 170.00, 143.49, 129.99, 128.57, 126.19, 118.05, 113.73, 54.86, 50.31, 31.34, 26.34, 19.14, 9.29. Anal. ($C_{16}H_{22}N_2O_3$·0.5H_2O) C, H, N.

1-[4-Methoxycarbonyl-2-(3-pentylamino)phenyl]-5,5-bis(hydroxymethyl)pyrrolidin-2-one (47). A solution of **44** (100 mg, 0.340 mmol) in 1 mL of dichloroethane and 0.5 mL of acetic acid was treated with 3-pentanone (200 mg, 2.30 mmol) and $NaCNBH_3$ (75.0 mg, 1.20 mmol). The resulting mixture was stirred at room temperature for 6 h, and 3-pentanone (200 mg, 2.30 mmol), $NaCNBH_3$ (75.0 mg, 1.20 mmol), and methanol (0.5 mL) were added. Stirring was continued for 12 h, and additional 3-pentanone (300 mg, 3.40 mmol) and $NaCNBH_3$ (150 mg, 2.30 mmol) were added. Stirring was then continued for an additional 16 h. The reaction mixture was quenched with 10 mL $NaHCO_3$ (saturated), and the product was extracted into ethyl acetate (3 × 50 mL). The organic layers were dried (Na_2SO_4) and evaporated to give an oil which was purified by flash chromatography on silica gel (5% ethanol in ether) to give **47** (105 mg, 82.2%) as a white solid: mp 183–185 °C; MS (electrospray) 365 (M + 1); ^{1}H NMR (300 MHz, CD_3OD) δ 7.31 (d, 1H, J = 1.7 Hz), 7.25 (d, 1H, J = 8.0 Hz), 7.2 (dd, 1H, J = 1.7 & 8.0 Hz), 3.9 (s, 3H), 3.45 (m, 4H), 3.27 (m, 1H), 2.6 (m, 1H), 2.5 (m, 1H), 2.3 (m, 2H), 1.6 (m, 3H), 1.45 (m, 1H), 0.97 (t, 3H), 0.93 (t, 3H); ^{13}C NMR (CD_3OD) δ 179.44, 169.05, 148.40, 132.45, 132.43, 125.68, 116.90, 112.88, 73.02, 64.06, 63.89, 56.34, 52.79, 32.16, 27.79, 27.69, 25.82, 11.11, 10.74. Anal. ($C_{19}H_{28}N_2O_5$) C, H, N.

1-[4-Carboxy-2-(3-pentylamino)phenyl]-5,5-bis(hydroxymethyl)pyrrolidin-2-one (14). A suspension of **47** (70 mg, 0.19 mmol) in 1 N NaOH (1 mL) was stirred at room temperature for 12 h. Acidification of the reaction mixture with glacial acetic acid resulted in a white precipitate, which was filtered and dried to give pure **14** (50 mg, 75%): mp 220–221 °C (water); MS (electrospray) 351 (M + 1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.02 (d, 1H, J = 1.5 Hz), 7.01 (d, 1H, J = 8.0 Hz), 6.93 (dd, 1H, J = 1.5 & 8.0 Hz), 5.3 (br, 3H), 3.2–3.0 (m, 5H), 2.3 (m, 1H), 2.2 (m, 1H), 2.05 (m, 2H), 1.4 (m, 3H), 1.2 (m, 1H), 0.8 (t, 3H, J = 7.3 Hz), 0.73 (t, 3H, J = 7.3 Hz). Anal. ($C_{18}H_{26}N_2O_5$) C, H, N.

X-ray Crystallography. Purified N9 neuraminidase 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 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 α = 183.8 Å. The crystals diffract strongly to at least 2 Å resolution on a rotating anode source.

The complexes of the inhibitor with neuraminidase 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 and 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 is 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_o's. The starting model for the refinement of the inhibitor complexes is the 1.9 Å refined uncomplexed neuraminidase 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. $F_o - F_c$ and $2(F_o - F_c)$ difference Fourier maps were calculated, and a model of the inhibitor molecule was fit 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 neuraminidase 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.[22] The neuraminidase from the H1N9 strain of influenza was obtained by the method described by Laver et al.[23] The assay employed a spectrofluorometric technique that uses 2′-(methylumbelliferyl)-α-D-acetylneuraminic acid as substrate. Cleavage of this substrate by neuraminidase produces a fluorescent product which can 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 and 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 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} value was calculated by plotting percent inhibition versus the inhibitor concentration, and the determination of each point was performed in duplicate. In all cases each IC_{50} value differed from its duplicate by less than 2-fold.

Acknowledgment. This work was supported in part by NIH Grant U01 AI31888 (M.L., W.J.B., G.M.A.) and by a research grant from BioCryst Pharmaceuticals, Inc.

References

(1) (a) Air, G. M.; Laver, W. G. The Neuraminidase of Influenza Virus. *Proteins: Struct., Funct., Genet.* **1989**, *6*, 341−356. (b) Ghate, A. N.; Air, G. M. Site-Directed Mutagenesis of Catalytic Residues of Influenza Virus Neuraminidase as an Aid to Drug Design. *Eur. J. Biochem.* **1998**, *258*, 320−331.

(2) Palese, P.; Jobita, K.; Ueda, M.; Compans, R. W. Characterization of Temperature-Sensitive Influenza Virus Mutants Defective in Neuraminidase. *Virology* **1974**, *61*, 397−410.

(3) Lin, C.; Eichelberger, M. C.; Compans, R. W.; Air, G. M. Influenza Type A Virus Neuraminidase Does Not Play a Role in Viral Entry, Replication, Assembly, or Budding. *J. Virol.* **1995**, *69*, 1099−1106.

(4) Von Itzstein, M.; Wu, Y.; Kok, G. B.; Pegg, M. S.; Dyason, J. C.; Jin, B.; Van Phan, T.; Synthe, M. L.; White, H. F.; Oliver, S. W.; Coleman, P. G.; Varghese, V. J.; Cameron, J. M.; Penn, C. R. Rational Design of Potent Sialidase-Based Inhibitors of Influenza Virus Replication. *Nature* **1993**, *363*, 418−423.

(5) (a) Burmeister, W. P.; Ruigrok, R. W. H.; Cusack, S. The 2.2 Å Resolution Crystal Structure of Influenza B Neuraminidase and its Complex with Sialic Acid. *EMBO J.* **1992**, *11*, 49−56. (b) Janakiraman, M. N.; White, C. L.; Laver, W. G.; Air, G. M.; Luo, M. Structure of Influenza Virus Neuraminidase B/Lee/40 Complexed with Sialic Acid and a Dehydro Analogue at 1.8 A Resolution: Implication for the Catalytic Mechanism. *Biochemistry* **1994**, *33*, 8172−8179. (c) Varghese, J. N.; Mckimm-Breschkin, J. L.; Caldwell, J. B.; Kortt, A. A.; Colman, P. M. The Structure of the Complex Between Influenza Virus Neuraminidase and Sialic Acid, the Viral Receptor. *Proteins: Struct., Funct., Genet.* **1992**, *14*, 327−332. (d) Bossart-Whitaker, P.; Carson, M.; Babu, Y. S.; Smith, C. D.; Laver, W. G.; Air, G. M. Three-Dimensional Structure of Influenza A N9 Neuraminidase and its Complex with the Inhibitor 2-Deoxy-2,3-dehydro-*N*-acetyl Neuraminic Acid. *J. Mol. Biol.* **1993**, *232*, 1069−1083. (e) Tulip, W. R.; Varghese, J. N.; Laver, W. G.; Colman, P. M. Refined Crystal Structure of the Influenza Virus N9 Neuraminidase-NC41 Fab Complex. *J. Mol. Biol.* **1992**, *227*, 122−148. (f) Varghese, J. N.; Epa, V. C.; Colman, P. M. Three-dimensional Structure of the Complex of 4-Guanidino-Neu5Ac2en and Influenza Virus Neuraminidase. *Protein Sci.* **1995**, *4*, 1081−1084.

(6) (a) Colman, P. M.; Varghese, J. N.; Laver, W. G. Structure of the Catalytic and Antigenic Sites in Influenza Virus Neuraminidase. *Nature (London)* **1983**, *303*, 41−44. (b) Air, G.; Laver, W. G.; Luo, M.; Stray, S. J.; Legrone, G.; Webster, R. G. Antigenic, Sequence, and Crystal Variation in Influenza B Neuraminidase. *Virology* **1990**, *177*, 578−587.

(7) Meindl, P.; Bodo, G.; Palese, P.; Schulman, J.; Tuppy, H. Inhibition of Neuraminidase Activity by Derivatives of 2-Deoxy-2,3-dehydro-*N*-acetylneuraminic Acid. *Virology* **1975**, *58*, 457−463.

(8) Holzer, C. T.; Von Itzstein, M.; Jin, B.; Pegg, M. S.; Stewart, W. P.; Wu, W.-Y. Inhibition of Sialidases from Viral, Bacterial and Mammalian Sources by Analogues of 2-Deoxy-2,3-didehydro-*N*-acetylneuraminic Acid Modified at the C-4 Position. *Glycoconjugate J.* **1993**, *10*, 40−44.

(9) Taylor, N. R.; Von Itzstein, M. Molecular Modeling Studies on Ligand Binding to Sialidase from Influenza Virus and the Mechanism of Catalysis. *J. Med. Chem.* **1994**, *37*, 616−624.

(10) Hayden, F. G.; Treanor, J. J.; Betts, R. F.; Lobo, M.; Esinhart, J. D.; Hussey, E. K. Safety and Efficacy of the Neuraminidase Inhibitor GG 167 in Experimental Human Influenza. *J. Am. Med. Assoc.* **1996**, *275*, 295−299.

(11) Ryan, D. M.; Ticeburst, J.; Dempsey, M. H.; Penn, C. R. Inhibition of Influenza Virus Replication in Mice by GG 167 is Consistent with Extracellular Activity of Viral Neuraminidase. *Antimicrob. Agents Chemother.* **1994**, *38*, 2270−2275.

(12) Sollis, S. L.; Smith, P. W.; Howes, P. D.; Cherry, P. C.; Bethell, R. C. Novel Inhibitors of Influenza Sialidase Related to GG 167. Synthesis of 4-Amino and Guanidino-4H-pyran-2-carboxylic Acid-6-propylamides; Selective Inhibitors of Influenza A Virus Salidase. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1805−1808.

(13) Smith, P. W.; Sollis, S. L.; Howes, P. D.; Cherry, P. C.; Cobley, K. N.; Taylor, H.; Whittington, A. R.; Scicinski, J.; Bethell, R. C.; Taylor, N.; Varghese, J.; Colman, P.; Singh, O.; Skarzynski, T.; Cleasby, A.; Woncott, A. Novel Ihibitors of Ifluenza Salidase Rlated to GG 167. Structure−Activity, Crystallographic and Molecular Dynamics Studies with 4H-Pran-2-carboxylic Acid 6-Carboxamides. *Bioorg. Med. Chem. Lett.* **1996**, *24*, 2931−2936.

(14) Kim, C. V.; Lew, W.; Williams, M. A.; Liu, H.; Zhang, L.; Swaminathan, S.; Bischofberger, N.; Chen, M. S.; Mendel, D. B.; Tai, C. Y.; Laver, W. G.; Stevens, R. C. Influenza Neuraminidase Inhibitors Possessing a Novel Hydrophobic Interaction in the Enzyme Active Site: Design, Synthesis, and Structural Analysis of Carbocyclic Sialic Acid Analogues with Potent Anti-Influenza Activity. *J. Am. Chem. Soc.* **1997**, *119*, 681−690.

(15) Jedrzejas, M. J.; Singh, S.; Brouillette, W. J.; Laver, G. W.; Air, G. M.; Luo, M. Structures of Aromatic Inhibitors of Influenza Virus Neuraminidase. *Biochemistry* **1995**, *34*, 3144−3151.

(16) Singh, S.; Jedrzejas, M. J.; Air, G. M.; Luo, M.; Laver, W. G.; Brouillette, W. J. Structure-Based Inhibitors of Influenza Virus Sialidase. A Benzoic Acid Lead with Novel Interaction. *J. Med. Chem.* **1995**, *38*, 3217−3225.

(17) Sudbeck, E. A.; Jedrzejas, M. J.; Singh, S.; Brouillette, W. J.; Air, G. M.; Laver, W. G.; Babu, Y. S.; Bantia, S.; Chand, P.; Chu, N.; Montgomery, J. A.; Walsh, D. A.; Luo, M. Guanidinobenzoic Acid Inhibitors of Influenza Virus Neuraminidase. *J. Mol. Biol.* **1997**, *267*, 584−594.

(18) Williams, M.; Bischofberger, N.; Swaminathan, S.; Kim, C. U. Synthesis And Influenza Neuraminidase Activity of Aromatic Analogues of Sialic Acid. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2251−2254.

(19) Kim, K. S.; Qian, L. Improved Method for the Preparation of Guanidines. *Tetrahedron Lett.* **1993**, *34*, 7677−7680.

(20) Chatterjee, B. G.; Rao, V. V.; Majumdar, B. N. G. Synthesis of Substituted β- and γ-Lactams. *J. Org. Chem.* **1965**, *30*, 4101−4104.

(21) Barney, C. L.; Huber, E. W.; Macarthy, J. R. A Convenient Synthesis of Hindered Amines And α-Trifluoromethyamines from Ketones. *Tetrahedron Lett.* **1990**, *31*, 5547−5550.

(22) von Itzstein, L. M.; Wu, W.-Y.; Phen, T. V.; Betty, J. Antiviral 4-Substituted-2-deoxy-2,3-didehydro Derivatives of α-D-Neuraminic Acid. EP Application 92309684.6, 1993.

(23) Laver, W. G.; Coleman, P. M.; Webster, R. G.; Hinshaw, V. S.; Air, G. M. Influenza Virus Neuraminidase with Hemagglutinin Activity. *Virology* **1984**, *137*, 314−323.

(24) Smith, P. W.; Sollis, S. L.; Howes, P. D.; Starkey, I. D.; Cobley, K. N.; Weston, H.; Scicinski, J.; Merritt, A.; Whittington, W. P.; Taylor, N.; Green, D.; Bethell, R.; Madar, S.; Fenton, R. J.; Morley, P. J.; Pateman, T.; Beresford, A. Dihydropyrancarboxamides Related to Zanamavir: A New Series of Inhibitors of Influenza Virus Sialidases. 1. Discovery, Synthesis, Biological Activity, and Structure−Activity Relationships of 4-Guanidino- and 4-Amino-4*H*-pyran-6-carboxamides. *J. Med. Chem.* **1998**, *41*, 787−797.

(25) Taylor, N. R.; Cleasby, A.; Singh, O.; Skarzynski, T.; Wonacott, A. J.; Smith, P. A.; Sollis, S. L.; Howes, P. C.; Bethell, R.; Colaman, P.; Varghese, J. Dihydropyrancarboxamides Related to Zanamavir: A New Series of Inhibitors of Influenza Virus Sialidases. 2. Crystallographic and Molecular Modeling Study of Complexes of 4-Amino-4*H*-pyran-6-carboxamides and Sialidase from Influenza Virus Types A and B. *J. Med. Chem.* **1998**, *41*, 798−807.

(26) Kim, C. U.; Lew, W.; Williams, M. A.; Wu, H.; Zhang, L.; Chem, X.; Excarpe, P. A.; Mendel, D. B.; Laver, W. G.; Stevens, R. C. Structure−Activity Relationship Studies of Novel Carbocyclic Influenza Neuraminidase Inhibitors. *J. Med. Chem.* **1998**, *41*, 2451−2460.

(27) Brouillette, W. J.; Atigadda, V. R.; Luo, M.; Air, G. M.; Babu, Y. S.; Bantia, S. Design of Benzoic Acid Inhibitors of Influenza Neuraminidase Containing a Cyclic Substitution for the *N*-Acetyl Grouping. *Bioorg. Med. Chem. Lett.* **1999**, in press.

JM980707K