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DESIGN OF BENZOIC ACID INHIBITORS OF INFLUENZA NEURAMINIDASE CONTAINING A CYCLIC SUBSTITUTION FOR THE *N*-ACETYL GROUPING

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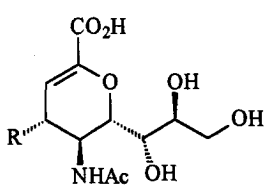
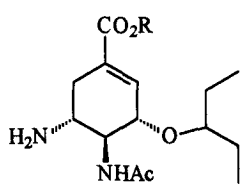
Abstract: A 2-pyrrolidinone ring containing a single hydroxymethyl side chain effectively replaces the *N*-acetylamino group of 4-(*N*-acetylamino)-3-guanidinobenzoic acid, a low micromolar inhibitor of influenza neuraminidase. This novel structural template affords new opportunities to evolve more potent benzoic acid inhibitors.

Influenza virus contains two surface glycoproteins that are essential to viral replication and infectivity. The hemagglutinin recognizes sialic acid conjugates on the surface of host cell membranes, and through binding these residues anchors the virus to the host cell surface. The neuraminidase (NA; also called sialidase) is a hydrolytic enzyme that cleaves sialic acid from conjugates on the host cell at the end of the viral replication cycle to facilitate spread of progeny virions.[1–3]

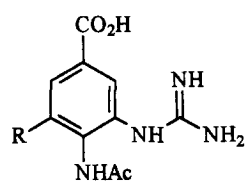
One of the earliest reported inhibitors of neuraminidase, which exhibits only moderate potency ($IC_{50} \approx$ 10 μM), is 2,3-didehydro-2,4-dideoxy-*N*-acetylneuraminic acid (Neu5Ac2en, **1**).[4,5] Recently, a simple analog of Neu5Ac2en, the 4-guanidino derivative (4-Guan-Neu5Ac2en, **2**, GG167, zanamavir), was reported to be an extremely potent inhibitor ($IC_{50} \approx 5$ nM) of both influenza A and B neuraminidase.[6] Zanamavir, although orally inactive and rapidly excreted from the body, has recently undergone human clinical trials which suggest efficacy for the prophylaxis and therapy of influenza infections when administered topically to the respiratory



1 R = OH
2 R = NHC(=NH)NH2



3 R = H
4 R = Et



5 R = H
6 R = NHC(=NH)NH2

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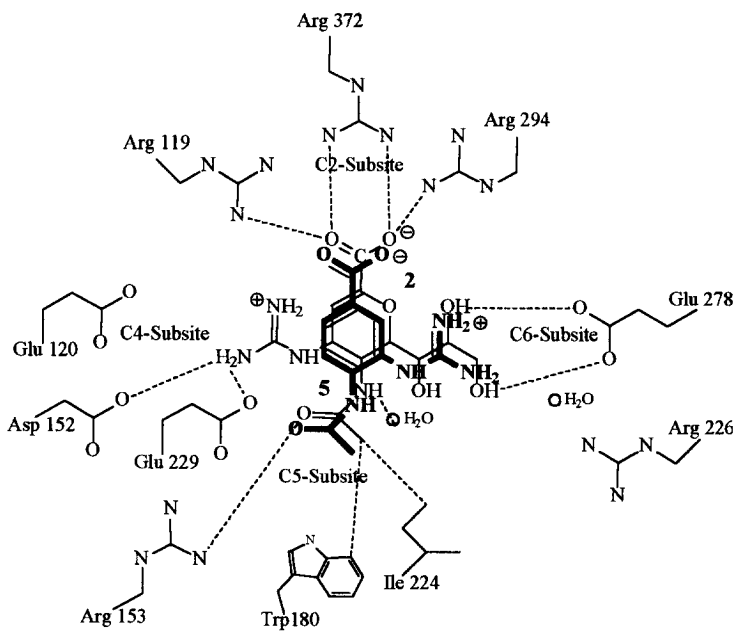
tract.[7] A second neuraminidase inhibitor based on a cyclohexene template (GS 4071, **3**) is also a potent inhibitor ($IC_{50} \approx 1$ nM), and its ethyl ester derivative (GS 4104, **4**) acts as a prodrug which exhibits high oral bioavailability.[8] GS 4104 is presently in phase III clinical trials.

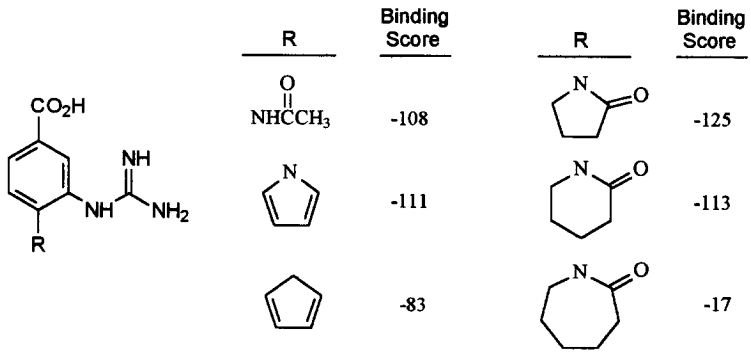
Compounds **2** (5 chiral centers) and **4** (3 chiral centers) are both stereochemically complex and require relatively complex synthetic preparations. Furthermore, **2** has negligible oral bioavailability and a short plasma half-life. We originally pursued the benzoic acid template for designing neuraminidase inhibitors in an effort to circumvent some of these problems.[9,10] Our early studies resulted in the synthesis of **5**, a simple achiral benzoic acid that inhibited influenza A and B neuraminidase ($IC_{50} \approx 10$ μM) as effectively as the much more complex sialic acid analog **1** (Neu5Ac2en).[10] Additionally, structural studies of the complex between **5** and neuraminidase revealed that the guanidino grouping oriented in a unique position in the binding site relative to **2**. The orientation of **2** (normal bonds) and **5** (bold bonds) in the influenza neuraminidase binding site are illustrated, along with selected conserved amino acid residues and contacts to inhibitor, in the cartoon below. These results suggested that **5** offered a novel lead from which to develop much more effective benzoic acid inhibitors.



Thus considerable effort was expended developing analogs of **5** that would exhibit enhanced inhibition. Unfortunately, while a number of compounds were, like **5**, low μM inhibitors, none of these exhibited significant enhancement in potency. Typical of these results is that for compound **6**, the bis-guanidino analog of **5**.[11] Since the guanidino grouping present in **2**, which interacts in the C4-subsite of neuraminidase, resulted

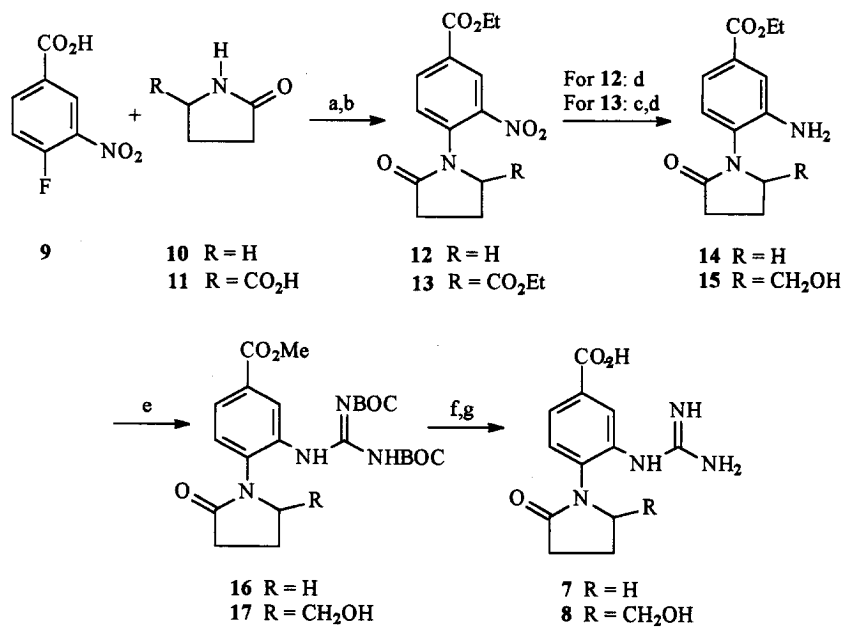
in a >1,000-fold enhancement in potency as compared to **1**, it was postulated that placing a second guanidino grouping on **5**, which would force occupancy of the C4-subsite, would generate a significantly better inhibitor. However, the inhibitory activity (IC_{50} = 250 μM) for **6** was worse than that for **5**, even though **6** oriented as expected in the neuraminidase binding site. The decreased activity of **6** was speculated to result from extra desolvation energy costs associated with bringing a triply charged ligand into the binding site and/or the requirement that the new guanidino grouping exist in a high energy conformation. This and other results led us to realize that the benzoic acid ring suffered limitations as a template due to the requirement that all substituents directly attached to the ring exist in a coplanar arrangement. While forming extra interactions between **5** and the protein was obviously essential to increasing activity, doing so from the benzene ring appeared too restrictive.

All of the effective neuraminidase inhibitors reported to date have consistently maintained two common substituents, the carboxylate that interacts in the C2 subsite and the *N*-acetyl grouping that interacts in the C5 subsite, as seen for compounds **1**–**6**. The contribution to optimum binding made by the *N*-acetyl grouping is illustrated by the fact that the analog of zanamavir (**2**, IC_{50} = 4 nM for type B NA) in which the *N*-acetyl grouping has been removed is essentially inactive (IC_{50} > 400 μM for type B NA).[12] While aware of the importance of the C5 subsite interactions, we reasoned that, for the benzoic acids, an alternative way to introduce new side chains that extend into the C4, C5, or C6 subsite would be to find a suitable replacement for the *N*-acetyl grouping, from which one or more side chains could be constructed. However, it was recognized that the C5 subsite, into which the *N*-acetyl grouping extends, is quite small. We therefore postulated that a small cyclic substituent might be a suitable replacement that would allow for incorporation of spatially oriented side chains that could interact in the C4, C5, or C6 subsites. In an effort to predict suitable candidates, we employed the software LeapFrog (Tripos Associates, Inc.), which is a *de novo* design program that utilizes molecular mechanics (the Tripos force field) to estimate relative binding scores for ligand protein interactions. Briefly, the crystal structure of the binding site of N9 (type A) neuraminidase was displayed as a complex containing **5**. All amino acid residues greater than 5 Å from any part of the ligand were deleted, as were the ligand and all water molecules. Hydrogens (using the BIO ADDH command) and partial charges (using the BIO LOAD CHARGE command) were added to the resulting binding site cavity. A box was built around the binding site fragment and a site point model was automatically generated. Site points not in the active site were manually deleted. The orientation of compound **5**, whose crystal structure coordinates relative to the present binding site model were available, was used as a template to construct candidate analogs containing *N*-acetyl substitutions. All candidates underwent energy minimization (Tripos force field) and atomic charges were calculated (AM1). The final compounds in the proper orientation were saved in one database, individually imported into the LeapFrog site point model, and the binding scores calculated (more negative values for more favorable binding). Selected results are illustrated below.



These calculations suggest that five-membered rings are accomodated well by the C5 subsite if the attachment atom of that ring is planar. (Note that attachment of cyclopentadiene at the allylic carbon gave a poor binding score). Six-membered rings also appear to be well accomodated, although seven-membered rings are very unfavorable. As a conservative choice we selected the 2-pyrrolidinone ring as meeting these requirements yet offering opportunities for side chain elaboration.

Scheme 1



9

10 R = H
11 R = CO_2H

12 R = H
13 R = CO_2Et

14 R = H
15 R = CH_2OH

16 R = H
17 R = CH_2OH

7 R = H
8 R = CH_2OH

Reagents: (a) NaH, HMPA; (b) methanol, 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

To validate this approach we proposed the preparation of, first, pyrrolidinone **7** and, subsequently, pyrrolidinone **8**. Their syntheses are summarized in Scheme 1. In this procedure nucleophilic aromatic substitution converted **9** and **10** or **11** to the pyrrolidinone products in 50–60% yields, and these were esterified to form **12** and **13** in 90% yields. Selective reduction of the aliphatic ester in **13** with $NaBH_4$ proceeded in 70% yield, and the nitro groups underwent catalytic hydrogenation in each case to give the amines **14** and **15** in 90% yields. These were each converted to the protected guanidino derivatives in 70% yield. Removal of the BOC groups and saponification gave the final products (80–90% overall).

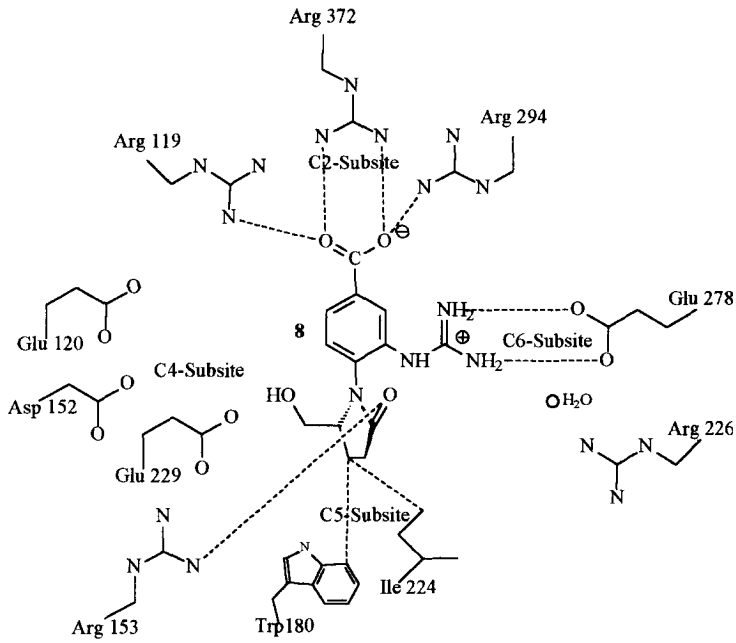
Table 1. In vitro inhibitory effects of benzoic acid analogs on influenza A and B neuraminidases (IC_{50}, μM)[a]

Compound	N9	B/Lee
5	10	10
6	250	–
7	250	1,000[b]
8[c]	20	10

[a]IC_{50} values are the mean of duplicate experiments. In all cases each IC_{50} value differed from its duplicate by less than two-fold. [b]Data for B/Mem/89. [c]Tested as racemic mixture.

Compound **7** was prepared first and was evaluated as an inhibitor of both influenza A and B neuraminidases. As shown in Table 1, compound **7** was considerably less effective than **5**, which is consistent with loss of a hydrogen bond between the NH of the *N*-acetyl grouping in **5** and an ordered water located directly below **5** in the C5 subsite. Using molecular modeling, we then designed a hydrogen bond donating side chain on **7**. Visual inspection in the protein structure suggested that incorporation of a hydroxymethyl substituent onto C5' on the pyrrolidinone of **7** should permit replacement of an ordered water located in the C4-subsite (the same water replaced by the guanidine in **2**). Subsequent enzymatic evaluation of racemic **8**, which added one hydrogen bonding group relative to **7**, revealed that **8** was essentially as effective as **5** (Table 1). As illustrated by the cartoon below, the crystal structure of **8** in complex with N9 NA revealed that only the S enantiomer was bound in the active site. The orientation of the hydroxymethyl side chain in **8** was found to occur as predicted in that the hydroxymethyl group replaced the C4 ordered water and the pyrrolidinone lactam carbonyl was oriented like the original *N*-acetyl grouping. Compound **8** thus represents a novel new lead compound that offers opportunities for construction of additional side chains to interact in the C5 or C6 subsite.

Furthermore, this is the first low μM inhibitor of influenza neuraminidase to contain a substituent that replaces the *N*-acetyl grouping.



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