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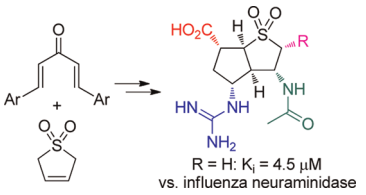
ABSTRACT



Rapid mutation of the influenza virus through genetic mixing raises the prospect of new strains that are both highly transmissible and highly lethal, and which have the ability to evade both immunization strategies (through mutation of hemagglutinin) and current therapies (through mutation of neuraminidase). Inspired by a need for next-generation therapeutics, a synthetic strategy for a new class of rigid, bicyclic inhibitors of influenza neuraminidase is reported.

The potential emergence of a readily transmissible influenza virus with a high mortality rate remains a potent threat to the global population. Since 2003, of the 608 laboratory-confirmed cases, the H5N1 "avian flu" has killed ~60% of individuals infected,[1] whereas the 1918 H1N1 "Spanish flu" had a much lower mortality rate but killed almost 3% of the human population due to its high transmissivity.[2] Recent studies suggest that mutations in the H5N1 genome can result in significantly enhanced infectivity in ferret models of human infection.[3]

The development of two distinct classes of antivirals (M2 proton channel inhibitors block viral unpacking, while neuraminidase inhibitors block the release of viral progeny from host cells) was once thought to protect against future influenza pandemics. However, their use has resulted in significant mutation-induced resistance. For example, M2 inhibitors amantadine and rimantadine are no longer recommended for use due to widespread resistance across nearly all strains of influenza.[4] Similarly, genetic variation in hemagglutinin can allow influenza strains to evade annual vaccination strategies.[5]

Neuraminidase inhibitors oseltamivir and zanamivir (Figure 1C) were once thought to be relatively immune to resistance, with less than 1% of resistant isolates identified prior to 2007.[6,7] However in the 2007–2008 flu season, an H274Y point mutation conferring resistance to oseltamivir was isolated from 12% of H1N1 viruses tested in the United States.[8,9] Early in the 2008–2009 flu season, the

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number of resistant isolates increased to 98.5%.[8,9] Fortunately, the 2009–2010 H1N1 "swine flu" and H5N1 "avian flu" pandemics did not contain the H274Y mutation and remained susceptible to oseltamivir.

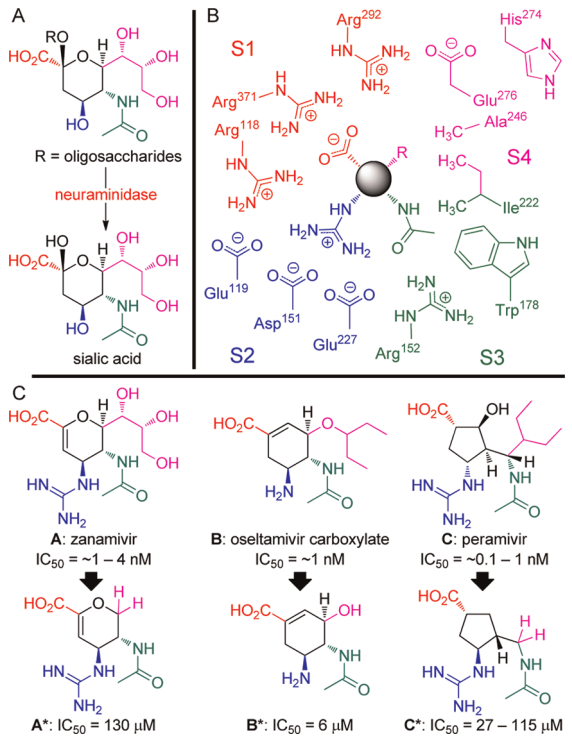


Figure 1. Neuraminidase function and inhibition. (A) Enzymatic function; (B) important interactions for inhibition; (C) structures of the three clinically used inhibitors and comparison with truncated analogs.

Zanamivir is less susceptible to mutation-induced resistance than oseltamivir but is orally inactive due to poor membrane solubility. A third neuraminidase inhibitor, peramivir, can be used as an injectible but is both orally inactive and relatively ineffective against the H274Y mutant.[10] The development of a new class of orally active neuraminidase inhibitors with a low susceptibility for resistance is of critical importance.

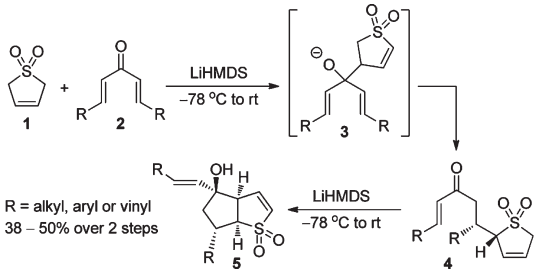
Various cyclic cores have been used for the generation of neuraminidase inhibitors, including aromatic rings,[11] dihydropyrans[12] (which led to the development of zanamivir), cyclohexenes[13] (which led to oseltamivir), cyclopentanes[14] (which led to peramivir), and tetrahydropyrroles.[15] The central scaffold does not make direct contact with the protein, but serves to position dependent functional groups for optimal engagement with four subregions (S1–S4) of the neuraminidase active site (see Figure 1B).

All potent neuraminidase inhibitors have a carboxylate group (or phosphonate)[16] to bind an arginine triad that is broadly conserved across the various subtypes of neuraminidase (the S1 region in Figure 1B), and most also incorporate an amine or guanidine function to interact with several acidic residues in the S2 pocket. The acetamide substituent on the natural substrate constitutes an important recognition element (partly due to a polar interaction between the carbonyl group and arginine-152, but mostly due to interaction of the acetamide methyl group with a lipophilic pocket in the S3 region), and this is likewise preserved in most inhibitors.

Much of the activity for **A**–**C** comes from filling the S4 pocket. For example, removal of the triol side chain from zanamivir (to give **A***, Figure 1C) increased the IC_{50} from 4 nM to 130 μM.[17] Similarly, early analogs of oseltamivir (**B***)[13] and peramivir (**C***)[14,18] lacking the 3-pentyl side chain were 3–6 orders of magnitude less potent.

Unfortunately, the S4 pocket is also responsible for the greatest threat to the clinical utility of oseltamivir and peramivir. The now-widespread mutation of histidine-274 to the larger tyrosine residue prohibits glutamic acid-276 from rotating out of the S4 binding site. As a result, the H274Y mutant maintains a more polar active site, which cannot effectively bind the lipophilic 3-pentyl groups of oseltamivir and peramivir, although the triol side chain of zanamivir can hydrogen bond to Glu276.[19]

Scheme 1. An Efficient Bicyclic Sulfone Synthesis



The search for next-generation inhibitors with an affinity for the H274Y mutant neuraminidase demands the inclusion of more polar groups targeting the S4 pocket (as for zanamivir) while maintaining oral activity (as for

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oseltamivir) and high potency (as for peramivir). This multifaceted goal prompted us to re-examine the structural properties of the enzyme-bound inhibitors. The published structures for neuraminidase-bound peramivir[20] reveal that the cyclopentane ring distorts significantly upon binding, placing the carboxylate and guanidinium functions into substantially equatorial orientations (Figure 2A). We hypothesized[21] that a bicyclic molecule which fixes this ring geometry in place would have a reduced entropy of binding and enhanced target selectivity.

We recently described an efficient synthesis of a family of rigid, orthogonally functionalized bicyclic sulfones (**5**, Scheme 1) from butadiene sulfone (**1**) and simple bisalkylidene ketones **2**. The synthesis proceeds through a tandem vinylogous 1,2-addition/anionic oxy-Cope reaction, followed by a second vinylogous 1,2-addition to afford the bicycle in moderate overall yield.[22] X-ray structural characterization of **5a** (R = CH_3) showed that the all-carbon ring places two substituents in pronounced pseudoequatorial geometries, with angles of projection that are similar to those found in the bound conformation of peramivir (i.e., compare Figure 2B with 2A). Comparison of coupling constants across several synthesized derivatives and examination of a second crystal structure (of a reduced, alkylated derivative of **5a**) confirmed that the bicyclic system was relatively rigid, but still maintained enough flexibility that inhibitors derived from **5** could adapt themselves to an active site.

Intriguingly, computational docking experiments conducted in MOLOC[23] showed that an acetamide group positioned β to the sulfone (installable through conjugate addition to the vinyl sulfone function in **5**) would overlay correctly with the *N*-acetyl group in peramivir (Figure 2C), while substituents installed α to the sulfone would be appropriately situated to probe the neuraminidase S4 site (Figure 2D). Presumably, alkyl substituents at the latter position would interact with the lipophilic S4 region of nonmutant neuraminidase, while more polar substituents might engage with the more polar binding site of the H274Y mutant. Other side chains might open the door to novel inhibitors of bacterial neuraminidases (which are often virulence factors for pathogenic bacteria)[24] or mammalian sialidases (which are recognized as potential anticancer targets).[25] As a first step toward the development of this new class of enzyme inhibitors, we targeted compounds designed to bind either to the S1 and S2 neuraminidase binding pockets (**10**, Scheme 2) or to each of the S1, S2, and S3 pockets (**14** and **15**).

To highlight the degree of orthogonal functionalization in intermediate **5**, we designed our synthetic routes in such a way that, for compound **10**, the S1-probing carboxylate would be attached to C-4 of the bicycle and the S2-probing guanidine would be attached to C-6, while for the regioisomeric analog **15**, the reverse would be true (see Scheme 2 for IUPAC numbering).

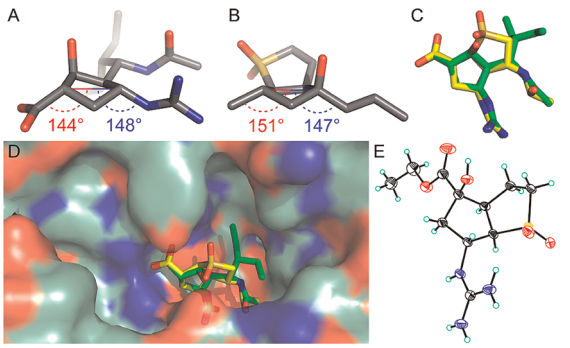

Figure 2. X-ray structural data and in silico docking results. (A) Structure of peramivir bound in the enzyme active site (from PDB 2HTU); (B) structure of **5a** (CCDC 766248); (C) overlay of enzyme-bound peramivir (green) with the docked structure of **15** (yellow; calculated in MOLOC); (D) peramivir and **15** pictured inside the neuraminidase active site; (E) small-molecule X-ray structure of the ethyl ester hydrochloride of **10**.

The synthesis of both targets began with the reaction of **2b** (available from *p*-anisaldehyde) with butadiene sulfone (**1**) to afford **5b**. In pursuit of the simpler target **10**, we first reduced the vinyl sulfone with Red-Al and then cleaved the exocyclic olefin under ozonolysis conditions. Pinnick oxidation provided α-hydroxy acid **7**. After conversion of the C-4 carboxylic acid to an ester, the aromatic ring was cleaved oxidatively and the resulting C-6 carboxylate was subjected to a Curtius rearrangement to provide **8**. Finally, the nitrogen protecting group (a legacy of the Curtius step) was removed, and the amine was subjected to guanidinylation conditions. The protecting groups on the guanidine were removed, and the ethyl ester was saponified to provide the first target compound, **10**, as a single diastereomer. An X-ray structure of the ethyl ester of **10** (as the hydrochloride salt) confirmed the peramivir-like conformation of the all-carbon ring (Figure 2E).

Although **10** does not contain a group capable of filling the lipophilic S3 binding pocket (which has evolved to recognize the methyl group of the natural substrate's *N*-acetyl function), we had hoped that one of the sulfone oxygens would bind to the arginine-152 residue at the top of this pocket. However, neuraminidase inhibition assays against an inactivated influenza virus showed that **10** had a K_i barely into the micromolar range, consistent with a

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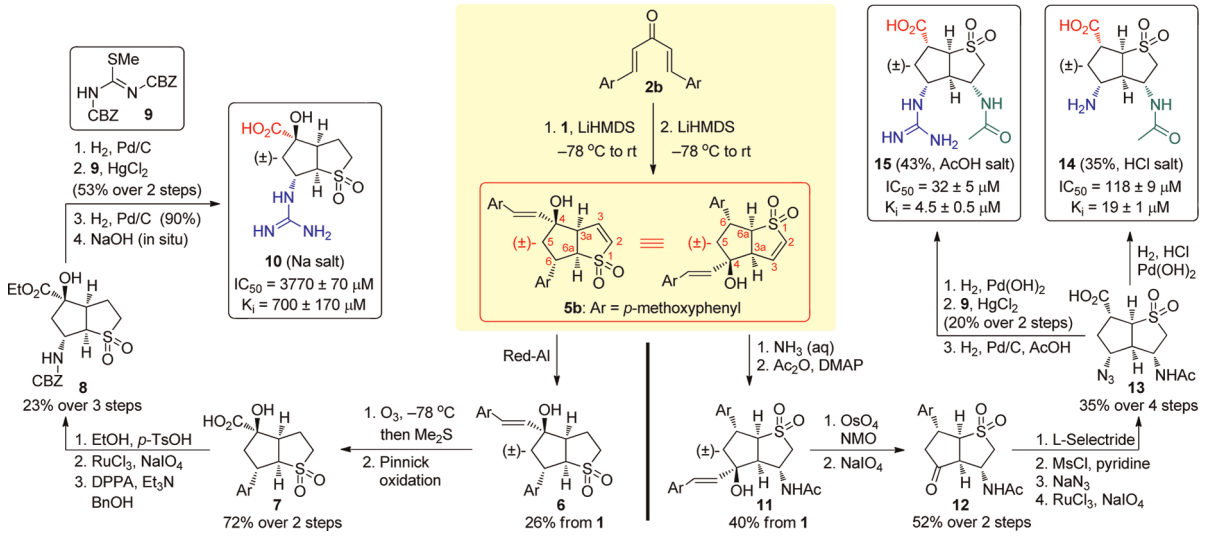
Scheme 2. Regiochemical Switch Employed in the Synthesis of Bicyclic Neuraminidase Inhibitors



compound that engages with only two of the four subregions of the active site.[26] Nonetheless, full kinetic characterization revealed **10** to be a competitive inhibitor of the enzyme (Figure 3A), suggesting that the compound was accessing the active site as designed.

The synthesis of **14** and **15** was more challenging, in that the allylic alcohol function in **5** (which lent itself naturally to the α-hydroxy acid in **10**) needed to be converted to an amine. Thus, after conjugate addition and acetylation to provide acetamide **11**, the alkene was dihydroxylated and the resulting triol was cleaved with $NaIO_4$ to generate ketone **12**. The ketone was selectively reduced with L-selectride and the resulting alcohol was mesylated and displaced (with inversion of configuration) by the azide anion. The aromatic ring was again removed oxidatively (without affecting the azide group) to give **13**, and the azide was reduced to provide **14**. Guanidinylation to afford **15** was accomplished without esterification.

Gratifyingly, compounds **14** and **15** were both substantially more potent than **10**: the amine displayed a K_i of 19 μM against the inactivated virus, while the guanidine had a K_i of 4.5 μM. Both compounds were determined to be competitive inhibitors of neuraminidase (Figure 3B and 3C). The increase in activity with the installation of the guanidine function provides additional evidence that the bicyclic compounds described here are binding in the expected geometry within the active site.[26]

Strictly speaking, it is wrong to compare IC_{50} values from different experiments. However, given that the measured activity for racemic **15** is up to 3-fold lower than the activities reported for the side chain deleted analogues of zanamivir and peramivir (**A*** and **C***), it appears likely that the rigidified bicyclic scaffold is providing some benefit. We are currently working to install functionality at the C-2 position, with the aim of developing potent and selective inhibitors of a variety of viral, bacterial, and mammalian neuraminidase targets. Results of these studies will be reported in due course.

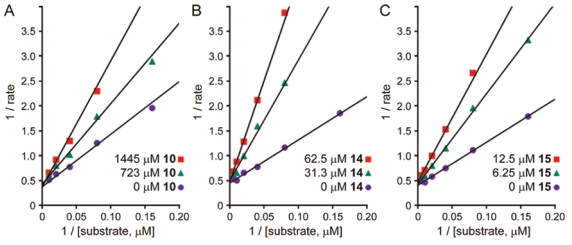


Figure 3. Lineweaver–Burk[27] plots confirming competitive neuraminidase inhibition for all three tested compouds in NP40-inactivated influenza A/Brisbane/59/2007(H1N1) virus.

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Supporting Information Available. Experimental details and spectral data for all new compounds, enzyme assay protocols, and crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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