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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

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Abstract: The design, synthesis, and in vitro evaluation of the novel carbocycles as transition-state-based inhibitors of influenza neuraminidase (NA) are described. The double bond position in the carbocyclic analogues plays an important role in NA inhibition as demonstrated by the antiviral activity of **8** (IC_{50} = 6.3 μM) vs **9** (IC_{50} > 200 μM). Structure−activity studies of a series of carbocyclic analogues **6a−i** identified the 3-pentyloxy moiety as an apparent optimal group at the C_3 position with an IC_{50} value of 1 nM for NA inhibition. The X-ray crystallographic structure of **6h** bound to NA revealed the presence of a large hydrophobic pocket in the region corresponding to the glycerol subsite of sialic acid. The high antiviral potency observed for **6h** appears to be attributed to a highly favorable hydrophobic interaction in this pocket. The practical synthesis of **6** starting from (−)-quinic acid is also described.

Introduction

Despite considerable progress in elucidating the molecular mechanism and cellular biology of influenza virus, influenza infection continues to be the most serious respiratory disease both in terms of morbidity and mortality.[1] Current available treatments have severe limitations. Amantidine and its analogue rimantidine are the only compounds licensed for the treatment and prophylaxis of influenza A infection. However, these compounds are not effective against influenza B viruses, and their clinical use has been limited by side effects and the rapid emergence of resistant viral strains.[2] Vaccine development has been partially successful in the control of influenza epidemics due to the highly variable mutation of influenza virus.[3]

The unique replication mechanism of influenza virus has allowed investigators to identify a number of potential molecular targets for drug design. Those targets include haemagglutinin,[4] neuraminidase,[5] M_2 protein,[6] and endonuclease.[7] Haemagglutinin (HA) and neuraminidase (NA) are two major surface glycoproteins expressed by both influenza A and B viruses. HA is known to mediate binding of viruses to target cells via terminal sialic acid residue in glycoconjugates. This binding is the first step of viral infection. In contrast to HA activity, NA catalyzes removal of terminal sialic acids linked to glycoproteins and glycolipids. Although biological consequences of this activity are not completely understood, it has been postulated that NA activity is necessary in the elution of newly formed viruses from infected cells by digesting sialic acids in the HA receptor.[8,9] NA may also promote viral movement through respiratory tract mucus, thus enhancing viral infectivity.[9,10] Therefore, NA has been considered to be a suitable target for designing agents against influenza viruses.

In earlier studies, 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en, **1**) was found to be an influenza NA inhibitor with a K_i of 4 μM.[11] Biochemical studies[12] indicate **1** is considered a transition state-like analogue binding to the active site of NA.[13,14] Recently, on the basis of structural information generated from the X-ray crystallographic study of **1** complexed with NA, the following rationally designed NA inhibitors were prepared: 2,3-dihydro-2,4-dideoxy-4-amino-*N*-acetylneuraminic acid (4-amino-Neu5Ac2en, **2**) and its guanidino analogue (4-

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guanidino-Neu5Ac2en, **3**).[13,17,18] In comparison to **1**, both **2** and **3** are more potent NA inhibitors with K_i values of 10^{-8} M and 10^{-10} M, respectively. Both the amino group in **2** and the guanidino group in **3** are suggested to form salt bridges with Glu119 in the NA active site, while the latter adds a strong charge−charge interaction with Glu227.[18] In addition, **3** also exhibited potent antiviral activity against a variety of influenza A and B strains in the cell culture assay.[19] Compound **3** is currently being evaluated in human clinical trials and has shown efficacy in phase II challenge studies in both prophylaxis and treatment of influenza virus infections.[21,22] However, poor oral bioavailability and rapid excretion precluded **3** as a potential oral agent against influenza infection and **3** has to be administered by either intranasal or inhaled routes in clinical trials.[20]
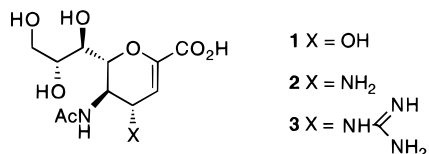
In the case of an influenza epidemic, oral administration may be a more convenient and economical method for treatment and prophylaxis. Therefore, it would be desirable to have a new class of orally active NA inhibitors as potential agents against influenza infection. Our first move toward this objective involved the design of a new class of compounds by using a carbocyclic template in place of the dihydropyran ring of the Neu5Ac2en system. It is expected that the carbocyclic ring would be chemically more stable than the dihydropyran ring and easier to modify for optimization of antiviral and pharmacological properties.

The validity of this approach was verified by the discovery of very potent NA inhibitors in this new carbocyclic series. New lipophilic side chains at the C_3 position of the carbocyclic system imparted potent NA inhibitory activity. X-ray crystallographic analysis of the carbocyclic analogue bound to NA confirmed that there was in fact hydrophobic space in the glycerol-binding subsite to accommodate bulky lipophilic groups. The discovery of this hydrophobic pocket in the active site of NA was exploited to increase the lipophilicity of inhibitors to optimize pharmacologic properties for potential oral bioavailability while maintaining potent antiviral activity.

This work constitutes an example of rational drug design based on information available from the crystal structure of inhibitors complexed with the enzyme in the active site and highlights the great importance of the hydrophobic interaction for the high-affinity binding of inhibitors toward the enzyme.

Design

NA has been classified into nine subtypes for type A influenza virus strains according to their serological properties. However, there are no subtypes in the type B virus. Although amino acid sequence homology among NA from both type A and type B virus strains has been found to be only 30%,[23] enzyme activity of NA among the different strains is the same, indicating the highly conserved nature of the active site of the enzyme.[24] The X-ray crystallographic structures of NA have been determined from three influenza subtypes: A/Tokyo,[25] A/Tern,[26] and B/Beijing.[27] The structures displayed a symmetrical folding pattern of six four-stranded antiparallel β-sheets arranged like blades of a propeller. NA exists as a mushroom-shaped spike with a boxlike head on top of a long stalk containing a hydrophobic region by which it is embedded in the viral membrane.

Crystallographic studies of NA reveal that, in the active site, the amino acids which line and surround the walls of the binding pocket are highly conserved among all influenza strains examined so far. The high-resolution crystallographic structure of sialic acid (**4a**) complexed with NA revealed that sialic acid binds the enzyme in a considerably deformed conformation due to the strong ionic interactions between the carboxylate of the substrate and Arg118, -292, and -371 in the active site of the enzyme.[28] In solution the carboxylate of sialic acid is axial, but the deformation of the ring on binding put the carboxylate into a pseudoequatorial position. This binding mode is very similar to that found in the X-ray crystal structure of Neu5Ac2en (**1**) complexed with NA.[26] In this case, the double bond of Neu5Ac2en constrains the pyranose ring of the sugar into a planar structure around the ring oxygen. On the basis of this structural information, it has been proposed that the catalytic mechanism for the cleavage of sialic acid from glycoconjugates (**4b**) implicates the formation of the C_2 carbonium cation **5** which is stabilized by the neighboring oxygen atom as shown in Scheme 1.[13,14] Furthermore, kinetic isotope studies have provided convincing evidence for the C_2 carbonium cation formation as an intermediate structurally similar to the transition state in the sialic acid cleavage by NA.[12]

Transition-state mimics frequently are potent inhibitors for the catalyzing enzyme. The concept of structural similarity to the transition state has found wide application in drug design over the years. The multitude of enzyme−inhibitor interactions are governed by steric as well as electronic factors. In theory, compounds that closely resemble the transition-state structure should give high binding affinity toward the target enzyme.[29] Using intermediate **5** as a key transition-state mimic is a reasonable approximation in view of the X-ray crystallographic studies described above. Considering the flat oxonium cation in **5** as an isostere of the double bond, the cyclohexene scaffold was selected as a replacement for the oxonium ring of **5** which would keep the conformational changes to a minimum (Scheme 1). In addition, the carbocyclic system was expected to be chemically versatile for the manipulation of side chains attached to the ring. Earlier attempts by us and others to mimic the intermediate **5** with the completely flat benzene ring did not lead to potent NA inhibitors,[30] suggesting the importance of

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**Scheme 1.** Rational Design of Carbocyclic Transition-State Analogues
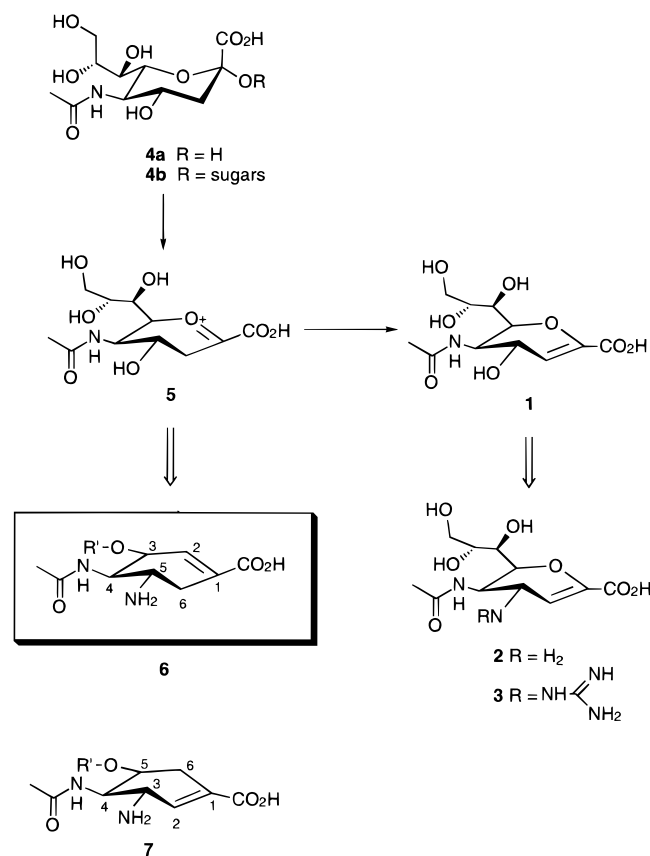
the stereochemistry of substituents around the ring in the design of inhibitors with high affinity for NA.

Importance of the Double Bond Position

In the initial stages of designing carbocyclic NA inhibitors, the olefinic isomers as shown in structures **6** and **7** were considered as two possible transition-state analogues. The isomer **6** is structurally closer to transition-state **5** than isomer **7**. However, it was difficult to assess a priori which isomer would be a better NA inhibitor, especially in light of the potent NA inhibitory activity displayed by Neu5Ac2en (**1**) and its basic analogues **2** and **3**, in which the double bond was located in the position corresponding to isomer **7**. Recently, carbocyclic analogues of the Neu5Ac2en system have been reported to have some interesting NA inhibitory activity.[31] The molecular modeling of isomers **6** and **7** indicated that these two molecules overlay well. Therefore, in order for us to assess the choice between isomers **6** and **7**, we felt that the synthesis of both isomers was necessary in order to compare NA activity before embarking on extensive structure-activity relationship studies. For this comparative study, simple isomers **8** and **9** were chosen.
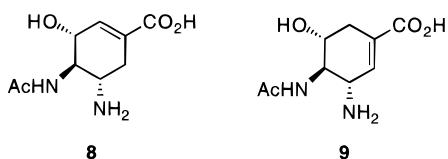
Synthesis and NA Activity of 8 and 9

A comparison of structural similarities revealed that (−)-shikimic acid (**11**) and **8** share common structural features. However, the conversion of **11** to **8** requires the effective stereochemical control for transforming the trans C_4 and C_5

Scheme 2[a]
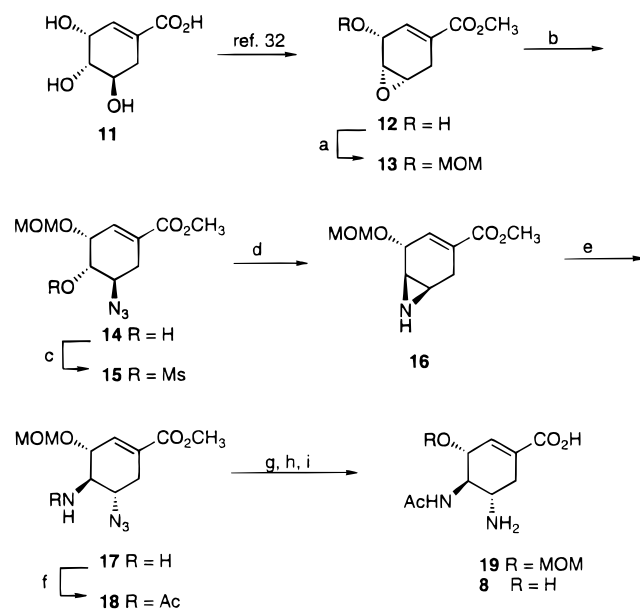
[a] Reagents: (a) MeOCH₂Cl, DIPEA, CH₂Cl₂; (b) NaN₃, NH₄Cl, MeOH/H₂O; (c) MeSO₂Cl, Et₃N, CH₂Cl₂; (d) Ph₃P, THF then Et₃N/H₂O; (e) NaN₃, NH₄Cl, DMF; (f) AcCl, pyridine; (g) H₂, Lindlar catalyst, EtOH; (h) KOH, THF/H₂O; (i) CF₃CO₂H, CH₂Cl₂.

hydroxyls of **11** to the trans C_4 and C_5 amino groups of **8**. The approach selected for this transformation, as outlined in Scheme 2, relied on the conversion of **11** to aziridine **16** followed by azide ion attack in a regio- and stereospecific manner as a key reaction. The synthesis began with the preparation of epoxide **12** from **11** as described in the literature.[32] Nucleophilic ring opening of MOM-protected epoxide **13** with sodium azide in the presence of ammonium chloride generated azido alcohol **14** in 86% yield. The ring opening of the epoxide was both regio- and stereospecific as depicted in **14**, and this could be attributed to the steric and electronegative inductive influence of the MOM group in **13**. Conversion of azide **14** to aziridine **16** was efficiently accomplished in 78% yield via a two-step sequence: (1) mesylation of the hydroxyl group in **14** and (2) reduction of the azide functionality in **15** with triphenylphosphine in the presence of triethylamine and water. The aziridine ring opening of **16** with sodium azide gave **17** exclusively. This selective ring opening was again a consequence of the favored azide ion attack at the C_5 position due to the steric and electronegative inductive effects of the MOM group. Finally, acetylation of **17** followed by reduction of the azide group in **18** and saponification of the methyl ester and deprotection of the MOM group in **19** with trifluoroacetic acid provided **8**.

Our initial attempts to utilize shikimic acid (**11**) for the synthesis of **9** proved to be rather difficult. Alternatively, (−)-quinic acid (**21**) was chosen as a starting material (Scheme 3). The cyclohexene intermediate **22**, readily available from **21** by literature methods,[33] possesses considerable structural similarity to **9**. The C_5 hydroxyl of **22** was protected as the pivaloyl ester, and mild acid hydrolysis of **23** furnished diol **24**. Exposure of **24** to thionyl chloride in the presence of triethylamine generated

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Scheme 3[a]
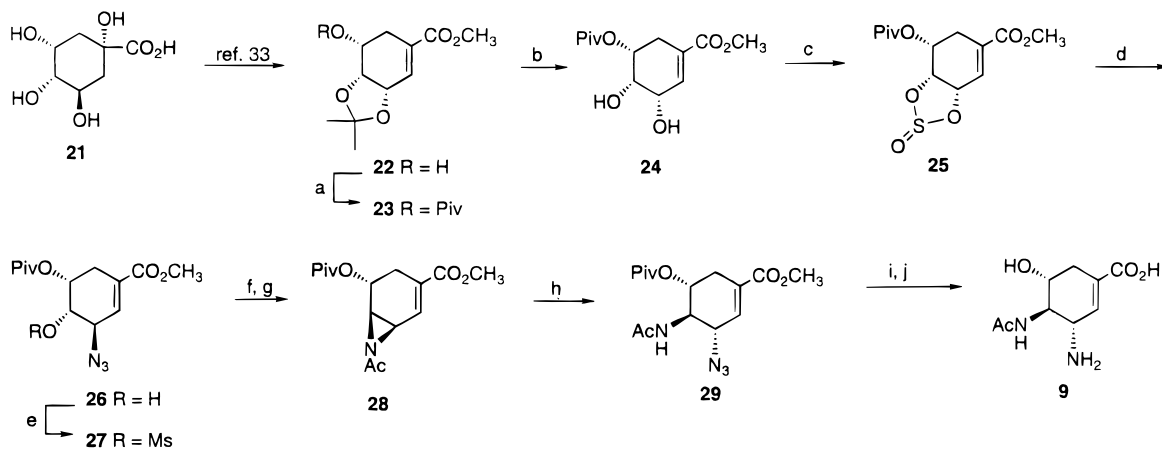
[a] Reagents: (a) $(CH_3)_3CCOCl$, pyridine; (b) CH_3CO_2H, H_2O; (c) $SOCl_2$, pyridine, CH_2Cl_2; (d) NaN_3, DMF; (e) CH_3SO_2Cl, Et_3N, CH_2Cl_2; (f) Ph_3P, THF then Et_3N/H_2O; (g) CH_3COCl, pyridine, CH_2Cl_2; (h) NaN_3, NH_4Cl, DMF; (i) KOH, CH_3OH, H_2O; (j) H_2, Lindlar catalyst.

cyclic sulfite **25** as a stable intermediate in 90% yield. The sulfite ring opening of **25** with sodium azide gave azide **26** as a single product. This complete regiospecific ring opening is a consequence of favored azide ion attack at the allylic C_3 position of **25**. The β-hydroxy azide moiety in **26** was then converted in a three-step sequence to aziridine **28**, analogous to the conversion of **14** to **16**. As expected, ring opening of acetylated aziridine **28** with sodium azide was completely regiospecific, giving azide **29** as the only product. Saponification of **29** followed by reduction of the azide functionality provided **9**.

The inhibitory activity of **8** and **9** was directly determined in a NA enzymatic assay. While **8** proved to be a potent NA inhibitor with an inhibitory concentration (IC_{50}) of 6.3 μM, **9** did not exhibit inhibitory activity at concentrations up to 200 μM. This result demonstrated that the double bond position in the design of carbocyclic NA inhibitors plays an important role in NA activity. However, further structural investigation is required in order to illustrate the binding difference(s) of **8** and **9** in the NA active site.

Replacement of the Glycerol Moiety with Lipophilic Side Chains

Crystallographic studies of Neu5Ac and its analogues bound to NA appear to indicate that the C_7 hydroxyl of the glycerol side chain does not interact with any amino acids of the NA active site.[5b] This suggested that the C_7 hydroxyl could be eliminated from the carbocyclic analogues without losing binding affinity to NA. Furthermore, it was also realized that, in the transition-state intermediate **5**, the oxonium double bond is highly polarized and electron deficient. Taking into account these features, in the carbocyclic structure, the CHOH group at the C_7 position of the glycerol side chain in the Neu5Ac system was replaced with the oxygen atom as shown in structure **6**. This C_3 oxygen atom would reduce the electron density of the double bond via the σ bond electronegative inductive effect. In addition to these rationales, our decision to have the C_3 oxygen atom was based on the synthetic practicality of modifying R′ groups to optimize NA inhibitory activity and pharmacological properties.
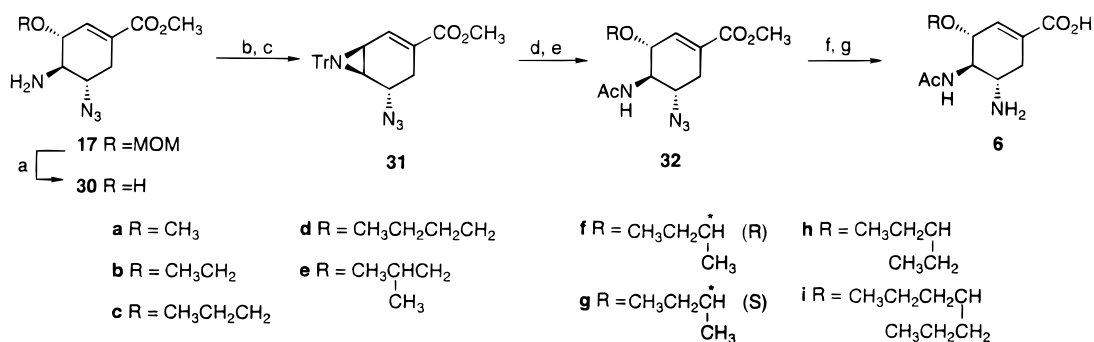
X-ray crystallographic structures of Neu5Ac and its analogues complexed with NA show that the two terminal hydroxyls of the glycerol side chain form a bidentate interaction with Glu276.[25,27] However, it is also noted that the C_8 of the glycerol chain makes hydrophobic contacts with the hydrocarbon chain of Arg224.[5b] Therefore, we hoped that the optimization of this

hydrophobic interaction would lead to new NA inhibitors with increased lipophilicity while maintaining potent NA inhibitory activity. This consideration is especially important for designing orally bioavailable drugs since balancing lipophilicity and water solubility could be as critical as the size of the molecule for its absorption from the intestinal tract. For optimization of hydrophobic interactions, the dimensions of the spacer would be expected to play an important role in binding affinity to the enzyme, as factors such as length, geometry, and conformational mobility. On the basis of this premise, we undertook a systematic modification of the R′ portion in **6** with various aliphatic side chains. Previous studies[5b] suggested that the carboxylate of Neu5Ac2en (**1**) and its analogues form strong ionic interactions with three guanidino groups of Arg118, -292, and -371. The acetamido moiety at the C_5 of Neu5Ac2en and its analogues interacts with Arg152 and Glu227, and the methyl group fits nicely into a hydrophobic pocket formed by Trp180, Ile224, and Arg226. The acetamido group was also demonstrated to be optimal for antiviral activity.[34] The basic amino and guanidino groups at the C_4 position of the Neu5Ac2en system were optimal for NA activity as described above.[11b,18] Therefore, in the carbocyclic structure **6**, the C_1 carboxylate, C_4 acetamido, and C_5 amino groups were kept constant, while the C_3 aliphatic group was optimized for antiviral activity.

Synthesis of Lipophilic Analogues 6

For structure−activity relationship studies of carbocyclic analogues **6**, a general and efficient route to introduce various alkyl ethers at the C_3 position was required. The approach we selected, as outlined in Scheme 4, relied on the aziridine opening of **31** with alcohols, which should in principle be highly regioselective due to the preferred nucleophilic attack at the C_3 allylic position. The requisite aziridine **31** was derived from the trans amino alcohol **30** by the two-step, one-pot process: (1) selective protection of the amino functionality with trityl chloride and (2) mesylation of the hydroxyl in the presence of triethylamine. Under these conditions, the mesylate intermediate was converted to aziridine **31** in 86% overall yield. Of a number of protecting groups tried on amine **30**, best results were obtained by using the trityl group. Treatment of **31** with various alcohols in the presence of 1.5 equiv of $BF_3 \cdot Et_2O$ followed by acetylation of the crude product provided the ethers **32** in 55−80% yield. As expected, no other regio- and stereoisomers were

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Scheme 4[a]

17 R =MOM	
30 R =H	

a R = CH$_3$	**d** R = CH$_3$CH$_2$CH$_2$CH$_2$
b R = CH$_3$CH$_2$	**e** R = CH$_3$CHCH$_2$ CH$_3$
c R = CH$_3$CH$_2$CH$_2$	

f R = CH$_3$CH$_2$CH CH$_3$ (R)	**h** R = CH$_3$CH$_2$CH CH$_3$CH$_2$
g R = CH$_3$CH$_2$CH CH$_3$ (S)	**i** R = CH$_3$CH$_2$CH$_2$CH CH$_3$CH$_2$CH$_2$

[a] Reagents: (a) CH$_3$OH, HCl; (b) TrCl, Et$_3$N, CH$_2$Cl$_2$; (c) CH$_3$SO$_2$Cl, Et$_3$N, CH$_2$Cl$_2$; (d) BF$_3$·Et$_2$O; ROH; (e) Ac$_2$O, DMAP, pyridine; (f) Ph$_3$P, THF then Et$_3$N/H$_2$O; (g) KOH, THF, H$_2$O.

Scheme 5[a]

33 R = H	
34 R = Ts	
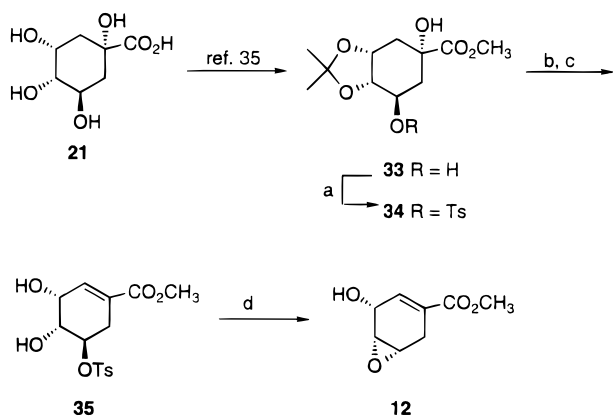
[a] Reagents: (a) TsCl, DMAP, pyridine; (b) SO$_2$Cl$_2$, pyridine; (c) *p*-TsOH, CH$_3$OH; (d) DBU, THF.

observed in this aziridine ring-opening reaction. Finally, reduction of the azide functionality and saponification of the ester group in **32** gave **6**. Thus the convergent and efficient synthesis of various ether analogues **6** has been achieved from intermediate **17** which is readily available from shikimic acid as shown in Scheme 2.

Although shikimic acid as a chiral starting material is convenient because of easy conversion to the key epoxy intermediate **12**, its high cost and low availability in large quantities made it impractical for scale-up of **12**. This became a formidable challenge when multi-kilograms of material were needed for evaluation of clinical candidates for further drug development. (−)-Quinic acid (**21**), which was used as a starting material for the synthesis of **9** (Scheme 3), appeared to be an ideal starting material because of its low cost and commercial abundance. Conversion of quinic acid to the acetonide intermediate **33** (Scheme 5) proceeded in high yield as reported.[35] At this point we focused on the selective dehydration of the C$_2$ hydroxyl in **34**. Although a similar dehydration method has been reported in the literature,[33] it was difficult to reproduce on a large scale in our hands.

Thus, tosylate **34** was reacted with sulfuryl chloride in pyridine, followed by acetonide cleavage in refluxing methanol in the presence of *p*-toluenesulfonic acid. The desired diol **35** was then directly crystallized out of the reaction mixture in 54% overall yield. This procedure was amenable to a several hundred gram scale. In the dehydration and acetonide removal sequence for the conversion of **34** to **35**, the other olefinic regioisomer of **35** was aromatized under the reaction conditions and easily separated by crystallization. Finally, epoxide **12** was produced

(35) Shing, T. K.; Tang, Y. *Tetrahedron* **1990**, *46*, 6575.

Table 1. Influenza Neuraminidase Inhibition and Plaque Reduction by Carbocyclic Analogues

6

| R	compd	enzyme[a] IC$_{50}$ (nM)	plaque[b] EC$_{50}$ (nM)
H	**8**	6300	ND[c]
CH$_3$	**6a**	3700	ND
CH$_3$CH$_2$	**6b**	2000	
CH$_3$CH$_2$CH$_2$	**6c**	180	ND
CH$_3$CH$_2$CH$_2$CH$_2$	**6d**	300	ND
(CH$_3$)$_2$CHCH$_2$	**6e**	200	ND
CH$_3$CH$_2$(CH$_3$)CH*	**6f**	10	80
	(R)-isomer		
	6g	9	135
	(S)-isomer		
(CH$_3$CH$_2$)$_2$CH	**6h**	1	16
(CH$_3$CH$_2$CH$_2$)$_2$CH	**6i**	16	ND
	2	150	2500
	3	1	15

[a] NA. [b] H1N1, A/ws. [c] ND = not determined.

in quantitative yield by treatment of **35** with 1,8-diazabicyclo-[5.4.0]undec-7-ene in tetrahydrofuran. This completed the synthesis of epoxide **12** from quinic acid in good overall yield without column chromatography.

Structure−Activity Relationships of Carbocyclic Analogues 6

Two methods were used to evaluate the carbocyclic analogues as inhibitors of NA (Table 1). The intrinsic activity of each compound was assessed by measuring the inhibition of enzymatic activity. Compounds that exhibited potent NA inhibitory activity were further evaluated in cell culture by a plaque reduction assay using an influenza A (H1N1) strain. As shown in Table 1, the length, size of branching, and geometry of the alkyl groups in **6** profoundly influence the NA inhibitory activity. In a series of linear alkyl analogues (**6a**−**d**), steady increases in the enzyme inhibitory activity were observed up to the *n*-propyl analogue **6c**. The over 20-fold increase in the NA inhibitory activity for **6c** compared to the methyl counterpart **6a** implicated a significant hydrophobic interaction of the *n*-propyl group with amino acids in the active site. Branching at the β-carbon of the *n*-propyl group (compound **6e**) resulted in no enhancement of NA inhibitory activity compared to that of **6c**. In contrast, when the methyl group was added at the α-position of the *n*-propyl group (compounds **6f** and **6g**), NA inhibitory activity increased almost 20-fold. This improved
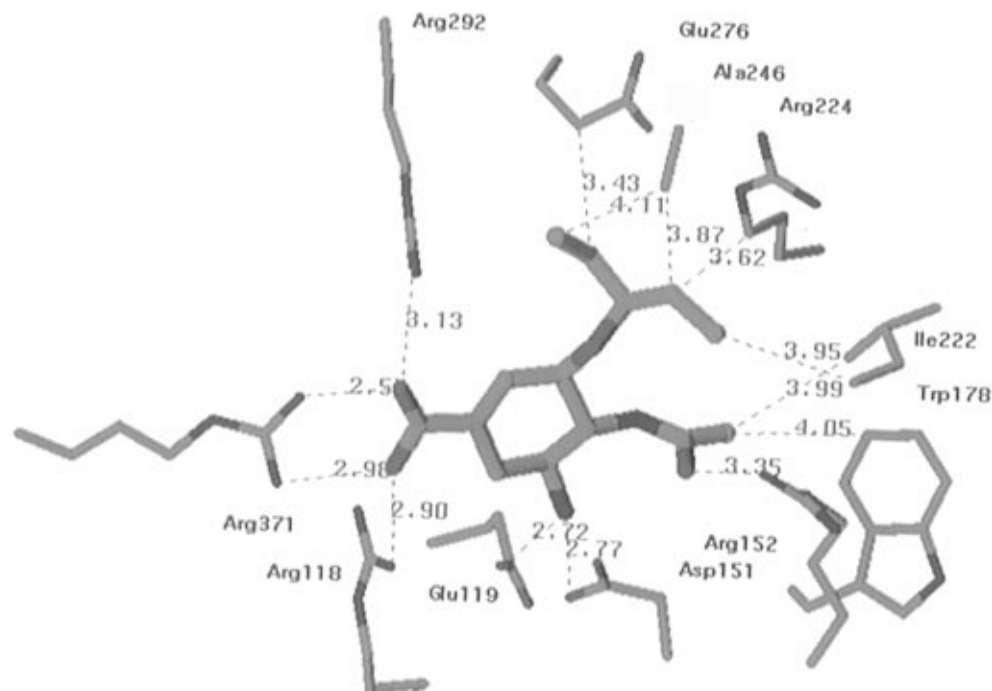
Figure 1. X-ray structure of **6h** bound to influenza neuraminidase.

potency undoubtedly arose from additional hydrophobic interactions in the NA active site generated by the α-methyl group. Remarkably, the absolute stereochemistry of the α-methyl group (**6f** and **6g**) did not influence the NA inhibitory activity as they both exhibited almost equal potency. This result led us to speculate that the methyl and ethyl groups in the *sec*-butyl analogues **6f** and **6g** contribute almost the same binding energy by an equal degree of interaction with amino acids in the active site. This hypothesis was further supported by the NA activity of 3-pentyl analogue **6h**, which possesses two identical branched ethyl groups and shows the NA inhibitory activity increase of almost 10-fold that of either isomers **6f** and **6g**. Further extension of the two ethyl groups to the 4-heptyl analogue **6i** resulted in a decrease of NA inhibitory activity, suggesting that the 3-pentyl group in **6h** might provide the optimum hydrophobic contact with the NA active site. Interestingly, the enzymatic and plaque reduction activities of **6h** were comparable to those of **3**, which exhibited over 100-fold increase in activity compared to that of amino analogue **2**. Replacement of the amino group in **6h** with the guanidino moiety resulted in a significant increase in its enzymatic and cell culture activity compared to **6h**.[36] Thus, carbocyclic NA inhibitors represented by **6h** and its guanidino analogue are more potent NA inhibitors than any inhibitors reported previously, including compounds **2** and **3**.

X-ray Structure Analysis of 6h

Information on protein−ligand interactions generated by X-ray crystallography plays a very important role in structure-based enzyme inhibitor design. In the course of studying structure−activity relationships of carbocyclic NA inhibitors, the crystal structure of **6h** bound to NA was investigated. As shown in Figure 1, the carboxylate of inhibitor **6h** was held strongly by three arginine residues (Arg292, Arg 371, Arg 118). The C_3 amino group forms strong charge−charge type hydrogen bond interactions with Glu119 and Asp151. The methyl group of the C_4 acetamide occupies a hydrophobic pocket generated

by Trp178 and Ile222 with the oxygen atom of the amide interacting with Arg152. These binding features were not significantly different from that found in the inhibitor−NA complex of Neu5Ac2en and other sialic acid based inhibitors.[25−27] The 3-pentyloxy side chain at the C_4 position was situated against a large hydrophobic surface created by the hydrocarbon chains of Glu276, Ala246, Arg224, and Ile222. Contrary to this, the glycerol side chain of sialic acid based inhibitors interact with the carboxylate of Glu276 through two terminal hydroxyls in a bidentate hydrogen bond donor−acceptor mode. In order to accommodate the large 3-pentyl group of **6h**, the carboxylate of Glu276 is forced to orientate outward from the hydrophobic pocket. The hydrophobic interaction observed for the 3-pentyloxy group in the NA active site illustrates well the other structure−activity relationships observed in the different alkyl chains in Table 1. As discussed above, the remarkable degree of correlation between the aliphatic side chains and NA inhibitory activity result from the different degree of hydrophobic interactions due to different length, geometry, and conformational rigidity of the alkyl chains. The incremental entropy gain resulting from increased hydrophobic interaction is believed to reflect the NA inhibitory activity of these compounds. Further detailed structural studies in this series are being investigated.

Conclusion

Carbocyclic analogues of sialic acid described in this study represent a rational drug design of enzyme inhibitors based on a transition-state mechanism. Similarity replacement of the oxonium transition state arising from the sialic acid cleavage by NA with a cyclohexene scaffold led to a new series of NA inhibitors. It was clearly demonstrated that, in the design of a transition-state analogue, the closer the structure is to the transitition state, the more potent the enzyme inhibitor, as indicated by the striking difference in NA inhibition of **8** vs **9**. The X-ray crystallographic study of **6h** bound to NA revealed the existence of a large hydrophobic pocket in the region corresponding to the glycerol subsite of sialic acid. The finding of this new hydrophobic pocket led us to a new series of potent carbocyclic NA inhibitors **6**, in which the clear structure−

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(36) Data not shown. A full account of structure−activity relationships of carbocyclic analogues related to **6** will be published separately.

activity relationship was observed among analogues with various alkyl chains (R′ in **6**). This result demonstrated that, in the design of enzyme inhibitors, consideration of hydrophobic interactions in the enzyme active site might lead to a new generation of structurally unique molecules. Finally, the ethyl ester of **6h** (designated as GS4104) exhibited good oral bioavailability in several animals (mice, rats, and dogs) and demonstrated oral efficacy in the mouse and ferret influenza model. On the basis of potent in vitro/in vivo activity and very favorable pharmacological properties, GS4104 has been selected as a clinical candidate for the oral treatment and prophylaxis of influenza infection.

Experimental Section

General. All reactions were conducted under a dry atmosphere of argon unless otherwise noted. All reaction solvents were anhydrous grade obtained from Aldrich Chemical Co. Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by Robertson Microlit Laboratories, Inc., Madison, NJ. High-resolution mass spectra were performed by the Mass Spectrometry Lab, University of California, Berkeley. Column chromatography was carried out using 230−400 mesh silica gel. NMR spectra were recorded at 300 MHz unless otherwise indicated; J values are reported in hertz. Reaction solutions were dried over $MgSO_4$ unless otherwise indicated.

Methyl (3R,4R,5S)-4,5-Epoxy-3-(methoxymethoxy)-1-cyclohex-ene-1-carboxylate (13). To a solution of **12** (4.0 g, 23.5 mmol) in CH_2Cl_2 (100 mL) was added N,N′-diisopropylethylamine (12.3 mL, 70.5 mmol) followed by chloromethyl methyl ether (3.6 mL, 47 mmol). The solution was refluxed for 3.5 h, and the solvent was evaporated. The residue was partitioned between ethyl acetate (200 mL) and water (200 mL). The aqueous phase was extracted with ethyl acetate (100 mL), and the combined organic extracts were washed with brine (100 mL), dried, filtered, and evaporated to give 4.9 g (97%) of a solid residue which was of suitable purity to use directly in the next step. An analytical sample was recrystallized (ether/hexane) to give **12** as colorless plates: mp 64−66 °C; ^{1}H NMR (CDCl$_3$) δ 6.73 (m, 1H), 4.87 (s, 2H), 4.59 (m, 1H), 3.75 (s, 3H), 3.57 (m, 1H), 3.48 (m, 4H), 3.07 (dd, 1H, $J = 1.2, 19.8$), 2.47 (dq, 1H, $J = 2.7, 19.5$). Anal. Calcd for $C_{10}H_{14}O_5$: C, 56.07; H, 6.59. Found: C, 56.15; H, 6.61.

Methyl (3R,4S,5R)-5-Azido-4-hydroxy-3-(methoxymethoxy)-1-cyclohexene-1-carboxylate (14). To a solution of **13** (4.9 g, 22.9 mmol) in methanol/water (8:1, 175 mL) were added sodium azide (7.44 g, 114.5 mmol) and ammonium chloride (2.69 g, 50.4 mmol), and the mixture was refluxed for 15 h. The reaction mixture was diluted with water (75 mL) to dissolve precipitated salts, and the solution was concentrated to remove methanol. The resulting aqueous phase containing an oily residue was diluted to a volume of 200 mL with water and was extracted with ethyl acetate (3 × 100 mL). The combined organic extracts were washed with brine (100 mL), dried, filtered, and evaporated. The crude was chromatographed (hexane/ethyl acetate, 1:1) to give **14** (5.09 g, 86%) as a pale yellow oil which was of sufficient purity to use in the next step without further purification. An analytical sample was prepared by chromatography (2.5% methanol in CH_2Cl_2): ^{1}H NMR (CDCl$_3$) δ 6.86 (m, 1H), 4.79 (s, 2H), 4.31 (br t, 1H, $J = 4.2$), 3.90−3.75 (m, 5H), 3.43 (s, 3H), 2.92 (d, 1H, $J = 6.6$), 2.87 (dd, 1H, $J = 5.4, 18.6$), 2.21−2.30 (m, 1H); ^{13}C NMR (CDCl$_3$) δ 166.2, 134.7, 130.3, 96.8, 72.5, 70.5, 58.6, 55.8, 52.1, 28.7. Anal. Calcd for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33. Found: C, 46.68; H, 5.89; N, 15.89.

Methyl (3R,4S,5R)-5-Azido-4-[(methylsulfonyl)oxy]-3-(meth-oxymethoxy)-1-cyclohexene-1-carboxylate (15). To a solution of **14** (6.47 g, 25.2 mmol) in CH_2Cl_2 (100 mL) at 0 °C was added triethylamine (4.4 mL, 31.5 mmol) followed by the addition of methanesulfonyl chloride (2.14 mL, 27.7 mmol). The reaction was stirred at 0 °C for 45 min and warmed to room temperature with stirring for 15 min. Solvent was evaporated, and the residue was partitioned between ethyl acetate (200 mL) and water (100 mL). The organic phase was washed with water (100 mL), saturated sodium bicarbonate (100 mL), and brine (100 mL). The aqueous washes were extracted with

ethyl acetate. The combined organic extracts were dried, filtered, and evaporated to afford crude mesylate **15** (8.56 g, 99%), which was of suitable purity to use directly in the next step. An analytical sample was prepared by chromatography eluting with ethyl acetate/hexane (1: 1) and isolated as a white oil: ^{1}H NMR (CDCl$_3$) δ 6.85 (m, 1H), 4.82 (d, 1H, $J = 6.9$), 4.73 (d, 1H, $J = 6.9$), 4.67 (dd, 1H, $J = 3.9, 9.0$), 4.53 (br t, 1H, $J = 4.2$), 3.78 (s, 3H), 3.41 (s, 3H), 3.15 (s, 3H), 2.98 (dd, 1H, $J = 6.0, 18.6$), 2.37 (m, 1H); ^{13}C NMR (CDCl$_3$) δ 165.6, 134.3, 129.6, 96.5, 78.4, 69.6, 55.8, 55.7, 52.1, 38.2, 29.1. Anal. Calcd for $C_{11}H_{17}N_3O_7S$: C, 39.40; H, 5.11; N, 12.53. Found: C, 39.55; H, 5.17; N, 12.75.

Aziridine (16). To a solution of **15** (8.56 g, 25 mmol) in THF (150 mL) at 0 °C was added triphenylphosphine (8.2 g, 31 mmol), initially adding a third of the amount while cooling and then after removing the ice bath adding the remaining amount over a period of 15 min. The reaction mixture was stirred at room temperature for 3 h, during which time a white precipitate formed. To this suspension was added triethylamine (5.2 mL, 37.5 mmol) and water (10 mL), and the mixture was stirred at room temperature for 12 h. The reaction mixture was concentrated to remove THF, and the residue was partitioned between CH_2Cl_2 (200 mL) and brine (200 mL). The aqueous phase was extracted with CH_2Cl_2, and the combined organic extracts were dried (Na_2SO_4), filtered, and evaporated to give a crude product which was purified by chromatography, eluting with ethyl acetate/methanol (9:1) to give **16** (4.18 g, 78%) as an oil containing 5% triphenylphosphine oxide: ^{1}H NMR (CDCl$_3$) δ 6.81 (m, 1H), 4.78 (s, 2H), 4.54 (m, 1H), 3.73 (s, 3H), 3.41 (s, 3H), 2.87 (dd, 1H), 2.64 (br s, 1H), 2.56−2.47 (m, 2H); ^{13}C NMR (CDCl$_3$) δ 166.9, 132.5, 128.0, 95.9, 69.5, 55.2, 51.6, 31.1, 27.7, 24.1.

Methyl (3R,4R,5S)-4-Amino-5-azido-3-(methoxymethoxy)-1-cyclohexene-1-carboxylate (17). To a solution of **16** (3.2 g, 15 mmol) in DMF (30 mL) was added sodium azide (4.9 g, 75 mmol) and ammonium chloride (1.6 g, 30 mmol), and the mixture was heated at 65−70 °C for 21 h. The reaction mixture was cooled to room temperature, diluted with ethyl acetate (100 mL), and filtered. The filtrate was evaporated, and the residue was partitioned between diethyl ether (100 mL) and brine (100 mL). The organic phase was washed again with brine, dried, filtered, and evaporated. The aqueous washings were extracted with ethyl acetate and treated in the same manner as described above. The crude product was chromatographed with 5% methanol in CH_2Cl_2 to give **17** (2.95 g) as an oil which contained a small amount of triphenylphosphine oxide from the previous step: ^{1}H NMR (CDCl$_3$) δ 6.82 (t, 1H, $J = 2.3$), 4.81 (d, 1H, $J = 7.2$), 4.77 (d, 1H, $J = 6.9$), 4.09−4.04 (m, 1H), 3.76 (s, 3H), 3.47 and 3.44 (m overlapping s, 4H), 2.94−2.86 (m, 2H), 2.36−2.24 (m, 1H); ^{13}C NMR (CDCl$_3$) δ 165.9, 137.3, 128.2, 96.5, 79.3, 61.5, 55.7, 55.6, 51.9, 29.5.

Amine **17** was characterized as the *N-tert*-butylcarbamate derivative. To a solution of **17** in ethyl acetate was added an equivalent volume of saturated sodium bicarbonate, followed by an excess of di-*tert*-butyl dicarbonate. The mixture was vigorously stirred at room temperature for 18 h. The organic phase was separated, washed with brine, dried, filtered, and evaporated. The residue was chromatographed with hexane/ethyl acetate (2:1) to afford the *N-tert*-butylcarbamate derivative as a white solid: mp 78−80 °C; ^{1}H NMR (CDCl$_3$) δ 6.79 (t, 1H, $J = 2.4$), 4.90 (br s, 1H), 4.70 (d, 1H, $J = 7.0$), 4.55 (br s, 1H), 4.01 (br s, 1H), 3.77 (s, 3H), 3.42 (m, 4H), 2.87 (dd, 1H, $J = 5.2, 17.8$), 2.31−2.22 (m, 1H). Anal. Calcd for $C_{15}H_{22}N_4O_6$: C, 50.84; H, 6.26; N, 15.81. Found: C, 50.73; H, 6.60; N, 15.58.

Methyl (3R,4R,5S)-4-Acetamido-5-azido-3-(methoxymethoxy)-1-cyclohexene-1-carboxylate (18). A solution of **17** (in CH_2Cl_2 15 mL) was treated with pyridine (4 mL) and acetyl chloride (150 μL). Aqueous workup followed by chromatography of the residue gave **18** (350 mg, 76%) as a pale oil: ^{1}H NMR (CDCl$_3$) δ 6.78 (s, 1H), 6.39 (br d, 1H, $J = 7.8$ Hz), 4.72 (d, 1H, $J = 6.9$ Hz), 4.66 (d, 1H, $J = 6.9$ Hz), 4.53 (br d, 1H, $J = 8.4$ Hz), 4.00−3.90 (m, 1H), 3.80−3.65 (m, 1H), 3.75 (s, 3H), 3.37 (s, 3H), 2.85 (dd, 1H, $J = 5.4, 17.7$ Hz), 2.35−2.20 (m, 1H), 2.04 (s, 3H).

(3R,4R,5S)-4-Acetamido-5-amino-3-(methoxymethoxy)-1-cyclo-hexene-1-carboxylic Acid (19). A solution of **18** (39 mg, 0.13 mmol) in ethanol was stirred with Lindlar catalyst (39 mg) under 1 atm of hydrogen. The reaction mixture was filtered through a Celite pad, which was washed with hot ethanol. The filtrate was evaporated to

give a pale foam (33 mg), which was dissolved in THF (1 mL) and was treated with aqueous 0.48 M potassium hydroxide (380 μL) for 1 h. The reaction was acidified to pH = 4.0 with Amberlite IR-120. The resin was filtered and washed with water, and the filtrate was evaporated to give a pale solid which was purified by C_{18} column chromatography eluting with water. The fractions containing the product were pooled and lyophilzed to give **19** (20 mg, 60%) as a white powder: ^{1}H NMR (D$_2$O) δ 6.65 (s, 1H), 4.87 (d, 1H, J = 7.5 Hz), 4.76 (d, 1H, J = 7.5 Hz), 4.47 (br d, 1H, J = 8.7 Hz), 4.16 (dd, 1H, J = 11.4, 11.4 Hz), 3.70−3.55 (m, 1H), 3.43 (s, 3H), 2.95 (dd, 1H, J = 5.7, 17.4 Hz), 2.60−2.45 (m, 1H), 2.11 (s, 3H).

(3R,4R,5S)-4-Acetamido-5-amino-3-hydroxy-1-cyclohexene-1-carboxylic acid (8). To **19** (4 mg, 0.015 mmol) was added 40% trifluoroacetic acid in CH$_2$Cl$_2$ (1 mL, cooled to 0 °C prior to addition). After being stirred at room temperature for 1.5 h, the reaction mixture was concentrated to give a white foam. Coevaporation from water several times followed by lyophilization gave trifluoroacetate salt **8** (4.9 mg, 100%) as a white solid: ^{1}H NMR (D$_2$O) δ 6.85 (m, 1H), 4.45 (m, 1H), 4.05 (dd, 1H, J = 11.4, 11.4 Hz), 3.65−3.55 (m, 1H), 3.00− 2.90 (m, 1H), 2.60−2.45 (m, 1H), 2.09 (s, 3H); HRMS (FAB) calcd for C$_9$H$_{15}$N$_2$O$_4$ (MH$^+$) 215.1032, found 215.1027.

Methyl (3S,4R,5R)-3,4-O-Isopropylidine-3,4-dihydroxy-5-(pivaloyloxy)-1-cyclohexene-1-carboxylate (23). To a solution of **22** (10.9 mmol) in CH$_2$Cl$_2$ (60 mL) at 0 °C was added pyridine (4.4 mL, 54.5 mmol) followed by addition of trimethylacetyl chloride (2.7 mL, 21.8 mmol). The mixture was warmed to room temperature and stirred for 14 h. The mixture was diluted with CH$_2$Cl$_2$, and the organic phase was washed with water and brine and dried. The solvent was evaporated, and the residue was purified by chromatography eluting with hexane/ethyl acetate (9:1) to give **23** (2.32 g, 68%) as an oil: ^{1}H NMR (CDCl$_3$) δ 6.72 (m, 1H), 5.04 (m, 1H), 4.76 (m, 1H), 4.40 (m, 1H), 3.77 (s, 3H), 2.72-2.49 (m, 2H), 1.37 (s, 3H), 1.35 (s, 3H), 1.23 (s, 9H).

Methyl (3S,4R,5R)-3,4-Dihydroxy-5-(pivaloyloxy)-1-cyclohexene-1-carboxylate (24). A solution of **23** (2.32 g, 2.3 mmol) in acetone/water (1:1, 100 mL) was heated at 55 °C for 16 h in the presence of *p*-toluenesulfonic acid (10 mg). The solvents were evaporated, and the residue was coevaporated sequentially from water and toluene to give **24** as an oil which was used without further purification: ^{1}H NMR (CDCl$_3$) δ 6.83 (m, 1H), 5.06 (m, 1H), 4.42 (m, 1H), 4.09 (m, 1H), 3.77 (s, 3H), 2.68-2.41 (m, 2H), 1.22 (s, 9H).

Cyclic Sulfite 25. To a solution of **24** (0.41 g, 1.5 mmol) in THF (8 mL) at 0 °C was added triethylamine (0.83 mL, 6.0 mmol), followed by dropwise addition of thionyl chloride (0.33 mL, 4.5 mmol). The mixture was warmed to room temperature and stirred for 3 h. The mixture was diluted with chloroform and was washed with water and brine and dried. The solvents were evaporated, and the residue was purified by chromatography eluting with hexane/ethyl acetate (5:1) to give **25** (0.430 g, 90%) as an oil: ^{1}H NMR (CDCl$_3$) δ 6.89−6.85 (m, 1 H), 5.48−4.84 (m, 3 H), 3.80, 3.78 (s, 3 H), 2.90−2.60 (m, 2 H), 1.25, 1.19 (s, 9 H).

Methyl (3R,4S,5R)-3-Azido-4-hydroxy-5-(pivaloyloxy)-1-cyclohexene-1-carboxylate (26). The mixture of **25** (0.40 g, 1.3 mmol) and sodium azide (0.41 g, 6.29 mmol) in DMF (10 mL) was stirred at room temperature for 20 h. The reaction mixture was then diluted with ethyl acetate, washed with saturated ammonium chloride, water, and brine, and dried. The solvents were evaporated to give **26** (0.338 g, 90%) as an oil: ^{1}H NMR (CDCl$_3$) δ 6.78 (m, 1H), 5.32 (m, 1H), 4.20 (m, 1H), 3.89 (m, 1H), 3.78 (s, 3H), 3.00−2.60 (m, 2H), 1.21 (s, 9H).

Methyl (3R,4S,5R)-3-Azido-4-[(methylsulfonyl)oxy]-5-(pivaloyloxy)-1-cyclohexene-1-carboxylate (27). To a solution of **26** (0.338 g, 1.1 mmol) in CH$_2$Cl$_2$ (11 mL) at 0 °C was added triethylamine (0.4 mL, 2.9 mmol) followed by dropwise addition of methanesulfonyl chloride (0.18 mL, 2.3 mmol). The mixture was stirred at 0 °C for 30 min and was diluted with CH$_2$Cl$_2$. The organic phase was washed with water and brine, dried, and evaporated. The residue was purified by chromatography eluting with hexane/ethyl acetate (3:1) to give **27** (0.38 g, 82%) as an oil: ^{1}H NMR (CDCl$_3$) δ 6.82 (m, 1H), 5.44 (m, 1H), 4.76 (dd, J = 7.3, 1.4 Hz, 1H), 4.48 (m, 1H), 3.80 (s, 3H), 3.11 (s, 3H), 2.82−2.61 (m, 2H), 1.21 (s, 9H).

N-Acetylaziridine (28). To a solution of **27** (0.380 g, 0.94 mmol) in THF (19 mL) was added triphenylphosphine (0.271 g, 1.04 mmol), and the solution was stirred at room temperature for 2 h. To this solution were added water (1.9 mL) and triethylamine (0.39 mL, 2.82 mmol), and the mixture was stirred for 14 h. The solvents were evaporated, the crude residue was dissolved in CH$_2$Cl$_2$ (20 mL) and cooled to 0 °C, and pyridine (0.68 mL, 8.4 mmol) was added followed by the dropwise addition of acetyl chloride (0.30 mL, 4.2 mmol). The mixture was stirred at 0 °C for 5 min and diluted with ethyl acetate. The organic phase was washed with water and brine and dried. The solvent was evaporated, and the residue was purified by chromatography eluting with hexane/ethyl acetate (3:1) to give **28** (0.205 g, 83%) as an oil: ^{1}H NMR (CDCl$_3$) δ 7.19 (m, 1H), 5.58 (m, 1H), 3.77 (s, 3H), 3.14 (m, 2H), 2.85 (dd, J = 7.0, 1.6 Hz, 1H), 2.34 (m, 1H), 2.16 (s, 3H), 1.14 (s, 9H); HRMS (EI) calcd for C$_{15}$H$_{21}$NO$_5$ (M$^+$) 295.1420, found 295.1420.

Methyl (3S,4S,5R)-4-Acetamido-3-azido-5-(pivaloyloxy)-1-cyclohexene-1-carboxylate (29). A mixture of **28** (0.200 g, 0.68 mmol), sodium azide (0.221 g, 3.4 mmol), and ammonium chloride (0.146 g, 2.7 mmol) in DMF (10 mL) was stirred at room temperature for 14 h. The reaction was diluted with ethyl acetate, washed with water and brine, and dried. The solvent was evaporated, and the residue was purified by chromatography eluting with hexane/ethyl acetate (2:1) to give a mixture of products which was dissolved in acetic anhydride (2 mL) and stirred for 2 h. Excess acetic anhydride was evaporated to afford **29** (149 mg, 65%) as an oil: ^{1}H NMR (CDCl$_3$) δ 6.76 (m, 1H), 5.53 (d, 1H, J = 8.5), 5.05 (m, 1H), 4.31 (m, 1H), 4.08 (m, 1H), 3.79 (s, 3H), 2.91 (m, 1H), 2.51 (m, 1H), 1.99 (s, 1H), 1.20 (s, 9H).

(3S,4S,5R)-4-Acetamido-3-amino-5-hydroxy-1-cyclohexene-1-carboxylic Acid (9). A solution of potassium hydroxide (123 mg, 2.2 mmol) in methanol/water (1:1, 4.4 mL) was added to **29** (149 mg, 0.44 mmol), and the mixture was stirred at room temperature for 3 h. The mixture was cooled to 0 °C and acidified with Amberlite IR-120 to pH = 3.5. The mixture was filtered, washed with methanol, and evaporated to give a solid residue (73 mg). The residue (8 mg) was dissolved in ethanol (2 mL), treated with Lindlar catalyst (15 mg), and stirred under 1 atm of hydrogen for 16 h. The reaction was filtered through Celite, and the catalyst was washed with hot methanol/water (1:1). The filtrate was evaporated, and the reside was purified by C$_8$ column chromatography eluting with water to give **9** (6 mg) as a white solid: ^{1}H NMR (D$_2$O) δ 6.28 (m, 1H), 4.06−3.85 (m, 3H), 2.83 (dd, 1H, J =17.7, 5.4), 2.35 (m, 1H), 2.06 (s, 3H); HRMS (FAB) calcd for C$_9$H$_{15}$N$_2$O$_4$ (MH$^+$) 215.1032, found 215.1025.

Methyl (3R,4R,5S)-4-Amino-5-azido-3-hydroxy-1-cyclohexene-1-carboxylate Hydrochloride (30). A solution of **17** (2.59 g, 10.2 mmol) in 5% hydrogen chloride in methanol (30 mL) was stirred for 3 h at room temperature, and additional 5% hydrogen chloride in methanol (10 mL) was added. After the mixture was stirred for 1 h, the solvent was evaporated to give **30** (2.52 g, 99%) as a tan solid after drying under high vacuum. An analytical sample was recrystallized from methanol/ether: ^{1}H NMR (CD$_3$OD) δ 6.73 (t, 1H, J = 2.1), 4.35 (m, 1H), 3.93 (s, 3H), 3.77 (s, 3H), 3.12−2.98 (m, 2H), 2.50−2.39 (m, 1H). Anal. Calcd for C$_8$H$_{13}$ClN$_4$O$_3$: C, 38.64; H, 5.27; N, 22.53. Found: C, 38.72; H, 5.21; N, 22.45.

N-Tritylaziridine (31). To a suspension of **30** (2.52 g, 10.1 mmol) in CH$_2$Cl$_2$ (50 mL) at 0 °C was added triethylamine (3.55 mL, 25.5 mmol) followed by the addition of solid trityl chloride (5.55 g, 12.8 mmol) in one portion. The mixture was stirred at 0 °C for 1 h. After being stirred for 2 h at room temperature, the reaction mixture was cooled to 0 °C, and triethylamine (3.6 mL, 25.5 mmol) and methanesulfonyl chloride (0.97 mL, 12.5 mmol) were added. The resulting mixture was stirred for 1 h at 0 °C and for 22 h at room temperature. The solvent was evaporated, and the residue was partitioned between ether (200 mL) and water (200 mL). The organic phase was washed with water (200 mL), and the combined aqueous phases were extracted with ether (200 mL). The combined organic extracts were washed with water and brine and dried. The crude product was chromatographed eluting with hexane/CH$_2$Cl$_2$ (1:1) to give **31** (3.84 g, 86%) as a white foam: ^{1}H NMR (CDCl$_3$) δ 7.4−7.23 (m, 16H), 4.32 (m, 1H), 3.81 (s, 3H), 3.06 (dt, 1H, J = 1.8, 17.1), 2.94−2.86 (m, 1H), 2.12 (m, 1H), 1.85 (t, 1H, J = 5.0); HRMS (FAB) calcd for C$_{27}$H$_{25}$N$_4$O$_2$ (MH$^+$) 437.1977, found 437.1976.

Representative Procedure: Alcoholysis of 31. Synthesis of Methyl (3R,4R,5S)-4-Acetamido-5-azido-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (32h). To a solution of **31** (15 g, 34 mmol) in 3-pentanol (230 mL) was added BF₃·Et₂O (6.27 mL, 51 mmol). The solution was heated at 70−75 °C for 2 h and then evaporated to give a residue which was dissolved in dry pyridine (2.0 mL) and treated with acetic anhydride (16 mL, 170 mmol) and (dimethylamino)pyridine (200 mg, 1.6 mmol). The reaction mixture was stirred at room temperature for 18 h and evaporated, and the residue was partitioned between ethyl acetate and 1 M HCl. The organic phase was washed with saturated sodium bicarbonate and brine and dried. The solvent was evaporated, and the residue was chromatographed eluting with hexane/ethyl acetate (1:1) to give **32h** (7.66 g, 69%) as a white solid. An analytical sample was recrystallized from hexane/ethyl acetate to afford **32h** as needles: mp 121−123 °C; ¹H NMR (CDCl₃) δ 6.79 (t, 1H, J = 2.1 Hz), 5.92 (d, 1H, J = 7.5), 4.58 (bd, 1H, J = 8.7), 4.35−4.25 (m, 1H), 3.77 (s, 3H), 3.36−3.25 (m, 2H), 2.85 (dd, 1H, J = 5.7, 17.4), 2.29−2.18 (m, 1H), 2.04 (s, 3H), 1.60−1.45 (m, 4H), 0.91 (t, 3H, J = 3.7), 0.90 (t, 3H, J = 7.3); ¹³C NMR (CDCl₃) δ 171.1, 166.2, 138.4, 127.7, 82.1, 73.8, 57.5, 57.2, 52.0, 30.4, 26.1, 25.5, 23.4, 9.5, 9.1. Anal. Calcd for C₁₅H₂₄N₄O₄: C, 55.54; H, 7.46; N, 17.27. Found: C, 55.57; H, 7.31; N, 17.30.

Methyl (3R,4R,5S)-4-acetamido-5-azido-3-methoxy-1-cyclohexene-1-carboxylate (32a): mp 145−146 °C (needles from hexane/ethyl acetate); ¹H NMR (CDCl₃) δ 6.89 (t, 1H, J = 2.4), 5.73 (d, 1H, J = 7.6), 4.40 (m, 1H), 4.12 (m, 1H), 3.78 (s, 3H), 3.58 (m, 1H), 3.42 (s, 3H), 2.89 (ddd, 1H, J = 0.7, 5.7, 17.6), 2.34−2.22 (m, 1H), 2.07 (s, 3H). Anal. Calcd for C₁₁H₁₆N₄O₄: C, 49.25; H, 6.01; N, 20.88. Found: C, 49.24; H, 5.98; N, 20.90.

Methyl (3R,4R,5S)-4-acetamido-5-azido-3-ethoxy-1-cyclohexene-1-carboxylate (32b): mp 141−143 °C (needles from hexane/ethyl acetate); ¹H NMR (CDCl₃) δ 6.86 (t, 1H, J = 2.3), 6.08 (d, 1H, J = 7.9), 4.45 (m, 1H), 4.12 (m, 1H), 3.77 (s, 3H), 3.72−3.51 (m, 2H), 2.87 (dd, 1H, J = 5.5, 17.1), 2.32−2.21 (m, 1H), 2.06 (s, 3H), 1.20 (t, 3H, J = 7.0). Anal. Calcd for C₁₂H₁₈N₄O₄: C, 51.06; H, 6.43; N, 19.85. Found: C, 50.79; H, 6.21; N, 19.63.

Methyl (3R,4R,5S)-4-acetamido-5-azido-3-(1-propoxy)-1-cyclohexene-1-carboxylate (32c): mp 147−148 °C (needles from hexane/ethyl acetate); ¹H NMR (CDCl₃) δ 6.85 (t, 1H, J = 2.1), 5.81 (d, 1H, J = 6.3), 4.74 (m, 1H), 4.10 (m, 1H), 3.77 (s, 3H), 3.69−3.37 (m, 3H), 2.87 (dd, 1H, J = 5.7, 17.1), 2.32−2.21 (m, 1H), 2.05 (s, 3H), 1.57 (m, 2H), 0.92 (t, 3H, J = 7.2). Anal. Calcd for C₁₃H₂₀N₄O₄: C, 52.69; H, 6.80; N, 18.91. Found: C, 52.67; H, 6.56; N, 18.89.

Methyl (3R,4R,5S)-4-acetamido-5-azido-3-(1-butoxy)-1-cyclohexene-1-carboxylate (32d): mp 136−137 °C (needles from hexane/ethyl acetate); ¹H NMR (CDCl₃) δ 6.86 (t, 1H, J = 2.3), 6.03 (d, 1H, J = 7.9), 4.43 (m, 1H), 4.09 (m, 1H), 3.77 (s, 3H), 3.68−3.41 (m, 4H), 2.87 (dd, 1H, J = 5.2, 17.6), 2.32−2.21 (m, 1H), 2.06 (s, 3H), 1.54 (m, 2H), 1.36 (m, 2H), 0.91 (t, 3H, J = 7.3). Anal. Calcd for C₁₄H₂₂N₄O₄: C, 54.18; H, 7.15; N, 18.05. Found: C, 54.21; H, 6.98; N, 17.94.

Methyl (3R,4R,5S)-4-acetamido-5-amino-3-(2-methylpropoxy)-1-cyclohexene-1-carboxylate (32e): isolated as a solid; ¹H NMR (CDCl₃) δ 6.86 (t, 1H, J = 2.2), 6.01 (d, 1H, J = 7.9), 4.40 (m, 1H), 4.07 (m, 1H), 3.77 (s, 3H), 3.63 (m, 1H), 3.43 (dd, 1H, J = 6.4, 8.8), 3.19 (dd, 1H, J = 6.7, 8.8), 2.87 (dd, 1H, J = 5.8, 17.3), 2.33−2.22 (m, 1H), 2.05 (s, 3H), 1.82 (m, 1H), 0.90 (2d, 6H).

Methyl (3R,4R,5S)-4-Acetamido-5-amino-3-(1(R)-methylpropoxy)-1-cyclohexene-1-carboxylate (32f). Isolated as a crystalline solid which was a 9:1 (**32f:32g**) diastereomeric mixture. Data for the major diastereomer are reported: ¹H NMR (CDCl₃) δ 6.79 (t, 1H, J = 2.2 Hz), 6.14 (d, 1H, J = 7.3 Hz), 4.55 (m, 1H), 4.33−4.23 (m, 1H), 3.77 (s, 3H), 3.56−3.45 (m, 1H), 3.40−3.27 (m, 1H), 2.85 (dd, 1H, J = 5.5, 17.5), 2.30−2.15 (m, 1H), 2.04 (s, 3H), 1.59−1.40 (m, 2H), 1.10 (d, 3H, J = 6.0 Hz), 0.91 (t, 3H, J = 7.4 Hz). Anal. Calcd for C₁₄H₂₂N₄O₄: C, 54.18; H, 7.15; N, 18.05. Found: C, 54.06; H, 7.11; N, 17.96.

Methyl (3R,4R,5S)-4-Acetamido-5-amino-3-(1S-methylpropoxy)-1-cyclohexene-1-carboxylate (32g). Isolated as a crystalline solid which was a 9:1 (**32g:32f**) diastereomeric mixture. Data for the major diastereomer are reported: ¹H NMR (CDCl₃) δ 6.78 (t, 1H, J = 2.2 Hz), 5.82 (d, 1H, J = 7.4 Hz), 4.61 (m, 1H), 4.35−4.26 (m, 1H), 3.76

(s, 3H), 3.56−3.48 (m, 1H), 3.31−3.25 (m, 1H), 2.86 (dd, 1H, J = 5.7, 17.5 Hz), 2.28−2.17 (m, 1H), 2.04 (s, 3H), 1.61−1.36 (m, 2H), 1.17 (d, 3H, J = 6.2 Hz), 0.91 (t, 3H, J = 7.4 Hz). Anal. Calcd for C₁₄H₂₂N₄O₄: C, 54.18; H, 7.15; N, 18.05. Found: C, 53.94; H, 7.10; N, 18.32.

Methyl (3R,4R,5S)-4-Acetamido-5-azido-3-(1-propylbutoxy)-1-cyclohexene-1-carboxylate (32i): isolated as a solid; ¹H NMR (CDCl₃, 500 MHz) δ 6.78 (t, 1H, J = 2.0), 6.05 (d, 1H, J =7.0), 4.55 (m, 1H), 4.29−4.23 (m, 1H), 3.76 (s, 3H), 3.46−3.42 (m, 1H), 3.36−3.30 (m 1H), 2.85 (dd, 1H, J = 6.0, 17.5), 2.26−2.16 (m, 1H), 2.03 (s, 3H), 1.47−1.24 (m, 8H), 0.91 (m, 6H).

Representative Procedure: Synthesis of 6 from 32. (3R,4R,5S)-4-Acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic Acid (6h). To a solution of azide **32h** (1 g, 3.1 mmol) in THF (30 mL) were added triphenylphosphine (1.21 g, 4.6 mmol) and water (5.6 mL). The solution was heated at 50 °C for 10 h, THF was evaporated, and the aqueous oily residue was partitioned between ethyl acetate and brine. The organic phase was dried, filtered, and evaporated. Purification of the residue by chromatography eluting with methanol/ethyl acetate (1:1) gave 830 mg of an oil which was dissolved in THF (15 mL) and treated with 1 N potassium hydroxide (4 mL, 4.16 mmol). The reaction mixture was stirred at room temperature for 40 min and acidified to pH 6 with Dowex 50WX8. The resin was filtered and washed with water and methanol. Solvents were evaporated, and the residue was purified by C₁₈ chromatography eluting with water and then with 5% acetonitrile in water. Fractions containing the desired product were pooled and lyophilized to afford **6h** (600 mg, 75%) as a white solid: ¹H NMR (D₂O) δ 6.50 (t, 1H, J = 2.1 Hz), 4.30−4.26 (m, 1H), 4.03 (dd, 1H, J = 9.0, 11.7 Hz), 3.58−3.48 (m, 2H), 2.88 (dd, 1H, J = 5.4, 16.8 Hz), 2.53−2.41 (m, 1H), 1.62−1.40 (m, 4H), 0.90 (t, 3H, J = 7.5 Hz), 0.85 (t, 3H, J = 7.5 Hz). Anal. Calcd for C₁₄H₂₄N₂O₄·1.5H₂O: C, 54.01; H, 8.73; N, 9.00. Found: C, 53.69; H, 8.70; N, 8.71.

(3R,4R,5S)-4-Acetamido-5-amino-3-methoxy-1-cyclohexene-1-carboxylic Acid Hydrochloride (6a). Amino acid **6a** was isolated as the hydrochloride salt after being treated with 1 N HCl and lyophilized: ¹H NMR (D₂O) δ 6.87 (s, 1H), 4.26−4.14 (m, 2H), 3.66−3.57 (m, 1H), 3.45 (s, 3H), 2.94 (dd, 1H, J = 5.7, 17.4), 2.59−2.47 (m, 1H), 2.09 (s, 3H).

(3R,4R,5S)-4-Acetamido-5-amino-3-ethoxy-1-cyclohexene-1-carboxylic acid (6b): isolated as an amorphous white powder; ¹H NMR (D₂O) δ 6.55 (s, 1H), 4.29 (m, 1H), 4.12 (dd, 1H), 3.82− 3.51 (m, 3H), 2.90 (dd, 1H), 2.58−2.43 (m, 1H), 2.07 (s, 3H), 0.90 (t, 3H).

(3R,4R,5S)-4-Acetamido-5-amino-3-propoxy-1-cyclohexene-1-carboxylic acid (6c): isolated as a solid; ¹H NMR (D₂O) δ 6.53 (t, 1H, J = 2.1), 4.29 (m, 1H), 4.16 (dd, 1H, J = 8.7, 11.1), 3.78−3.72 (m, 2H), 3.62 (ddd, 1H), 2.95 (dd, 1H), 2.58−2.52 (m, 1H), 2.11 (s, 3H), 1.58 (q, 2H, J = 7.3), 0.91 (t, 3H, J = 7.3).

(3R,4R,5S)-4-Acetamido-5-amino-3-butoxy-1-cyclohexene-1-carboxylic acid (6d). Amino acid **6d** was isolated as a hydrochloride salt after being treated with 1N HCl and lyophilized: ¹H NMR (D₂O) δ 6.81 (s, 1H), 4.28 (m, 1H), 4.15 (dd, 1H), 3.84−3.75 (m, 1H), 3.66−3.53 (m, 2H), 2.95 (dd, 1H), 2.59−2.45 (m, 1H), 2.10 (s, 3H), 1.61−1.50 (m, 2H), 1.42−1.23 (m, 2H), 0.90 (t, 3H).

(3R,4R,5S)-4-Acetamido-5-amino-3-(2-methylpropoxy)-1-cyclohexene-1-carboxylic acid (6e): isolated as an amorphous white powder; ¹H NMR (D₂O) δ 6.61 (br s, 1H), 4.25 (m, 1H), 4.13 (dd, 1H, J = 9.0, 11.3), 3.62−3.54 (m, 2H), 3.30 (dd, 1H, J = 7.2, 9.3), 2.91 (dd, 1H, J = 5.5, 17.2), 2.56−2.45 (m, 1H), 2.03 (s, 3H), 1.85−1.77 (m, 1H), 0.88 (2d, 6H).

(3R,4R,5S)-4-Acetamido-5-amino-3-(1(R)-methylpropoxy)-1-cyclohexene-1-carboxylic acid (6f): isolated as an amorphous white powder; ¹H NMR (D₂O) δ 6.52 (s, 1H), 4.28 (d, 1H, J = 8.7), 4.04 (dd, 1H, J = 8.8, 11.5), 3.74−3.65 (m, 1H), 3.50−3.60 (m, 1H), 2.90 (dd, 1H, J = 5.5, 17.2), 2.50−2.40 (m, 1H) 2.10 (s, 3H), 1.60−1.45 (m, 2H), 1.14 (d, 3H, J = 6.2), 0.91 (t, 3H, J = 7.4).

(3R,4R,5S)-4-Acetamido-5-amino-3-(1(S)-methylpropoxy)-1-cyclohexene-1-carboxylic acid (6g): isolated as a white solid; ¹H NMR (D₂O) δ 6.48 (t, 1H), 4.27 (m, 1H), 4.04 (dd, 1H, J = 8.9, 11.6), 3.71−3.65 (m, 1H), 3.59−3.50 (m, 1H), 2.90 (dd, 1H, J = 5.5, 17.2), 2.50−2.40 (m, 1H), 2.10 (s, 3H), 1.60−1.45 (m, 2H), 1.14 (d, 3H, J = 6.2 Hz), 0.91 (t, 3H, J = 7.4 Hz).

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(1-propylbutoxy)-1-cyclohexene-1-carboxylic acid (6i): isolated as a white solid; ^{1}H NMR (D$_2$O, 500 MHz) δ 6.62 (t, 1H, J = 2.5), 4.32 (m, 1H), 4.06 (dd, 1H, J = 8.5, 11.5), 3.69−3.65 (m, 1H), 3.61−3.56 (m, 1H), 2.93 (dd, 1H, J = 5.5, 17.0), 2.54−2.48 (m, 1H), 1.57−1.22 (m, 8H), 0.95−0.90 (m, 6H).

Methyl (1*R*,3*R*,4*R*,5*R*)-1-Hydroxy-3,4-*O*-isopropylidine-3,4-dihydroxy-5-[(*p*-tolylsulfonyl)oxy]cyclohexane-1-carboxylate (34). To a solution of **33** (29.8 g, 121 mmol) and (dimethylamino)pyridine (500 mg) in pyridine (230 mL) was added *p*-toluenesulfonyl chloride (27.7 g, 145 mmol). The mixture was stirred at room temperature for 72 h. The solvent was evaporated and the residue was diluted with water, and extracted with ethyl acetate. The combined organic extracts were washed with water, brine, and dried. Evaporation and purification by chromatography eluting with hexane/ethyl acetate gave tosylate **34** (44.6 g, 92%) as a viscous oil; ^{1}H NMR δ 7.84 (d, 2H, J = 8.4), 7.33 (d, 2H, J = 8.1), 4.76 (m, 1H), 4.42 (m, 1H), 4.05 (dd, 1H, J = 5.5, 7.5), 3.80 (s, 3H), 2.44 (s, 3H), 2.35 (m, 1H), 2.24 (m, 2H), 1.96 (m, 1H), 1.26 (s, 3H), 1.13 (s, 3H). Anal. Calcd for C$_{15}$H$_{24}$O$_8$S: C, 53.90; H, 5.91; S, 8.01. Found: C, 53.86; H, 5.91; S, 7.88.

Methyl (3*R*,4*R*,5*R*)-3,4-Dihydroxy-5-[(*p*-tolylsulfonyl)oxy]-1-cyclohexene-1-carboxylate (35). To a solution of **34** (44.6 g, 111.5 mmol) in CH$_2$Cl$_2$ (450 mL) at −78 °C was added pyridine (89 mL) followed by slow addition of sulfuryl chloride (26.7 mL, 335 mmol). The mixture was stirred at −78 °C for 5 h, and methanol (45 mL) was added dropwise. The mixture was warmed to room temperature and stirred for 12 h. Ethyl ether was added, and the organic phase was washed with water and brine and dried. The crude residue (44.8 g) was dissolved in methanol (500 mL), *p*-toluenesulfonic acid (1.06 g, 5.6 mmol) was added, and the solution was refluxed for 4 h. The reaction mixture was cooled to room temperature and evaporated, and the residue was dissolved in methanol (500 mL) and refluxed for 4 h. Solvent was evaporated, and the residue was chromatographed eluting with hexane/ethyl acetate to give 26.8 g of a solid which was recrystallized from hexane/ethyl acetate to give **35** (20.5 g, 54%) as a white solid; ^{1}H NMR (CDCl$_3$) δ 7.82 (d, 2H, J = 8.3), 7.37 (d, 2H, J = 8.3), 6.84 (m, 1H), 4.82 (dd, 1H, J = 5.8, 7.4), 4.50 (m, 1H), 3.90 (dd, 1H, J = 4.4, 8.2), 3.74 (s, 3H), 2.79 (dd, 1H, J = 5.5, 18.2), 2.42 (dd, 1H, J = 6.6, 18.2). Anal. Calcd for C$_{15}$H$_{18}$O$_7$S: C, 52.60; H, 5.30; S, 9.37. Found: C, 52.75; H, 5.15; S, 9.21.

Methyl (3*R*,4*R*,5*S*)-4,5-Epoxy-3-hydroxy-1-cyclohexene-1-carboxylate (12). To a solution **35** (20.0 g, 58.5 mmol) in THF (300 mL) at 0 °C was added 1,8-diazabicyclo[5.4.0]undec-7-ene (8.75 mL, 58.5 mmol). The reaction mixture was warmed to room temperature and stirred for 12 h. Solvent was evaporated, and the residue was purified by chromatography eluting with hexane/ethyl acetate (1:3) to give epoxide **12** (9.72 g, 100%) as a white solid which was identical to an authentic sample by NMR.[32]

Neuraminidase Enzyme Assay. Neuraminidase enzyme activity was determined using minor modifications to the literature method.[37] Influenza A/PR/8/34 (H1N1), purified on sucrose density gradients, was used as the source of enzyme and 4-(methylumbelliferyl)-α-D-*N*-acetylneuraminic acid was used as substrate in a reaction buffer containing 33 mM MES, pH 6.5, and 4 mM calcium chloride. Virus was mixed with various inhibitor concentrations and incubated at room temperature for 30 min before substrate was added to a final concentration of 10 μM. Reactions were stopped after 8 min at 37 °C with the addition of 1.5 volumes of 0.014 mM sodium hydroxide in 83% ethanol. Fluorescence was quantitated in a Perkin-Elmer fluorimeter (Model LS50B) with an excitation wavelength of 360 nm, emission wavelength of 448 nm, and slit width of 2.5.

Plaque Reduction Assay. Plaque reduction assays were performed as previously described.[38] Confluent monolayers of MDCK cells in six-well tissue culture plates were inoculated with 30 pfu of influenza A/WS/33 virus. After 1 h, the inoculum was removed, and the cells were overlaid with DMEM medium containing Earle's salts, 15 mM Hepes, pH 7.4, 0.2% BSA, 2 μg of TPCK-treated trypsin/mL, and 1% agarose. After 2−3 days the overlay was removed, and the plaques were visualized by staining the cell monolayer with 0.1% crystal violet in 20% methanol.

Crystallization and Data Collection. Isolation, purification, and crystallization of N9 neuraminidase has previously been reported.[39] Crystals of the neuraminidase−inhibitor **6h** complex were obtained by soaking the neuramindase crystals overnight in 5 mM inhibitor **6h** solution (5 mM inhibitor, 2 volumes 1.4 M KH$_2$PO$_4$, 1 volume of 3 M K$_2$HPO$_4$). Cubic-shaped crystals with approximate dimensions 0.2 × 0.2 × 0.2 mm were used to collect room temperature data at the Stanford Synchrotron Radiation Laboratory, beamline 7-1 (λ = 1.08 Å) using a MAR30 Image Plate System. The crystal belong to the cubic space group I432, a = 182.9 Å. A complete data set to 2.7 Å resolution was collected using 1° oscillations and a total of 30°. The reflections were indexed using DENZO 1.5.11 and merged/scaled using the program SCALEPACK.[40] A total of 51 334 reflections were collected; 12 383 reflections were unique. The reduced data set was 98% complete to 2.7 Å resolution with an R_{merge} of 7.1%.

Structure Solution and Refinement. The neuraminidase complexed with inhibitor **6h** structure was solved by molecular replacement using the influenza A subtype N9 neuraminidase crystal structure containing residues 82−469 (PDB ID 1INY).[41] Alternate rounds of model building with the molecular graphics program O version 6.0.3[42] and refinement of the atomic coordinates using X-PLOR 3.1[43] were performed until convergence of the free-*R* and *R* factor was achieved. Examination of $F_o − F_c$ maps showed unambiguous density for the orientation of inhibitor **6h**, sugar molecules, and calcium ion. Addition of four *N*-acetyl-D-glucosamine sugars, five α-D-mannose sugars, one calcium ion, and one inhibitor **6h** molecule reduced the free-*R* and *R* factor further. The model was refined through positional and overall *B* factor refinement in X-PLOR until the minimization was complete. The final model has an *R* factor of 16.3% (R_{free} = 22.8) in the 6−2.7 Å resolution range, with rms deviations of 0.012 Å and 1.83° for the bond lengths and angles, respectively. The coordinates will be deposited in the Protein Data Bank, Brookhaven National Laboratory.

Structure Analysis. Visual inspection of the structures was aided with the programs O[42] and INSIGHTII[44] on an INDIGO Silicon Graphics workstation. The stereochemistry of the models were examined with PROCHECK 2.1.4.[45]

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