Design, Synthesis, and Structural Analysis of Influenza Neuraminidase Inhibitors Containing Pyrrolidine Cores

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The discovery of (±)-(2*S*,3*R*,4*R*)-2-(trifluoroacetamido)methyl-3-amino-1-(*N*′-ethyl-*N*′-isopropylcarbamyl)pyrrolidine-4-carboxylic acid (A-192558, **20e**) as a potent inhibitor of influenza neuraminidase (NA) is described. Efficient syntheses of two core structures, *cis*-3-(allyloxycarbonyl)amino-1-(9′-fluorenylmethoxycarbonyl)pyrrolidine-4-carboxylic acid (**7**) and *tert*-butyl (±)-(2*S*,3*R*,4*R*)-2-aminomethyl-3-bis(*tert*-butyloxycarbonyl)amino-1-(*N*′-ethyl-*N*′-isopropylcarbamyl)pyrrolidine-4-carboxylate (**18b**), were developed. Starting with these core structures and using available structural information of the NA active site as the guide, analogues were synthesized in both the tri- and tetrasubstituted pyrrolidine series by means of high-throughput parallel synthesis in solid or solution phase for expeditious SAR. These studies accelerated the identification of (±)-(2*S*,3*R*,4*R*)-2-(trifluoroacetamido)methyl-3-amino-1-(*N*-ethyl-*N*-isopropylcarbamyl)pyrrolidine-4-carboxylate (**20e**, A-192558) as the most potent NA inhibitor in this series (IC_{50} = 0.2 μM against NA A and 8 μM against NA B). The X-ray crystallographic structure of A-192558 bound to NA revealed the predicted interaction of the carboxylic group with the positively charged pocket (Arg118, Arg292, Arg371) and interaction of the trifluoroacetamino residue with the hydrophobic pocket (Ile222, Trp178) of the enzyme active site. Surprisingly, the ethyl and isopropyl groups of the urea functionality induced a conformational change of Glu276, turning the Glu276/Glu277 hydrophilic pocket, which normally accommodates the triglycerol side chain of substrate sialic acid, into an induced hydrophobic pocket.

Introduction

Influenza infection has been the cause of some of the worst epidemics in human history and continues to be a major health concern. In the United States alone, 20 000−40 000 people die from the flu, and the illness costs as much as $12 billion a year in health care and lost productivity.[1] Despite these facts, effective and safe antiinfluenza therapeutics are lacking, making antiinfluenza a high-priority and attractive area of drug discovery. In recent years, virology studies of influenza virus have resulted in an improved understanding of the replication mechanism of the virus. Several molecular targets have been identified for drug intervention including haemaagglutinin, neuraminidase (sialidase), M2 protein, and endonuclease.[2] Among those potential targets, neuraminidase (NA) appears to be particularly attractive.[3] NA is one of two glycoproteins expressed on the virus surface and catalyzes the cleavage of sialic acid residues from glycoproteins, glycolipids, and oligosaccharides. The catalytic activity of NA is essential for influenza virus replication and infectivity. It has been shown that NA is required for elution of newly synthesized virons from infected cells and that it facilitates the movement of the virus through the mucus of the respiratory tract.[3] The active site of NA is highly conserved across all influenza A and B virus strains, rendering broad-spectrum antiinfluenza agents possible. Indeed, the effectiveness of NA inhibitors as antiinfluenza agents has been demonstrated both in animal models and in human clinical trails by several research groups and highlighted by recent FDA approval of zanamivir and Tamiflu.[4−7]

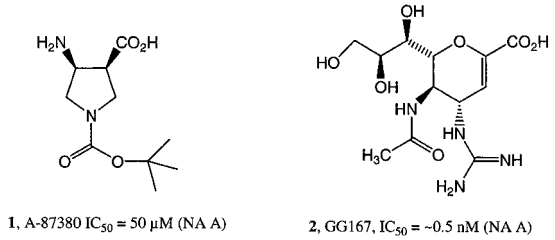

Figure 1. Structure of A-87380 and GG167.

Our effort in this area started with the identification of *cis*-*N*-Boc-3-aminopyrrolidine 4-carboxylic acid (**1**, Figure 1) as a modestly active NA inhibitor (IC_{50} = 50 μM against NA A/Tokyo). Compound **1** was evaluated structurally as a NA inhibitor lead by comparing **1** with NA inhibitors known in the literature at the time, primarily GG167 (**2**, Figure 1) and the related compounds[4,5] and by computer modeling. On the basis of the literature data,[8,9] we have derived an "airplane" model of the NA active site as illustrated in Figure 2 to summarize the basic structural requirements of a potent

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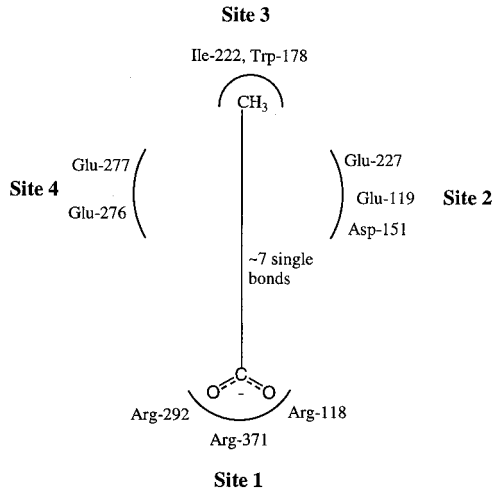
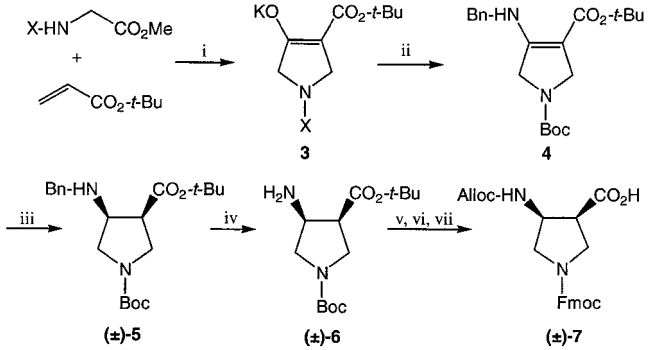


Figure 2. Working model of the NA active site shown in two-dimensional illustration. The center of site 2 is about 6 Å from site 1 and about 4 Å from site 3, while site 4 is about 6 Å from site 1 and 5 Å from site 3. Sites 1 and 3 are separated by 9−10 Å or about 7 single bond lengths. With GG167, the carboxylate and acetyl groups bind to sites 1 and 3, respectively, while the guanidino group interacts with site 2 and the triglycerol side chain interacts with site 4.

NA inhibitor. The active site of NA has four main well-conserved binding sites. The positively charged site 1 consists of Arg118, Arg292, and Arg371 and interacts with the carboxylate of sialic acid or GG167 via charge−charge interaction and hydrogen bonding. The negatively charged site 2 consists of Glu119, Glu227, and Asp151 and interacts with the guanidino group of GG167. The small hydrophobic pocket consisting of Ile222 and Trp178 (site 3) accommodates the acetyl group of sialic acid or GG167, and site 4, consisting of Glu276 and Glu277, binds to the triglycerol side chain of sialic acid or GG167. In the two-dimensional sense, sites 3 and 1 are situated at the head and tail of the "airplane" respectively, separated by 9−10 Å or 6−7 single bond lengths, while sites 2 and 4 are situated at two wings of the "airplane". In the three-dimensional sense, these binding pockets are clearly off-set from the plane defined by the ring of the cyclic nucleus (as illustrated by GG167) since aromatic (planar) mimics of GG167 are poor inhibitors of NA.[10] Literature data indicated that occupation of all four pockets is necessary for potent inhibition.[8]

Analysis of compound **1** using this model clearly indicated some structural deficiencies. Aside from the fact that a five-membered ring is used to present ligands for an enzyme active site that normally accommodates a six-membered ring in both the substrate and the most potent known inhibitor (GG167),[11] compound **1** has only three substituents and therefore can interact with three out of the four binding pockets. Nevertheless, compound **1** has a rigid (cyclic) scaffold, a positively charged group, and a carboxylate, all in common with GG167. Thus, we concluded that compound **1** can serve as a valid lead and set out to optimize the structure, with the ultimate goal of discovering clinically effective oral NA inhibitors. Our strategy was to combine the strength of traditional medicinal chemistry approaches with combinatorial chemistry. Specifically, structural (X-ray crystallography) analysis, computer modeling, and tradition synthesis was used to explore a variety of scaffolds, while

Scheme 1[a]



[a] Reagents and conditions: (i) *t*-BuOK, THF, rt overnight, 85−95%; (ii) $BnNH_3OAc$, HOAc, MeOH, reflux 1 h, 70−80%; (iii) $NaBH_4$, HOAc, 10 °C, 92%; (iv) H_2, Pd/C, 90%; (v) allyl chloroformate, DIEA, DCM, overnight; (vi) TFA, rt 3 h; (vii) Fmoc-Cl, $NaHCO_3$, dioxane−H_2O. Overall yield for steps v−vii was 93%.

combinatorial chemistry, particularly high-throughput parallel synthesis, was used for rapid and comprehensive optimization of a given scaffold.[11] In this paper, we report our effort on the tri- and tetrasubstituted pyrrolidine series using this approach, which led to the discovery of A-192558.

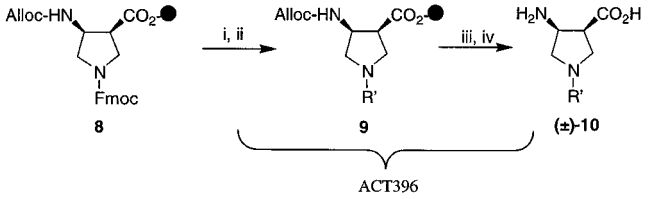
Chemistry

Synthesis of the *cis*-3-Aminopyrrolidine-4-carboxylic Acid Core. Our synthesis of the required trisubstituted pyrrolidine core **7** is shown in Scheme 1. Tandem Michael addition and Dieckmann condensation of Boc-glycine methyl ester and *tert*-butyl acrylate following a modification of the procedure of Kuhn and Oswald gave the desired pyrrolidine β-keto ester **3** in good yield.[12] Compound **3** was converted to *N*-benzyl enamine **4** by treating with benzylamine acetate in methanol. Reduction of enamine **4** was accomplished with $NaBH_4$ in acetic acid in excellent yield, leading to a separable mixture of *cis*- and *trans*-*N*-benzylamino esters in 35:1 ratio, from which the *cis*-isomer **5** was isolated. Debenzylation of **5** by hydrogenolysis then gave compound **6**. The *cis*-configuration of compound **6** was readily established from the large NOE observed between protons on C3 and C4. Further manipulation of protecting groups gave the orthogonally protected diamino acid **7**.

High-Throughput Parallel Synthesis of Trisubstituted Pyrrolidine Analogues. Since a free carboxylic acid residue is required for NA inhibitory activity, our SAR effort for the trisubstituted pyrrolidine core was focused on substitution on two amino groups. With the orthogonal protected core **7** in hand, solid-phase synthesis appeared perfectly suited for this purpose.

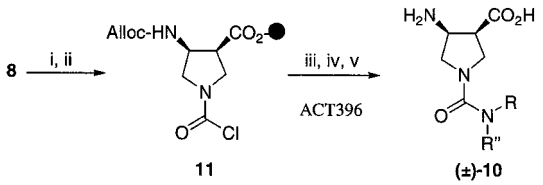
Compound **7** was coupled to standard *p*-hydroxymethylphenoxymethyl resin (Wang resin) following the standard procedure, giving resin-bound core **8**. Conceptually, a combinatorial library using mix-and-split synthesis method can be prepared to co-optimize substitution on both the ring nitrogen and the exocyclic amino group. However, we chose to take the parallel synthesis approach based on a number of considerations. Structurally, substitution on the exocyclic amino group may not be tolerated, and therefore, large num-

Scheme 2[a]



[a] Reagents and conditions: (i) 20% piperidine, DMF, rt 1 h; (ii) RCO_2H, DIC, DCM; or ROCOCl, DIEA, DCM; or RSO_2Cl, DIEA, DCM; or RR″NCOCl, DIEA, DCM; 2−24 h; (iii) $Pd(PPh_3)_4$, Bu_3SnH, DCM, H_2O, 15 min; (iv) 95% TFA, 2.5 h, rt.

Scheme 3[a]



[a] Reagents and conditions: (i) 20% piperidine, DMF, rt 1 h; (ii) 20% phosgene, DCM, DIEA, 0 °C, 1 h; (iii) RNHR″, pyridine, DCM, 3−8 h; (iv) $Pd(PPh_3)_4$, Bu_3SnH, DCM, H_2O, 15 min; (v) 95% TFA, 2.5 h, rt.

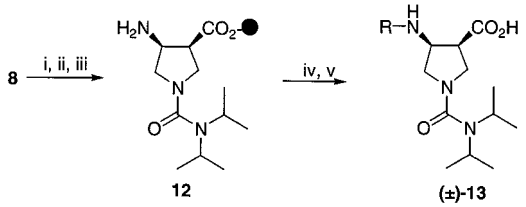
bers of analogues with subsitution at this position may not be warranted. Technically, parallel synthesis of discrete compounds allows routine characterization of the products as well as purification if necessary and, therefore, is well-suited for SAR studies.

Thus, resin-bound core **8**, after deprotection of the ring nitrogen, was functionalized with commercially available carboxylic acids, chloroformates, sulfonyl chlorides, or carbamyl chlorides to give resin-bound amides, carbamates, sulfonamides, or ureas (**9**), respectively (Scheme 2). Additionally, the resin-bound core **8**, after removal of the Fmoc protection with piperidine, was converted to the corresponding carbamyl chloride on resin using a method which we have described[13] and then reacted with primary or secondary amines, allowing preparation of a broader range of ureas (Scheme 3). The Alloc group of the resin-bound compounds **9** was then removed with $Pd(PPh_3)_4$ and *n*-Bu_3SnH in DCM with a catalytic amount of water. The desired products **10** were obtained by cleavage with TFA. All the operations of solid-phase synthesis, including the cleavage step, were carried out fully automated in batches of 32 on a commercial instrument (Advanced ChemTech 396), allowing preparation of about 550 analogues over the course of 2 months. All the products were analyzed by HPLC with both UV and an evaporative light scattering detectors and mass spectrometry before accepted for enzyme inhibition assay.

To examine the effect of substitution on the exocyclic amino group, the substitution on the ring nitrogen was fixed as *N*,*N*-diisopropylurea (vide infra) and the exocyclic amino group was converted into a guanidino moiety using *N*,*N*′-bis(Boc)-1-guanylpyrazole (**13a**) or *N*,*N*-dimethylurea using *N*,*N*-dimethylcarbamyl chloride (**13e**), or alkylated via reductive amination (**13b**−**d**) (Scheme 4). Guanidination of primary amines on a solid support with *N*,*N*′-bis(Boc)-1-guanylpyrazole has been described recently.[14]

Synthesis of Tetrasubstituted Pyrrolidines. The synthesis of the desired tetrasubstituted pyrrolidine core

Scheme 4[a]



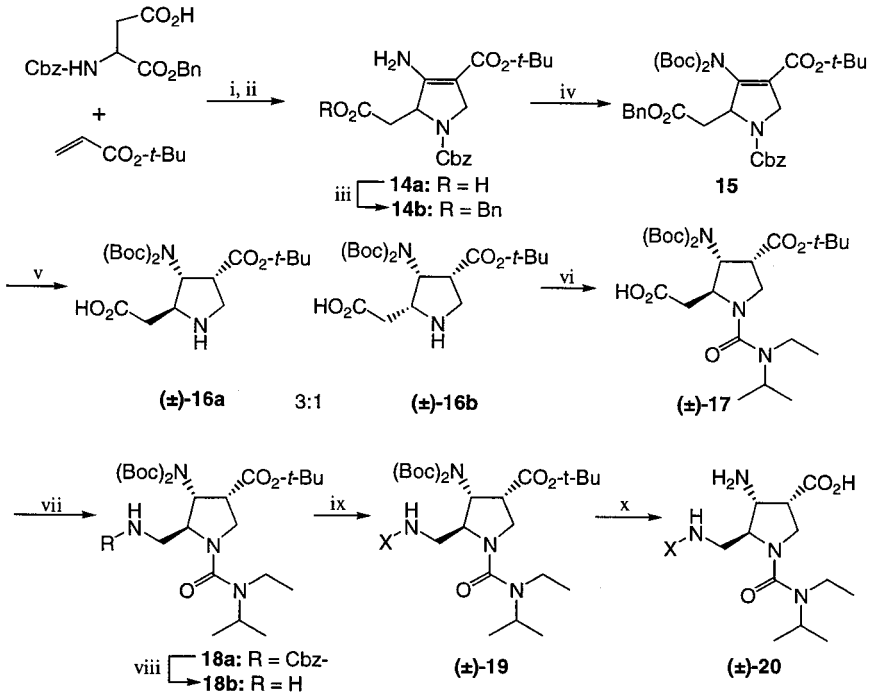
[a] Reagents and conditions: (i) 20% piperidine, DMF, rt 1 h; (ii) *N*,*N*-diisopropylcarbamyl chloride, pyridine, DCM, 4 h; (iii) $Pd(PPh_3)_4$, Bu_3SnH, DCM, H_2O, 15 min; (iv) *N*,*N*′-bis(Boc)-1-guanylpyrazole, DIEA, DMF, 50 °C (**13a**); RCHO, DMA, $NaBH(OAc)_3$, HOAc (**13b**−**d**); Me_2NCOCl, DIEA, DCM (**13e**); (v) 95% TFA, 2.5 h, rt.

structure **18** also utilized the tandem Michael addition and Dieckmann condensation between amino acid and acrylate as the key step (Scheme 5).[15] Initially, *N*-Cbz-serine methyl ester with or without side chain hydroxyl protection was tried as the condensation partner, but the reaction was complicated by β-elimination of the hydroxyl group. This problem was circumvented by employing benzyl *N*-Cbz-α-glutamate, which reacted with *tert*-butyl acrylate in the presence of *t*-BuOK to give *tert*-butyl *N*-Cbz-2-(carboxymethyl)-3-oxopyrrolidine-4-carboxylate in acceptable yield. This very polar material was treated directly with ammonium acetate in refluxing methanol, leading to enamino ester **14a** which was subsequently converted to the corresponding benzyl ester **14b** with benzyl bromide. Convertion of acid **14a** to ester **14b** seemed an unnecessary step, but it facilitated purification of the material at this stage.[17] The enamino group of **14b** was then protected as the bis(Boc) derivative to give **15**, and hydrogenation of **15** with Pd/C in acetic acid gave two diastereomers **16a**,**b** in good yield. The diastereomeric amino acids **16a**,**b** were inseparable chromatographically, and the ratio was determined by NMR studies to be 3:1. This observed selectivity in favor of the desired (±)-(2*S*,3*R*,4*R*) diastereomer contradicted what would be predicted based on steric factors. We postulated that the debenzylation of the side chain benzyl ester precedes the reduction of the double bond and the free carboxylate group partially controls the relative sterochemistry by chelating with the catalyst, giving the observed stereoselectivity. In contrast, hydrogenation of the analogous enamine **21** (derived from *N*-Cbz-L-leucine methyl ester and *tert*-butyl acrylate following similar procedures) under identical conditions gave two separatable diastereomers **22a**,**b** in a ratio of 1:1.2, as predicted based on steric consideration (Scheme 6).

We again chose to fix the ring nitrogen substitution based on the results of optimizing the trisubstituted pyrrolidines **10** (Scheme 3 and Table 1). Accordingly, a mixture of **16a**,**b** was converted to the corresponding *N*-ethyl-*N*-isopropylureas, from which the desired (±)-(2*S*,3*R*,4*R*) diastereomer **17** (higher R_f, major isomer) was isolated. Curtius rearrangement using diphenylphosphoryl azide in the presence of benzyl alcohol gave the Cbz-protected amine **18a** which upon hydrogenolysis gave the free amine **18b**.

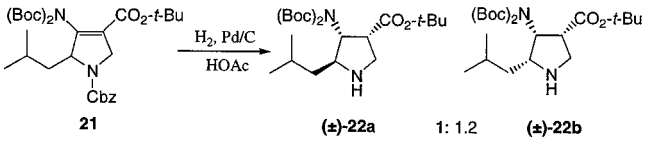
With compound **18b** on hand, a library of analogues then became readily accessible. Since we intended to project a ligand into the hydrophobic pocket (site 3, Figure 1) via the C2 aminomethyl group and the small

Scheme 5[a]



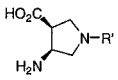
[a] Reagents and conditions: (i) *t*-BuOK, THF, rt 24 h; (ii) H_4NOAc, MeOH, reflux 3 h; (iii) BnBr, DIEA, DCM, reflux 2 days (30−50% yield for steps i−iii); (iv) $(Boc)_2O$, DMAP, DCM, 24 h (40−50% yield); (v) H_2, Pd, HOAc (80% yield); (vi) *N*-ethyl-*N*-isopropylcarbamyl chloride, pyridine, DCM, 4 h (82% yield); (vii) DPPA, Et_3N, BnOH, toluene, 100 °C, 16 h; (viii) H_2, Pd (70% yield for steps vii and viii); (ix) RCO_2H, EDC, DCM; or ROCOCl, DIEA, DCM; or RSO_2Cl, DIEA, DCM; or RR″NCOCl, DIEA, DCM; 2−24 h; (x) 95% TFA, 2.5 h, rt.

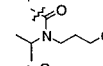
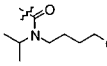
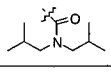
Scheme 6



volume of this pocket, a relatively small set of analogues was required to fully optimize the structure. Consequently, solution-phase parallel synthesis starting with **18b** was apparently the method of choice for library synthesis. Thus, **18b** was treated with a set of amine-reactive reagents (carboxylic acids, sulfonyl chlorides, chloroformates, and carbamyl chlorides) to give **19** (a library of 70 compounds). Compounds **19** were purified by extraction and chromatography using silica gel cartridges. TFA deprotection then gave the designed inhibitors **20**. All samples of **20** were analyzed with HPLC and mass spectrometry. Samples with HPLC purity better than 75% were accepted for biological testing (70 compounds out of 76 attempted syntheses met this criteria).

Table 1. NA Inhibitory Activity of Selected Trisubstituted Pyrrolidines (±)-**10**



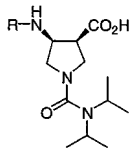
no.	R'	IC_{50} (μM) (NA A/Tokyo)	no.	R'	IC_{50} (μM) (NA A/Tokyo)
10a		22	10h		21
10b		25	10i		2.1
10c		32	10j		2.0
10d		26% inhibition at 50 μM	10k		19
10e		1.6	10L		1.3
10f		4	10m		46
10g		25% inhibition at 50 μM	10n		1.3

Results and Discussion

Trisubstituted Pyrrolidine Series. On the basis of computer modeling, we anticipated that the $-CO_2H$ group of our lead structure **1** interacts with the positively charged site 1 of the NA active site (Figure 2), the exocyclic NH_2 group binds to the negatively charged site 2, and the Boc group interacts with the hydrophobic site 3 via one of the methyl groups. That leaves the polar site 4 unoccupied. Consequently, we focused our initial effort primarily on optimizing the ring nitrogen substitution of **1**, with the goal of identifying substituents that can interact with both sites 3 and 4 of the NA active site. Accordingly, larger numbers (∼550) of analogues of **1** were synthesized, including amides (∼250), carbamates (∼25), sulfonamides (∼25), and ureas (∼250). Automated solid-phase parallel synthesis (Schemes 2 and 3) turned out to be an excellent approach for this purpose due to its efficiency and simplicity.

In selecting monomers for the functionalization of the ring nitrogen of **8**, considerations were given to achieving both diversity and the structural requirements of NA active site. This was particularly important for carboxylic acids (amide formation) and amines (urea

Table 2. NA Inhibitory Activity of Selected Trisubstituted Pyrrolidines (±)-**13**



no.	R	IC_{50} (μM) (NA A/Tokyo)	no.	R	IC_{50} (μM) (NA A/Tokyo)
13a		13	13d	$PhCH_2$-	<10% inhibition at 50 μM
13b	CH_3-	<10% inhibition at 50 μM	13e		<10% inhibition at 50 μM
13c	n-C_4H_9-	<10% inhibition at 50 μM	13f		21% inhibition at 50 μM

formation), for which large pools were commercially available. Thus, large numbers of aliphatic carboxylic acids and amines with subtle structural variations were employed to fine-tune the interaction with the hydrophobic pocket (Figure 2). At the same time, a variety of monomers with aromatic or polar functional groups were chosen in order to discover unexpected active structures.

The vast majority of compounds **10** synthesized were inactive as NA inhibitors (IC_{50} > 100 μM). Compounds **10** with IC_{50} lower than 50 μM against NA A/Tokyo are summarized in Table 1, along with several closely related inactive compounds. Of all the amides synthesized, the only active compound was the 2-ethylbutyric acid derivative **10a** (IC_{50} = 22 μM). The ureas turned out to be more productive, with nearly a dozen compounds showing IC_{50} below 50 μM. The most active compounds (**10e**,**l**) showed IC_{50} of about 1.5 μM, representing about 30-fold improvement in potency over our lead **1**. Further examination of the data in Table 1 indicated a clear preference for α-branched amides or ureas derived from aliphatic secondary amines. In the urea series, isopropyl was clearly optimal as one of the N-substituents (**10b**−**f**), while the other N-substituent can be a variety of groups larger than methyl. It is noteworthy that ureas of primary amines, including propylamine, are inevitably inactive. Similar observations have been reported in pyrancarboxamides related to GG167.[6b] No active compounds were identified from the carbamate or sulfonamide series, most likely a reflection of the fact that all the chloroformates and sulfonyl chlorides employed are either aromatic or aliphatic with straight chains.

Compounds **10h**−**n** were specially designed for concomitant interaction with both the hydrophobic site 2 (via the isopropyl group) and the polar site 4 of the active site. Evidently, this strategy failed to yield the desired outcome. Despite the variation in both charge characteristics and the chain length of the second N-substituent, the activity of these compounds fluctuates within a range of 1.3−20 μM. This result seemingly suggests that the polar residues have no interaction with the enzyme and point toward the solvent. This was proven by X-ray crystal structure (vide infra).

A limited number of compounds with substitution on the exocyclic amino group were prepared (Scheme 4 and

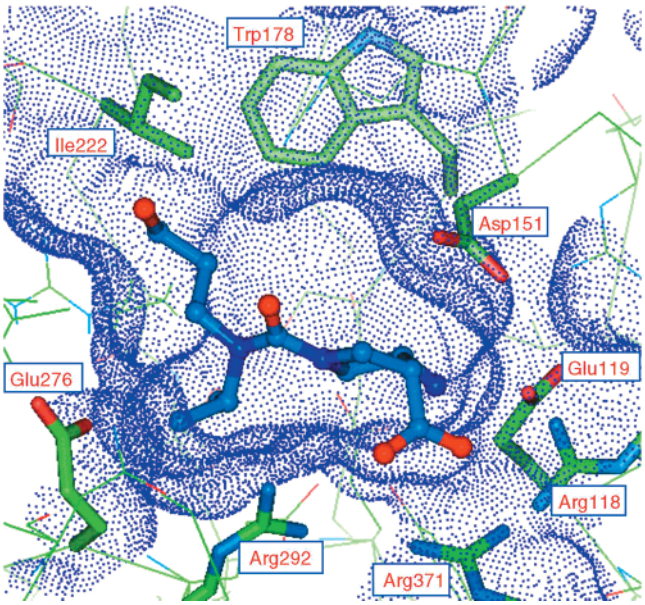


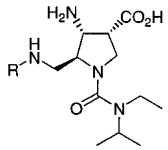
Figure 3. X-ray crystallographic structure (active site only) of the complex of trisubstituted pyrrolidine **10i** with NA A.

Table 2). For the GG167 (Figure 1) series of compounds, it has been reported that conversion of 4-amino-Neu5Ac2en to 4-guanidino-Neu5Ac2en (GG167) resulted in a 40-fold increase in NA inhibitory activity.[8b] In contrast, when **10f** (IC_{50} = 4 μM) was converted to the corresponding guanidine (**13a**), a small loss of activity was observed. Alkylation or acylation of the exocyclic amino group led to inactive compounds.

To gain insight of the interaction of this series of compounds with the NA active site, the structure of compound **10i** bound to N9 A/Tern NA was solved using X-ray crystallography to a resolution of 2.8 Å (Figure 3). As expected, the carboxylate of **10i** interacts strongly with the three arginines of site 1 (Arg118, Arg292, Arg371) through charge−charge interaction. The exocyclic 3-amino group forms hydrogen bonds with Glu119 of site 2 and interacts electrostatically with Asp151. The most surprising discovery from this structure is, however, the disposition of the urea functionality of **10i**. Contrary to our initial expectation, the isopropyl group does not occupy the hydrophobic pocket formed by the side chains of Ile222 and Trp178 (site 3, Figure 2). Instead, this group is projected into site 4 and forces a conformational change of the side chain of Glu276, creating a new (induced) hydrophobic pocket. Since our observation, this same phenomenon has also been reported with several compounds containing six-membered rings with hydrophobic residues replacing the triglycerol side chain of GG167 (Figure 1).[4,6] The 3′-hydroxypropyl group, the second N-substituent of the urea functionality of **10i**, indeed points toward the solvent.

Tetrasubstituted Pyrrolidine Series. Even though our effort with the trisubstituted pyrrolidine series led to only 30-fold enhancement in NA inhibitory activity (Table 1), we were encouraged by the findings of the X-ray structure of the **10i** and NA A complex (Figure 3). It has been reported that GG167 is not orally bioavailable.[5] The extreme hydrophilicity of the compound (calculated log P = −7.1) is undoubtedly a contributing factor. Thus, placing a hydrophobic group

Table 3. NA Inhibitory Activity of Selected Tetrasubstituted Pyrrolidines (±)-**20**



no.	R	IC_{50} (μM) (NA A/Tokyo)	IC_{50} (μM) (NA B/Memphis)
20a	CH_3CO-	7.5	340
20b	CH_3CH_2CO-	16	26% inhib. at 1 mM
20c	CH_2=CHCO-	96	Not determined
20d	$(CH_3)_2CHCO$-	39% inhib. at 0.5 mM	Not determined
20e	CF_3CO- (A-192558)	0.28	8
20f	CH_3SO_2-	130	38% inhib. at 0.5 mM

(e.g. isopropyl) into site 4 to replace polar residues such as the triglycerol side chain of GG167 represented an important step toward the goal of orally bioavailable NA inhibitors. Furthermore, the X-ray structure (Figure 3) revealed that compound **10i** did not occupy the hydrophobic site 3. This hydrophobic interaction was known to be very important, contributing about 3 orders of magnitude to the binding affinity of GG167.[8b] Thus, it was conceivable that modified pyrrolidines permitting this interaction would bring the desired enhancement in potency. From the structure shown in Figure 3, it appeared that a lipophilic residue placed at the C2 position of the pyrrolidine ring *trans* to the 3-amino and 4-carboxylate would be capable of interacting with site 3. The tetrasubstituted pyrrolidine core **18b** was designed on the basis of this premise.

With the synthesis of tetrasubstituted pyrrolidine core **18b** accomplished (Scheme 5), solution-phase parallel synthesis was utilized to prepare a library of 70 derivatives **20**, including amides, sulfonamides, and some ureas. The SARs of these compounds are summarized in Table 3. Not surprisingly, compounds **20** with an acetamido (**20a**) or trifluoroacetamido (**20e**) group were found to be the only actives from the series. The activity falls off rapidly with the increasing size of the C2 substituents, reflecting the small size as well as the rigidity of site 3 of the NA active site (Figure 2). The trifluoroacetamino-functionalized **20e** is 26- and 40-fold more active than the acetamino-functionalized **20a** against NA A and NA B, respectively.

The most active tetrasubstituted pyrrolidine, **20e** (A-192558), was soaked into a crystal of N9 A/Tern NA, and the structure of the complex was determined, as shown in Figure 4. The expected interaction of the carboxylate with the positively charged site 1 was readily observed. The urea functionality again interacts with site 4 hydrophobically by inducing a conformational change of Glu276, as with compound **10i** (Figure 3). The C2 trifluoroacetamido group occupies the hydrophobic pocket (site 3), meeting our design goal. However, the pyrrolidine ring of **20e** unexpectedly rotated by about 90° relative to the trisubstituted **10i**.

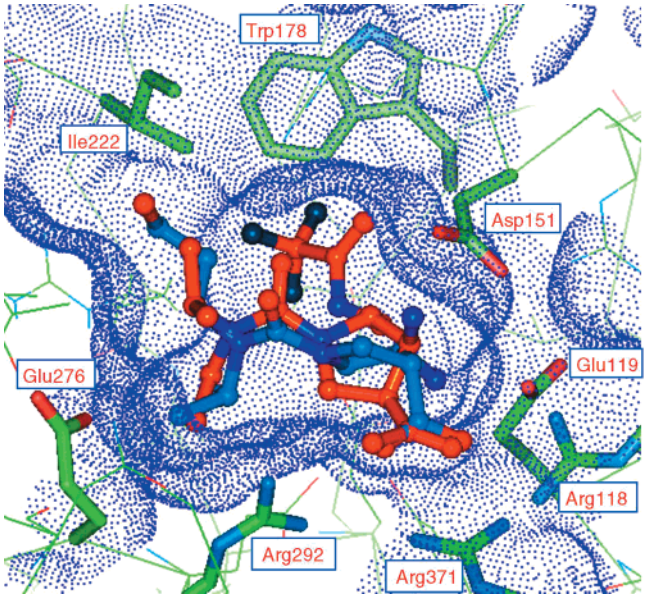


Figure 4. X-ray crystallographic structure (active site only) of the complex of tetrasubstituted pyrrolidine **20e** with NA A. Compound **10i** was also shown for comparison.

This rotation turned the exocyclic amino group away from a position for optimal interaction with all three acidic amino acid residues of site 2 (Asp151, Glu119, Glu227), diminishing binding contribution from this site.

The data in Table 3 also indicate that the tetrasubstituted pyrrolidines are substantially more potent inhibitors of NA A than NA B, an undesirable feature. This phenomenon has been reported in other series of NA inhibitors where the induced hydrophobic pocket is seen.[6,7] Therefore, one of the challenges for future work is to improve the potency against both NA A and NA B simultaneously.

Conclusion

Starting from the initial lead **1**, a sub-micromolar NA inhibitor **20e** was discovered employing a combination of structure-based drug design and combinatorial chemistry (parallel synthesis). Compound **20e** represents a 250-fold improvement in terms of activity against NA A over the initial lead **1**.

Several reasons can be cited to explain why the tetrasubstituted pyrrolidines **20** failed to show further improvement in NA inhibitory activity. Fixing the substitution on the ring nitrogen of **20** as the *N*-ethyl-*N*-isopropyl might be one of the factors. This residue was optimized for the trisubstituted series **10** but may not be optimal for the tetrasubstituted series **20**. We addressed this question by synthesizing a combinatorial library which varied substitutions on both the ring nitrogen and the C2 aminomethyl group simultaneously, as described elsewhere.[18] Another important factor is the questionable contribution to binding from the exocyclic amino group. As mentioned previously, the X-ray crystallographic structures (Figures 3 and 4) indicated that this amino group was not in the optimal position for maximum interaction with site 2 in either the tri- or tetrasubstituted series. Indeed, control compounds in which the exocyclic amino group of **20e** was removed had essentially identical NA inhibitory activity

as the parent **20e**, indicating that the exocyclic amino group makes negligible contribution to binding.[19] This indicates that even the tetrasubstituted pyrrolidine **20e** still acts as a three-site binder, consistent with its high-nanomolar activity. These observations clearly illustrate the challenge of designing potent NA inhibitors based on a five-membered ring scaffold: how to occupy all four binding pockets of the NA active site simultaneously in an optimal way. Indeed, our group came to the realization that designing compounds using GG167 as a template and focusing on solving the oral bioavailability problem would be a more productive avenue,[20] as reported subsequently by the Gilead group.[6,7] Within the perimeter of the five-membered ring scaffold, the only way to address this challenge is to synthesize a series of core structures varying the substitution pattern in order to identify a structure that can project all four required substituents into the corresponding binding pockets. The results of these efforts will be described in future reports.

Experimental Section

General Methods. Commercially available solvents were used as received and reagents were purchased from Aldrich Chem. Co. (Milwaukee, WI), unless noted otherwise. Column chromatography was performed with the solvent systems indicated using E. Merck silica gel 60 (70−230 mesh). Unless noted otherwise, all the reactions were conducted under an atmosphere of nitrogen. ^{1}H NMR spectra were recorded on a GE AE-300 (300 MHz) using tetramethylsilane as an internal standard. Elementary analyses were performed by Robertson Micolit Laboratories, Madison, NJ. Abbreviations: Cbz, (benzyloxy)carbonyl; Fmoc, fluorenylmethyloxycarbonyl; DMF, *N*,*N*-dimethylformamide; NMP, *N*-methylpyrrolidone; DMAP, 4-(dimethylamino)pyridine; EDCI, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid.

***tert*-Butyl *N*-Boc-3-oxopyrrolidine-4-carboxylate Potassium Salt (3).** A solution of Boc-glycine methyl ester (37 g, 0.199 mol) and *tert*-butyl acrylate (29.75 mL, 0.199 mol) in 400 mL of anhydrous THF (Aldrich SureSeal Grade) was cooled to 0 °C. Potassium *t*-butoxide (25.86 g, 0.219 mol) was added portionwise. The mixture was allowed to warm to ambient temperature and stirred with a mechanical stirrer for 24 h. Hexane (300 mL) was added to the thick mixture and the solid was collected by filtration and washed twice with diethyl ether. The dry solid weighed 56 g and was used directly for the next step. MS (DCI-NH_3): *m*/*z* 303 (M + NH_4), base peak. This material is a mixture of two regioisomers.

***tert*-Butyl *N*-Boc-3-benzylamino-3-pyrroline-4-carboxylate (4).** A solution of compound **3** (41 g, 0.127 mol) and benzylamine acetate (106 g, 0.63 mol) in 400 mL of MeOH and 7.3 mL of HOAc was refluxed for 1.5 h. The mixture was concentrated and the residue was partitioned between 300 mL of water and 300 mL of EtOAc. Two layers were separated and the aqueous layer was extracted with EtOAc (3 × 135 mL). The combined organic solution was dried ($MgSO_4$), filtered and concentrated to give a pale yellow solid. Column chromatography on silica gel eluting with 10% EtOAc in hexane gave **4** as a white solid (37 g, 78.7%). Further elution with 30% EtOAc in hexane give the minor regioisomer (6.0 g). MS (DCI-NH_3): *m*/*z* 375 (M + H), *m*/*z* 319 (M − C_4H_9). ^{1}H NMR ($CDCl_3$): 1.47 (s, 9H), 1.48 (s, 9H), 4.13 (m, 2H), 4.20 (m, 1H), 4.25 (m, 1H), 4.30 (m, 3H), 7.30 (m, 5H). Anal. ($C_{21}H_{30}N_2O_4$) C, H, N.

***tert*-Butyl *cis*-*N*-Boc-3-benzylaminopyrrolidine-4-carboxylate (5).** A three-necked flask, fitted with a thermometer and rubber septum, was charged with HOAc and cooled to 10−15 °C. $NaBH_4$ (10.8 g, 0.284 mol) was added portionwise slowly so that the internal temperature was kept below 20 °C. The solution was stirred for 30 min. Compound **4** (35.5 g, 94.8 mmol) was then added in one portion. The solution was stirred at 10−15 °C for 6 h, when TLC (30% EtOAc−hexane) indicated complete conversion of the starting material. Acetic acid was evaporated. The residue was cooled with an ice bath and 200 mL of saturated aqueous Na_2CO_3 was carefully added. The mixture was then extracted with DCM (4 × 200 mL). The combined organic solution was dried ($MgSO_4$), filtered and concentrated. The crude product was purified by chromatography (silica gel, 10% EtOAc−hexane, then 30% EtOAc) to give **5** (33.0 g, 92%, white solid) and 1.3 g (3.7%) of the *trans*-isomer. MS (DCI-NH_3): *m*/*z* 377 (M + H), base peak. ^{1}H NMR ($CDCl_3$): 1.48 (s, 9H), 1.49 (s, 9H), 2.17 (bs, 1H), 3.08−3.12 (m, 1H), 3.45−3.55 (m, 4H), 3.60−3.78 (m, 1H), 3.85 (s, 2H), 7.32 (m, 5H). In DMSO-d_6, strong NOE was observed between C3 H (δ 3.38) and C4 H (δ 3.09). Anal. ($C_{21}H_{32}N_2O_4$) C, H, N.

***tert*-Butyl *cis*-*N*-Boc-3-aminopyrrolidine-4-carboxylate (6).** Compound **5** (11.5 g, 30.6 mmol) was hydrogenated in MeOH (250 mL) with 10% Pd/C overnight. After filtration of the catalyst, evaporation of the solvent gave the crude product which was purified by column chromatography (5% MeOH in EtOAc) to give a white solid (7.8 g, 90.5%). MS (DCI-NH_3): *m*/*z* 287 (M + H), base peak. ^{1}H NMR ($CDCl_3$): 1.48 (s, 9H), 1.49 (s, 9H), 3.00 (m, 1H), 3.30 (m, 1H), 3.50−3.70 (m, 3H), 3.90 (m, 1H). Anal. ($C_{14}H_{26}N_2O_4$) C, H, N.

***cis*-3-(Allyloxycarbonyl)amino-1-(9′-fluorenylmethoxycarbonyl)pyrrolidine-4-carboxylic Acid (7).** To a solution of compound **6** (8.0 g, 27.9 mmol) in DCM (140 mL) was added diallyl dicarbonate (11.5 mL, 30 mmol) and the solution was stirred for 4 h. The solvent was evaporated and the residue was directly purified by column chromatography (5−30% EtOAc in hexane) to give 11.1 g of a white solid (~100%). MS (DCI-NH_3): *m*/*z* 371 (M + H), *m*/*z* 388 (M + NH_4). ^{1}H NMR ($CDCl_3$): 1.47 (s, 9H), 1.48 (s, 9H), 3.12 (m, 1H), 3.30−3.48 (m, 1H), 3.50−3.70 (m, 3H), 4.4−4.5 (m, 1H), 4.58 (d, 2H), 5.20−5.38 (d. d, 2H, 5.80−6.00 (m, 1H).

The material from above was treated with 100 mL of 95% TFA−water at ambient temperature for 2.5 h. The solvent was evaporated under vacuum. The oily material was dissolved in 250 mL of 10% Na_2CO_3 and 150 mL of dioxane. *N*-(9-Fluorenylmethoxycarbonyloxy)succinimide (10.4 g, 30.7 mmol) was added. The solution was stirred at room temperature overnight, washed with diethyl ether (2 × 300 mL), and adjusted to pH 1−2 with concentrated HCl. The mixture was then extracted with EtOAc (3 × 150 mL). The combined organic solution was dried ($MgSO_4$), filtered and concentrated. Column chromatography gave a white solid, 11.4 g (93% from **6**). MS (FAB+ K): *m*/*z* 475 (M + K), *m*/*z* 513 (M + 2K − H). ^{1}H NMR ($CDCl_3$): 3.25−3.35 (m, 1H), 3.40−3.50 (m, 1H), 3.65−3.80 (m, 3H), 4.15−4.25 (m, 1H), 4.35−4.45 (d, 2H), 4.50−4.70 (m, 2H), 5.15−5.40 (m, 2H), 5.55−5.65 (m, 1H), 5.80−5.98 (m, 1H), 6.85 (bs, 1H), 7.28−7.34 (t, 2H), 7.35−7.42 (t, 2H), 7.54, 7.56 (d, 2H), 7.74, 7.76 (d, 2H).

Loading of 7 to *p*-Hydroxymethylphenoxymethyl Resin (8). A mixture of *p*-hydroxymethylphenoxymethyl resin (Wang resin, 0.73 mmol/g, 15.0 g, 10.95 mmol; NovaBiochem, San Diego, CA) and 100 mL of DCM was shaken for 0.5 h. Compound **7** (7.17 g, 16.43 mmol) was then added, followed by diisopropylcarbodiimide (2.57 mL, 16.43 mmol) and DMAP (134 mg, 1.1 mmol). The mixture was shaken overnight. The liquid was drained and the resin was washed 3 times with DCM. The resin was then treated with 10% Ac_2O in DCM (100 mL) for 0.5 h, drained, and washed again with DCM 3 times, and dried in vacuo. The dry resin weighed 19.2 g. Fmoc test gave a loading of 0.52 mmol/g.[21]

***N*-Acylated *cis*-3-(Allyloxycarbonyl)aminopyrrolidine-4-carboxylate Wang Resin (9).** For removal of the Fmoc, resin **8** (19.0 g, 0.52 mmol/g) was treated with 190 mL of 25% piperidine in NMP for 1 h. After draining, the resin was washed with DMF (3 × 100 mL), MeOH (3 × 100 mL) and DCM (3 × 100 mL) and dried.

On an Advanced ChemTech 396 multiple peptide synthesizer (Advanced ChemTech, Louisville, KY), 80−100 mg of the above deprotected resin was placed in each reaction vessel (RV) (typically in batches of 32 reactions). The instrument was programmed to add 1.0 mL of 80% DCM−NMP. For coupling

of carboxylic acids, the instrument was programmed to add 3 equiv of selected carboxylic acids in 0.25 mL of NMP and 3 equiv of diisopropylcarbodiimide to the corresponding RV. For coupling of chloroformates, sulfonyl chlorides or carbamyl chlorides, the instrument was programmed to add 3 equiv of each monomer in 0.25 mL NMP and 3 equiv of diisopropylethylamine to the corresponding RV. The RV block was then programmed to shake intermittently for 2−24 h, followed by programmed washing with NMP (5 × 1 mL), MeOH (5 × 1 mL) and DCM (5 × 1 mL). Randomly chosen resins were subjected to the Chloranil Test to confirm the completion of acylation.[22]

***N*-Acylated *cis*-3-Aminopyrrolidine-4-carboxylic Acid (10).** At the end of synthesis of resins **9** described above, 1.5 mL of a solution of $(PPh_3)_4Pd$ in DCM (10 mg/mL) was manually added to each RV via a syringe, followed by manual addition of 0.1 mL of water and 0.1 mL of *n*-Bu_3SnH. The instrument was programmed to shake the RV block for 0.5 h, wash the RVs with NMP (5 × 1.0 mL), MeOH (5 × 1.0 mL) and DCM (5 × 1.0 mL). The instrument was then fitted with a cleavage block and programmed to deliver 1.5 mL of TFA to each RV, shake the RV block for 2.5 h, and drain the TFA solutions into receiving vessels. The TFA solutions were then concentrated on a speedvac to dryness. Each resulting products were split into three portions, one for HPLC analysis, one for MS analysis and the major portion for registration and biological testing.

***cis*-3-Amino-1-(*N′*-ethyl-*N′*-isopropylcarbamyl)pyrrolidine-4-carboxylic acid (10e):** 1.06 (t, J = 7.1 Hz, 3H), 1.16 (2 d, J = 6.4 Hz, 6H), 3.09−3.23 (m, 2H), 3.43−3.50 (m, 1H), 3.53−3.58 (d.d, J = 4.1 and 11.9 Hz, 1H), 3.74−3.83 (m, 2H), 3.86−3.99 (hep., J = 6.4 Hz, 1H), 4.10−4.15 (m, 1H). HRMS: $C_{11}H_{22}N_3O_3$, calcd 244.1661, found 244.1664.

Preparation of Carbamyl Chloride on Resin (11). This was carried out according to previously described procedure.[13]

***cis*-3-Amino-1-(*N′*,*N′*-diisopropylcarbamyl)pyrrolidine-4-carboxylic Acid on Wang Resin (12).** Resin **8** was deprotected with piperidine as described above. The deprotected resin (2.0 g) was treated with 5 equiv each of diisopropylcarbamyl chloride and diisopropylethylamine in DCM (8 mL) in a peptide flask overnight. The mixture was then drained and the resin was washed with NMP, MeOH and DCM (3 times each) and vacuum-dried. Chloranil test was negative. This resin was then subjected to the $(PPh_3)_4Pd-H_2O-Bu_3SnH$ procedure described above to remove the Alloc group, and then washed and dried.

Preparation of 13. 13a: To a mixture of resin **12** (100 mg, 0.05 mmol) and NMP (1.5 mL) were added *N*,*N′*-bis(Boc)-1-guanylpyrazole (77.5 mg, 0.25 mmol) and DIEA (44 μL, 0.25 mmol). The mixture was shaken at 50 °C overnight, when the ninhydrin test of the resin turned negative. The mixture was then filtered and the resin was washed with NMP, MeOH and DCM (3 times each) and vacuum-dried. This resin was then treated with 95% TFA−water to effect deprotection and cleavage. The TFA solution was concentrated to give an oil from which 9.0 mg of a solid was obtained upon trituration with Et_2O−hexane. MS (DCI-NH_3): m/z 300 (M + H) and m/z 317 (M + NH_4). The parent amine was not observed.

13b−d: To a mixture of resin **12** (100 mg, 0.05 mmol) and 5% HOAc in NMP (1.5 mL) was added 20 equiv of the required aldehyde. The mixture was shaken for 2 h and drained, and this treatment was repeated one more time. The resin was then suspended in 2.0 mL of NMP and $NaBH_3CN$ (16 mg, 0.25 mmol) was added. The mixture was then shaken overnight and drained. The resin was washed with NMP, MeOH and DCM (3 times each) and vacuum-dried. TFA cleavage (rt, 2.5 h) gave the desired products which were characterized by HPLC and MS.

***tert*-Butyl *N*-Cbz-2-(benzyloxycarbonyl)methyl-3-amino-3-pyrroline-4-carboxylate (14b).** A solution of *N*-Cbz-L-Asp-α-OBn (NovaBiochem, San Diego, CA; 100 g, 0.278 mol) and *tert*-butyl acrylate (90 mL, 0.56 mol) in 500 mL of THF was cooled to 0 °C and *t*-BuOK (72 g, 0.56 mol) was added portionwise over 0.5 h. After stirring overnight at ambient temperature, the solvent was evaporated. The residue was taken up in 300 mL of water and the solution was adjusted to pH 2−3, followed by extration with EtOAc (4 × 200 mL). The EtOAc solution was then dried, filtered and concentrated to give a brown oil (∼130 g). MS (DCI-NH_3): m/z 395 (M + H) of the keto ester as base peak.

The crude keto ester from above was mixed with 500 mL of MeOH and 107 g of NH_4OAc. The solution was refluxed for 5 h. After evaporation of the MeOH, the residue was mixed with water (100 mL) and extracted with EtOAc (4 × 150 mL). The combined EtOAc solution was dried, filtered and concentrated to give crude **14a** as a brown oil, which was directly used for the next step.

To a solution of crude **14a** from above (∼89 g, ∼0.237 mol) in 500 mL of DCM were added benzyl bromide (49 g, 0.28 mol) and DIEA (40.9 mL, 0.47 mol). The solution was refluxed for 2 days. DCM was evaporated and the residue was dissolved with water (200 mL) and EtOAc (300 mL). Two layers were separated and the aqueous layer was further extracted with EtOAc (3 × 100 mL). The combined EtOAc solution was worked up by drying, filtration and solvent removal. Flash chromatography of the crude product on a silica gel column using 20% EtOAc in hexane gave **14b** as a clear oil (36 g, 27.8% for three steps). Another product with lower R_f was also isolated (15 g) which was identified as the benzyl *N*-Cbz-2-(benzyloxycarbonyl)methyl-3-amino-3-pyrroline-4-carboxylate. **14b** MS (DCI-NH_3): m/z 467 (M + H), m/z 484 (M + NH_4). ^{1}H NMR ($CDCl_3$): 1.43 (s, 9H), 2.62 (d, 1H), 2.66 (d, 1H), 3.38 (d, 1H), 4.10 (d, 1H), 4.24 (d, 1H), 5.16 (bs, 4H), 6.0 (bs, 2H), 7.20−7.42 (m, 10H).

***tert*-Butyl *N*-Cbz-2-(benzyloxycarbonyl)methyl-3-bis-(butyloxycarbonyl)amino-3-pyrroline-4-carboxylate (15)** To a solution of 1**4b** (14 g, 30 mmol) in 100 mL of DCM at 0 °C was added di-*tert*-butyl dicarbonate (20 g, 90 mmol), followed by DMAP (0.65 g). The solution was stirred at ambient temperature overnight and concentrated. Flash chromatography on silica using 15% EtOAc as the eluent gave **15** as a thick oil which solidified upon standing (9.8 g, 49.0%). MS (DCI-NH_3): m/z 667 (M + H), m/z 684 (M + NH_4), m/z 567 (M − Boc + H), base peak. ^{1}H NMR ($CDCl_3$): 1.43 (s, 27H), 2.70−2.82, 3.00−3.08 (m, 2H), 4.22−4.40 (m, 1H), 4.40−4.50 (m, 1H), 5.00 (bs.1H), 5.10−5.30 (m, 4H), 7.20−7.40 (m, 10H).

(±)-(2*S*,3*R*,4*R*)- and (±)-(2*R*,3*R*,4*R*)-*tert*-Butyl 2-Carboxymethyl-3-bis(butyloxycarbonyl)amino-3-pyrrolidine-4-carboxylate (mixture of 16a,b). A solution of compound **15** (19 g, 28.5 mmol) in HOAc (500 mL) was mixed with 40 g of 10% Pd/C and the mixture was hydrogenated at 4 atm of H_2 overnight. After removal of the catalyst, the solution was concentrated and dried in vacuo to give a solid (14 g). This crude mixture of **16a**,**b** was used directly for the next step.

***tert*-Butyl (±)-(2*S*,3*R*,4*R*)-2-Carboxymethyl-3-bis(*tert*-butyloxycarbonyl)amino-1-(*N′*-ethyl-*N′*-isopropylcarbamyl)pyrrolidine-4-carboxylate (17).** To a solution of **16** (16.0 g, 31.7 mmol) in DCM (150 mL) was added DIEA (11 mL, 79 mmol), followed by *N*,*N*-isopropylethylcarbamyl chloride (5.2 g, 38 mmol). The solution was stirred at ambient temperature for 24 h when complete reaction was observed. The solution was then washed with cold 0.01 N HCl (3 × 80 mL) and brine (3 × 80 mL). After drying ($MgSO_4$), the solution was concentrated and the residue purified by flash chromatography on a silica gel column using 60% EtOAc in hexane with 2% HOAc as the solvent, giving the desired (±)-(2*S*,3*R*,4*R*) isomer **16** (higher R_f, 9.0 g, 50.9%, ∼90% purity) and the (±)-(2*R*,3*R*,4*R*) isomer (lower R_f, 3.5 g). **16** MS (DCI-NH_3): m/z 558 (M + H), base peak. ^{1}H NMR ($CDCl_3$): 1.04−1.18 (m, 5H), 1.22 (d, 3H), 1.45 (s, 9H), 1.50 (s, 18H), 2.60−2.80 (d.q, 2H), 2.90−3.00 (m, 1H), 3.30−3.40 (m, 2H), 3.50−3.60 (t, 1H), 3.70−3.80 (d. d, 1H), 3.90−4.00 (m, 1H), 4.60−4.70 (m, 1H), 4.70−4.80 (m, 1H).

***tert*-Butyl (±)-(2*S*,3*R*,4*R*)-2-(Benzyloxycarbonyl)aminomethyl-3-bis(*tert*-butyloxycarbonyl)amino-1-(*N′*-ethyl-*N′*-isopropylcarbamyl)pyrrolidine-4-carboxylate (18a).** Compound **17** (4.0 g, 7.2 mmol), triethylamine (5.0 mL, 35.9 mmol), benzyl alcohol (6.5 mL, 63 mmol) and diphenylphosphoryl azide (1.5 mL, 11.6 mmol) were mixed with 50 mL of

toluene. The solution was stirred at 100 °C for 48 h, when TLC indicated complete reaction. The solution was diluted with 100 mL of EtOAc and washed with cold 0.01 N HCl (2 × 150 mL) and brine (2 × 150 mL). After drying ($MgSO_4$), the solvent was evaporated and the residue was purified by flash chromatography (20% EtOAc−hexane, then 30% EtOAc−hexane), giving **18b** as a white solid (2.9 g, 61.0%). MS (DCI-NH_3): *m/z* 663 (M + H), base peak. ^{1}H NMR ($CDCl_3$): 1.04−1.18 (m, 5H), 1.20 (d, 3H), 1.45 (s, 9H), 1.50 (s, 18H), 2.80−2.96 (m, 1H), 3.20−3.42 (m, 4H), 3.43−3.56 (bt, 1H), 3.72−3.82 (bt, 1H), 4.50−4.60 (m, 1H), 4.62−4.72 (m, 1H), 5.08 (s, 2H), 5.50 (bs, 1H), 7.35 (bs, 5H).

***tert*-Butyl (±)-(2*S*,3*R*,4*R*)-2-Aminomethyl-3-bis(*tert*-butyloxycarbonyl)amino-1-(*N*′-ethyl-*N*′-isopropylcarbamyl)-pyrrolidine-4-carboxylate (18b).** A solution of **18a** (2.5 g, 3.8 mmol) in 100 mL of 4.4 wt % of formic acid in MeOH was purged with nitrogen. Palladium black (0.2 g) was added. After 15 min of stirring at ambient temperature, TLC indicated complete reaction. The catalyst was removed by filtration and the filtrate was concentrated. The residue was further dried under vacuum to give a thick oil (2.1 g, ∼100%). MS (DCI-NH_3): *m/z* 529 (M + H), base peak. This material was used directly for solution-phase synthesis of library of compounds **20**.

Synthesis of Library of Compounds 20. (a) Reaction of 18b with Acid Chlorides, Sulfonyl Chlorides, and Carbamyl Chlorides. Reactions were carried out in batches of 48 or smaller numbers when appropriate. A stock solution of **18b** in DCM (25 mg/mL) was aliquotted into the reaction vessels (screw-cap tubes) at 1 mL/tube. Pyridine (5 equiv) was added, followed by the desired acid chlorides, sulfonyl chlorides, and carbamyl chlorides (1 equiv). The solutions were agitated by shaking on a shaker. After 6 h at ambient temperature, the reaction mixtures were directly purified by chromatography using 2.0-g silica gel cartridges (Alltech, Deerfield, IL) in batches of 24 with a 24-port vacuum manifold, eluting with 50% EtOAc in hexane. The fractions containing compound **19** were concentrated in a speedvac. The dried compounds **19** were then treated with 2.0 mL of 80% TFA in DCM for 4 h. Evaporation of TFA/DCM gave the TFA salt of compounds **20** which were analyzed by reverse-phase HPLC and MS (typical yield: 10 mg). The vast majority of compounds **20** prepared were over 90% pure by evaporative light scattering detection (ELS) on HPLC, even though samples with purity over 75% purity were all accepted for NA inhibition testing.

(b) Reaction of 18b with Carboxylic Acids. These reactions were carried out following the same procedure as described above with 25 mg of **18b** per reaction, except that 1 equiv of EDCI and catalytic amount (3 mg) of HOBt were used as the coupling reagents.

(±)-(2*S*,3*R*,4*R*)-2-(Trifluoroacetamido)methyl-3-amino-1-(*N*′-ethyl-*N*′-isopropylcarbamyl)pyrrolidine-4-carboxylic Acid (20e). To a solution of **18b** (0.47 g, 0.89 mmol) in 10 mL of DCM were added HOBt (24 mg, 0.1 mmol), EDCI (0.2 g, 1.1 mmol) and TFA (82 μL, 1.1 mmol). The solution was stirred at room temperature overnight and then concentrated. The residue was directly purified by flash chromatography on a silica gel column eluting with 10−25% EtOAc in hexane, giving **19** (X = CF_3CO−) as a white solid (0.51 g, 91%). MS (DCI-NH_3): *m/z* 625 (M + H), base peak. ^{1}H NMR ($CDCl_3$): 1.04−1.20 (m, 5H), 1.25 (d, 3H), 1.45 (s, 9H), 1.55 (s, 18H), 2.90−3.00 (m, 1H), 3.28−3.40 (m, 3H), 3.50−3.62 (m, 2H), 3.75−3.85 (dd, 1H), 3.85−3.96 (m, 1H), 4.50−4.60 (dd, 1H), 4.70−4.80 (m, 1H), 8.28 (bs, 1H). This material (**19**, X = CF_3CO−, 0.28 g, 0.45 mmol) was treated with 10 mL of 80% TFA in DCM for 6 h. After evaporation of the solvents, the residue was treated with 10 mL of 1 N HCl in diethyl ether, resulting in a white solid (**20e**, HCl salt) (175 mg, 97%). HPLC (ELS) indicated 98% purity. MS (DCI-NH_3): *m/z* 369 (M + H), base peak. ^{1}H NMR (DMSO-d_6): 0.9−1.1 (m, 5 H), 1.10−1.18 (d, 3H), 2.80−3.00 (m, 1H), 3.10−3.30 (m, 3H), 3.50−3.60 (m, 2H), 3.70−3.80 (m, 1H), 3.80−3.90 (m, 1H), 4.35−4.45 (d.d, 1H), 8.30−8.50 (bs, 2H), 9.32−9.42 (bs, 1H). HRMS: $C_{14}H_{24}N_4O_4F_3$, calcd 369.1750, found 369.1760.

NA Inhibition Assay. NA from A/Tokyo/3/67 virus was prepared by digestion of the virus with Pronase and purified as described previously.[23] NA inhibition assays were conducted in 50 mM sodium citrate, 10 mM calcium choride, 0.1 mg/mL BSA (pH 6.0) buffer with 5% DMSO. Reaction mixtures included NA, inhibitor, and 30 μM 4-methylumbelliferylsialic acid substrate in a total volume of 200 μL and were contained in white 96-well U-shaped plates. The reactions were initiated by the addition of enzyme and allowed to proceed for 30 min at room temperature. The fluorescence for each well of the plate was measured once each minute during the reaction period by a Fluoroskan II plate reader (ICN Biomedical) equipped with excitation and emission filters of 355 ± 35 and 460 ± 25 nm, respectively. The plate reader was under the control of DeltaSoft II software (Biometallics) and a Macintosh computer. Reaction velocities were linear over the 30-min reaction period. Compounds were initially tested for influenza NA inhibition at 0.5 mM. Those compounds which exhibited more than 50% inhibition were evaluated further using eight concentrations of compound. The reaction velocities for the dose−response study were fit to the following equation using a nonlinear regression program (Kaleidagraph) to determine the overall K_i value:[24] $(1 - V_i/V_o) = [I]/\{[I] + K_i(1 + [S]/K_m)\}$, where V_i and V_o represent inhibited and uninhibited reaction velocities, respectively, and $K_m = 40$ μM which was established from previous experiments.

X-ray Crystallography. Isolation, purification, and crystallization of N9 Tern NA has been described previously.[25] The crystals were exposed to the compounds of interest by soaking in solutions that contained 2.3 M $NaPO_4$, pH 7.5, and ligand concentration approximately 10 mM. The soakings were carried out at 4 °C and for 12-h duration. Once soaked, the crystals were then transferred serially to a cryoprotectant, 2.3 M $NaPO_4$, which contained 13% (v/v) and then 26% (v/v) glycerol. The crystals were then mounted on an R-Axis II-C area detector, which used X-rays generated by a Rigaku RU-200 rotating anode X-ray generator fitted with Yale focusing mirrors. Crystals were typically exposed for 20 min/0.5° oscillation, and a total of 25° of data were collected. Data were integrated and scaled using the HKL suite of programs.[26] Maps were calculated using the XPLOR suite of programs[27] and then displayed in the QUANTA molecular modeling program.[28]

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Supporting Information Available: Additional analytical data for compounds in Tables 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

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